SECOND EDITION

Essentials of MEDICAL MICROBIOLOGY

First edition widely appreciated among faculty and students

Student feedback

- Revolutionary book; crystal clear concepts and crisp presentation.
- Proud to have this book; far better than any others; boon for examination.
- Very well written book. A must for UGs, PGs and PGMEE aspirants.
- Very nice book. I wish I would have known about this book earlier.
- Gem of a microbiology textbook. Up-to-date info., short and precise.
- Absolutely brilliant, thanks authors for this wonderful creation.
- All Microbiology MCQs of PGMEE exam (2015-2018) sessions—came from this book.

First Indian clinical microbiology book

- Newer sections included-Hospital infection control and Clinical infective syndromes.
- Updates made in recent advances in laboratory diagnosis, treatment, prophylaxis and epidemiology.
- Newer topics such as newer sterilization methods, automations and Zika virus included.
- Bulleted format in concise, simple and lucid language.

Apurba Sankar Sastry
Sandhya Bhat K

JAYPEE

Essentials of
MEDICAL MICROBIOLOGY

SECOND EDITION

Apurba S Sastry MD DNB MNAMS PDCR
Hospital Infection Control Officer
Officer in-charge HICC
Antimicrobial Stewardship Lead
Associate Professor
Department of Microbiology
Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry, India

Sandhya Bhat MD DNB MNAMS PDCR
Professor
Department of Microbiology
Pondicherry Institute of Medical Sciences (PIMS)
(A Unit of Madras Medical Mission)
Puducherry, India

Co-Editors

Anand Bhimaray Janagond MD DNB
Associate Professor
Department of Microbiology
S Nijalingappa Medical College
Bagalkot, Karnataka, India

Deepashree R MD DNB MNAMS
Senior Resident
Department of Microbiology
JIPMER, Puducherry, India

Forewords

Elmer W Koneman MD
Author of the famous book 'Koneman's Color Atlas and Textbook of Diagnostic Microbiology'

Sujatha Sistla MD
Professor and *Head*, Department of Microbiology, JIPMER, Puducherry, India

JAYPEE

JAYPEE BROTHERS MEDICAL PUBLISHERS
The Health Sciences Publisher
New Delhi | London | Panama

Jaypee Brothers Medical Publishers (P) Ltd

Headquarters

Jaypee Brothers Medical Publishers (P) Ltd
4838/24, Ansari Road, Daryaganj
New Delhi 110 002, India
Phone: +91-11-43574357
Fax: +91-11-43574314
Email: jaypee@jaypeebrothers.com

Overseas Offices

J.P. Medical Ltd
83 Victoria Street, London
SW1H 0HW (UK)
Phone: +44 20 3170 8910
Fax: +44 (0)20 3008 6180
Email: info@jpmedpub.com

Jaypee-Highlights Medical Publishers Inc
City of Knowledge, Bld. 235, 2nd Floor, Clayton
Panama City, Panama
Phone: +1 507-301-0496
Fax: +1 507-301-0499
Email: cservice@jphmedical.com

Jaypee Brothers Medical Publishers (P) Ltd
17/1-B Babar Road, Block-B, Shaymali
Mohammadpur, Dhaka-1207
Bangladesh
Mobile: +08801912003485
Email: jaypeedhaka@gmail.com

Jaypee Brothers Medical Publishers (P) Ltd
Bhotahity, Kathmandu
Nepal
Phone: +977-9741283608
Email: kathmandu@jaypeebrothers.com

Website: www.jaypeebrothers.com
Website: www.jaypeedigital.com

Inquiries for bulk sales may be solicited at: jaypee@jaypeebrothers.com

Essentials of Medical Microbiology

First Edition: 2016

Second Edition: **2019**

ISBN: 978-93-5270-479-8

Printed at : Paras offset Pvt. Ltd., New Delhi

Dedicated to

Our Beloved Parents, Family Members
And, above all, the Almighty

"Life is the most difficult exam. Many fail because they tend to copy others, not realizing that everyone has a different question paper."

Gold medalists are not made up of gold. They are made up of determination and hard work and ready to kill themselves to achieve their goal.

Golden Rules of Goal Setting

Dear Students

Here are some important tips which will help you in setting your goals in studies:

1. Set Goals That Motivate You: This means making sure that they are important to you, and that there is value in achieving them
2. Set SMART Goals
   - Specific: Your goal must be clear and well defined, not vague or generalized
   - Measurable: Goals must have measurable objectives
   - Attainable : Make sure that your goals are achievable and within your limit
   - Relevant: Will take you to the direction you want your life and career to go
   - Time Bound: You must know when you have the deadline and can celebrate success
3. Set Goals in Writing: Written commitment in presence of your close people (parents, close friends) will always push and remind you whenever you tend to deviate from your goal
4. Make an Action Plan: Do not focus only on the outcome, but make planning of all small steps that collectively take to the outcome. This is especially important if your goal is big and demanding, or long-term
5. Monitor Yourself: Compliance to the action plan should be monitored at least weekly (for one month goal) or monthly (for a yearly goal), depending upon your goal size.

Remember,

"Success is not final; failure is not fatal: It is the courage to continue that counts."

—Winston S Churchill

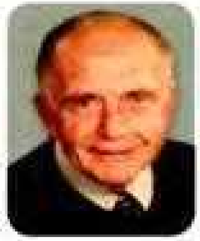
"There are two types of people who will tell you that you cannot make a difference in this world: those who are afraid to try and those who are afraid you will succeed."

—Ray Goforth

"Success ke piche mat bhago. Kabil bano kabil. Kamyabi toh sali jhak maar ke peeche ayegi."

—Aamir Khan

Foreword



The second edition of *Essentials of Medical Microbiology* is indeed a masterpiece. I am truly amazed for the amount of time that the authors would have devoted in the preparation of the outstanding writing of this book.

The intent of the authors of this text, "Essentials", is to bring reality of interactions between men and microbes, in specific to meet the diverse needs of medical school students in India and world. This book will also be of great help for preparation of postgraduate entrance examinations and for postgraduate students. This text also provides valuable guidelines for microbiology technologists who are employed as faculty in training centers or practitioners in microbiology laboratories within and across national borders.

This book is a perfect blend of conventional methods and newer automated and molecular methods used in diagnostic microbiology. The content of the book is reliable, updated, and according to the current practice in diagnostic microbiology. The descriptive diagrams and laboratory/clinical photographs are most supportive of the well-focused text.

Of note is the application in "Essentials" are those chapters in bacteriology, virology and mycology, where the focus is on the species of bacteria, viruses, and fungi more commonly encountered in clinical laboratories, as related to human infections they cause. In each case, a presentation is made of information on specimen collection and transport, laboratory tests including the antimicrobial susceptibility testing. Also included are an array of pictures of human clinical diseases caused by bacteria, viruses, and fungi. Immunology section has been well explained in a much lucid and easy-to-understand language.

Of much assistance at the end of each chapter, directed both to faculty and students in microbiology teaching courses, and for use by technologists who might decide to change practice in one of the microbiology subspecialties, is series of case-based essay questions focused on key practice applications as covered in each chapter.

I am sure this book is the future of Microbiology in India and will serve as a standard book of Microbiology for Indian medical school students. In fact, it will also cater to the need of medical school students of rest of the world. This book definitely needs promoting for a wide circulation.

In conclusion, this "Essentials" treatise, as having joined and even exceeded other current texts in microbiology for medical school students, can be highly recommended.

Elmer W Koneman MD
Professor Emeritus
University of Colorado School of Medicine
Aurora, Colorado
United States
Author of the famous book 'Koneman's Color Atlas and Textbook of Diagnostic Microbiology'

Foreword

I am happy to know that the second edition of *Essentials of Medical Microbiology* is to be released. This book has come at a much needed time when there is a strong demand for a Clinical Microbiology textbook written from an Indian perspective. The first edition was very well appreciated among faculty and students across the country. The second edition is a fully updated book with a simple lucid presentation style similar to its earlier edition.

Hospital infection control has been incorporated as a new section which comprises of many new chapters such as HAI surveillance, Biomedical Waste Rule 2016, needle stick injury, antimicrobial stewardship, and environmental surveillance.

Clinical infective syndromes has been made into a separate section with each syndrome divided into a separate chapter. Each chapter begins with clinical case scenarios.

Updates have been made in recent advances in laboratory diagnosis, treatment guidelines and vaccine prophylaxis of each infectious disease. Latest updates in epidemiology of infectious diseases have been incorporated such as recent outbreaks and geographical distribution with special emphasis given to the latest data on Indian epidemiology.

Recent updates in health care program/guideline such as Revised National Tuberculosis Control Program (RNTCP), National AIDS Control Organization (NACO) and Global Polio Eradication Initiative (GPEI) programs, etc., have been incorporated. More emphasis has been given to automation in microbiology, newer molecular methods, newer immunoassays pertaining to microbiology such as ELFA technology and recent outbreaks such as Zika virus and Nipah virus infections.

Sterilization and disinfection chapter has been updated by incorporating the common methods used in hospitals and adding a note on CSSD (Central Sterile Supply Department). Changes have been made to the nomenclature of several organisms. More number of tables, flowcharts, real images and schematic diagrams have been incorporated for better understanding.

I really appreciate the efforts made by my colleague Dr Apurba S Sastry and his wife Dr Sandhya Bhat, for this commendable work and I am sure that this book will be widely read by both undergraduate and postgraduate students of microbiology.

Sujatha Sistla MD
Professor and Head
Department of Microbiology
Jawaharlal Institute of Postgraduate
Medical Education and Research (JIPMER)
Puducherry, India

Preface to the Second Edition

It is a proud moment for us. The excitement reaches its crazy heights as our sleepless nights of last six months have come to an end and the second edition of *Essentials of Medical Microbiology* is released.

The first edition went viral among students and faculty; was highly appreciated. Many have described it as 'block buster', 'revolutionary book', 'the game changer book', 'boon for exam', 'catering to the need of all: UG, PGMEE aspirant and PGs', etc. The second edition has been designed in a similar fashion taking all your feedback and suggestions into consideration. The newer concepts and recent advances incorporated are as follows:

- **Section 1:** *General Microbiology section* has been updated with addition of topics such as recent Nobel laureates, latest changes in nomenclature, newer sterilization methods/topics (plasma sterilization, ETO, etc., with an update on sterilization indicators, a special note on CSSD), newer automations (MALDITOF, VITEK, BacT/ALERT VIRTUO, newer molecular methods (LAMP, Real-time PCR, Biofire FilmArray, etc.), newer antimicrobial agents such as fifth generation cephalosporins, fosfomycin and colistin, latest antimicrobial susceptibility testing (AST) methods including automated AST methods.
- **Section 2:** *Immunology section* has been updated with newer serological methods such as ELFA technology (Vidas system), use of chemiluminescence immunoassay in diagnostic microbiology, IgG avidity, ELISA, etc. All the schematic figures are redrawn to make the concept crystal clear.
- **Section 3:** In *Systematic Bacteriology section*, many topics are thoroughly updated such as pneumococcal vaccine, MRSA, VRSA, VRE, anthrax vaccine, diphtheria vaccine, epidemiology of meningococcal meningitis, *Clostridioides difficile* infection, elimination of neonatal tetanus, tuberculosis (thorough updation on newer investigations such as GeneXpert, MGIT, TrueNat, Drug Susceptibility Testing methods, latest RNTCP guideline on diagnosis and treatment of TB), leprosy (including a note on newer leprosy vaccine introduced by Government of India), melioidosis, scrub typhus, yaws (including yaws eradication program), leptospirosis, etc. The epidemiology, laboratory diagnosis, and treatment have been thoroughly updated in all the chapters. Many newer photographs have been incorporated for better understanding.
- **Section 4:** *Virology section* needs a special mention. We must say that this section underwent a major update. As we all know that virology is the emerging area of microbiology with lots of changes keep happening every year. We updated all the chapters with latest epidemiology, laboratory diagnosis and treatment data from standard references with incorporation of many new photographs, tables, and flow charts. The topics which underwent major update include influenza (epidemiology, laboratory diagnosis with special emphasis on interpretation of real-time PCR, vaccine), measles and rubella (epidemiology and laboratory diagnosis), arboviruses (new topics such as Zika virus, SFTS virus; update of existing topics such as chikungunya, dengue, JE virus, yellow fever, etc.), hepatitis (update in epidemiology, laboratory diagnosis, treatment of HBV, and HCV), HIV (update in epidemiology, NACO strategy, progressors, treatment and PEP), Nipah virus (including the Kerala outbreak, 2018), poliomyelitis (update in vaccine schedule, epidemiology and end game strategy and GPEI), rabies (epidemiology, laboratory diagnosis and prophylaxis) and many more.
- **Section 5:** *Mycology section* has been updated with recent advances/changes in epidemiology, nomenclature of fungi, laboratory diagnosis, and treatment of various fungal diseases. Many new photographs have been incorporated.
- **Section 6:** *Hospital Infection Control* has been added as a new section in this edition. It was a long-time demand from the readers from all the parts of our country. Infection control practices are very poorly followed in India and lack of awareness at undergraduate level is the main reason behind it. As first of its kind, we have introduced this section which addresses several areas of infection control such as hospital-acquired infection surveillance, biomedical waste (according to 2016 guideline, with 2018 amendment), needle stick injury, antimicrobial stewardship and environmental surveillance (it is a revised chapter of the first edition chapter, 'Bacteriology of Water, Air, Surface, Milk and Food'). We propose the faculty to keep at least 6–7 dedicated theory hours and two hours of practical classes for this section. Basic knowledge on infection control practice is the minimum thing expected from any MBBS doctor.

- **Section 7:** *Clinical Microbiology (Infective Syndromes)* chapter of first edition has been expanded to a new section with each syndrome has been divided into a separate chapter. Each chapter begins with clinical case scenarios. This section will bridge the gap between bench microbiology and clinical microbiology. This is a major requirement in today's date. The subject 'Microbiology' in India is moving towards 'Clinical Microbiology and Infectious Disease'. Having a clinical-oriented section is of great help, which will provide the students and readers a bird's eye view on clinical aspects of various infectious diseases.
- **Annexures:** Quality control in microbiology has been added as a new annexure. All the previous annexures have been thoroughly updated.
- **MCQs:** More number of MCQs have been incorporated in every chapter as requested by most readers.
- Inclusion of more tables, flowcharts, real images and schematic diagrams—for better understanding.
- **Most features of the first edition have been maintained:** such as concept of more content-less pages, concise, bulleted format and to-the-point text, simple and lucid language, separate boxes for summary of laboratory diagnosis and treatment for quick review, clinical case-based essay questions at the end of each chapter.

As you know, human errors are inevitable; and no book is immune from it. We would request all the readers to provide any errata found and also valuable suggestions and updates via e-mail.

We are confident and hoping that you all will fall in love with this revised second edition of the book.

Apurba S Sastry
drapurbasastry@gmail.com

Sandhya Bhat
sandhyabhatk@gmail.com

Preface to the First Edition

The association of man and microorganisms, and their fight to survive each other is never-ending. With the increasing complexity of interaction between man and microbes, the relevance of studying medical microbiology has increased manifold. Our book titled *Essentials of Medical Microbiology* attempts to provide smart ways to master the world of microbes relevant to the mankind.

The idea of yet another book on the subject, but with a quite different approach was born after several discouraging and unsatisfying experiences with several existing books regarding many needs of the enthusiastic students of the subject and a strong desire to make medical microbiology more interesting, up-to-date, clinically relevant and yet palatable to mainly undergraduate students of medicine and also the postgraduate aspirants and students. This book was conceptualized and brought to reality to meet the strongly felt diverse needs of the Indian students, such as gaining essential concepts, acquiring contemporary knowledge, approaching university exams with ease and confidence, scoring high in postgraduate entrance examinations, etc.

The book focuses on providing good foundation in clinically important concepts and principles of microbiology. Enough (over 300) tables and flowcharts have been included along with the text. Over 200 schematic diagrams have been drawn to simplify difficult concepts, and they are easy to reproduce where necessary as in examinations. Plenty of clinical photographs (over 400) included in the book will create a real life-like picture in the minds of the reader and also are meant to help solving image-based MCQs in postgraduate entrance examinations. It has more content in fewer pages, making the book handy. The concise bulleted format and to-the-point text used in this book will be helpful in rapid revision before the examinations. Best attempts have been made to keep the language simple yet lucid to help easy comprehension. Summary of laboratory diagnosis and treatment in separate boxes makes quick review possible. Highlighted boxes are incorporated to cover the important concepts. In a nutshell, this book is carefully written targeting to meet the varied needs of undergraduate students with an approach that will orient them to build concepts and to clear undergraduate examinations as well as to equip them for postgraduate entrance examinations in future.

General Microbiology section deals with principles of microscopy, morphology, physiology, culture identification of bacteria, concepts of bacterial genetics, etc. Principles of sterilization, antimicrobial chemotherapy and susceptibility testing are also explained in detail.

It is our humble hope that this book would change the general feeling of the students regarding immunology as being a difficult section into immunology as an interesting and enjoyable topic. In this section, topics such as *immunity, antigen, antibody, complement* and *structure of immune system* are explained in a simple and logical manner. Chapters like *Immune Response* and *Antigen-Antibody Reaction* have been fully updated according to the current need. Appropriate diagrams and flowcharts are incorporated to make critically tough content easy to grasp. Topics such as *autoimmunity, immunodeficiency* and *immunization* provide complete and latest information compiled in tabular form at one place.

Systematic Bacteriology section deals with individual bacterial pathogens in detail. Flow of information follows a very logical and clinically relevant course. More stress is given to the knowledge that helps in clinical setting and a careful attempt has been made to reduce the obsolete and not-so-useful core microbiology content. Sections like *laboratory diagnosis, treatment* and *prophylaxis* are most updated and referenced from internationally accepted literature and guidelines.

Virology is another section where the readers will find a different approach from the existing books. The updated and succinct information provided in this section with emphasis on *pathogenesis* and *laboratory diagnosis* will be useful to the students.

This book also addresses to the long-time complaint of the undergraduate students about unavailability of a concise and pictorialized *Mycology* section. Written in a clear and concise manner with appropriate and beautiful schematic pictures, images and illustrations, this section will surely make the students enjoy reading.

Applied Microbiology covers important aspects of various clinical infective syndromes with special reference to the approach towards the diagnosis. Useful information regarding hospital-acquired infections and biomedical waste management have been incorporated. The annexure incorporated at the end covers the recent topics, such as *emerging pathogens*, *bioterrorism* and *laboratory-acquired infections*.

Clinical case-based essay questions and MCQs are given at the end of each chapter to orient and prepare students for the examinations. Advanced and newer postgraduate entrance-oriented topics like H1N1, ebola, polio eradication, bacterial drug resistance mechanisms (such as ESBL, VRSA, VRE), automations and molecular methods in microbiology, etc. are incorporated.

We hope that the undergraduates, postgraduate aspirants, and postgraduate students will relish reading this book and find it useful. We also hope that we have made a good start in addressing the varied needs of students and faculty teaching medical microbiology with a single comprehensive book. We will feel glad to receive your valuable feedback, which will enable us to improve further.

Apurba S Sastry
drapurbasastry@gmail.com

Sandhya Bhat
sandhyabhatk@gmail.com

Acknowledgments

The release of second edition of *Essentials of Medical Microbiology* would not have been possible without our close association with many people. We take this opportunity to extend our sincere gratitude and appreciation to all those who made this book possible.

Hearty acknowledgments to our teachers, departmental staff, family members and others, for their blessings and support

1. We would like to sincerely thank **Dr Elmer W Koneman**, author of *Color Atlas and Textbook of Diagnostic Microbiology* and Professor Emeritus, University of Colorado School of Medicine, United States, for his willingness to write an encouraging foreword.
2. We express heart-felt gratitude to **Dr Sujatha Sistla**, Professor and Head, Department of Microbiology, JIPMER, for giving the foreword, for her guidance during manuscript preparation and giving permission to reproduce photographs from the department. She is an extremely friendly and knowledgeful person. She has been our '**Google Search**'; clarifying all our doubts all the time. I (Dr Apurba) am greatly indebted to you mam for your timely support and guidance.
3. We are extremely thankful to **Dr S Vivekanandam**, Director, JIPMER, Puducherry and also the previous Director **Dr Vishnu Bhat**, for giving the permission to revise this textbook.
4. We are grateful to **Dr Renu G Boy Varghese**, Director-Principal, Pondicherry Institute of Medical Sciences (PIMS), Puducherry, for giving permission to revise this textbook
5. We would like to express our special word of thanks to **Dr Reba Kanungo**, Dean Research, Professor and Head, Department of Microbiology, Pondicherry Institute of Medical Sciences (PIMS), for giving permission to reproduce photographs from the department. I (Dr Sandhya) am truly grateful to you mam for your wholehearted support.
6. **Other Faculty of Department of Microbiology, JIPMER—** Dr Jharna Mandal (Additional Professor), Dr Rakesh Singh (Additional Professor), Dr Rahul Dhodapkar (Additional Professor), Dr Noyal M Joseph (Associate Professor), Dr Rakhi Biswas (Associate Professor), Dr Nonika Rajkumari (Assistant Professor).
7. **Other Faculty of Department of Microbiology, PIMS—** Dr Shashikala (Professor); Dr Sheela Devi (Professor); Dr Johny Asir (Associate Professor); Dr Vivian Joseph P (Associate Professor), Dr Sujitha E (Associate Professor); Dr Anandhalakshmi (Associate Professor), Dr Arthi E (Associate Professor), Mrs Patricia Anita (Assistant Professor), Dr Ramya SR (Assistant Professor), Dr Meghna (Assistant Professor) and Mrs Desdemona Rasitha (Tutor).
8. **Residents and postgraduates, JIPMER—** Dr Deepashree, Dr Ramya, Dr Suman, Dr Prasanna, Dr Kavita, Dr Sunil, Dr Radha, Dr Rachna, Dr Sushmita, Dr Sneha, Dr Akshatha, Dr Sruthi, Dr Subhashree, Dr Dhanalakshmi, Dr Sarumathi, Dr Anitha, Dr Kalpana, Dr Debdutta, Ms Amritha, Ms Manisha, Ms Kavipriya and Ms Sugalya. I (Dr Apurba) would like to keep in record the immense help provided by all my residents. I must say that you guys are highly talented, will be the bright future of Indian Microbiology.
9. **HICC, JIPMER—** Infection control nurses and other office staff such as Ms Ilaveni and Mr Venkat.
10. **Special thanks to our teachers—**Dr SC Parija (JIPMER), Dr BN Harish (JIPMER), Dr ER Nagaraj (SSMC, Tumkur), Dr Sharadadevi Mannur (SSMC, Tumkur) and Dr Renushree (SSMC, Tumkur).
11. **For providing photographs—**We are extremely thankful to all people/institutes/companies who have agreed to provide valuable photographs.
12. **Microbiology Faculty from our previous working place—**
    - Meenakshi Medical College, Chennai, Tamil Nadu—Dr Senthamarai, Dr Sivasankari, Dr Kumudavathi, Dr Somasundar and others
    - ESIC Medical College and PGIMSR, Chennai—Dr Manisha S Mane and others
    - Sri Siddhartha Medical College, Tumkur, Karnataka, India (all faculty members).

13. **Our friends**—Dr Godfred, Dr Sadia, Dr Ramakrishna, Dr Mridula, Dr Srinivas Acharya, Dr Chaya, Dr Manisha, Dr Ira, Dr Sreeja, Dr Wajid and others.
14. **Family**— Parents, brother, sister and other family members, maternal and paternal cousins and all other well-wishers.

Sincere acknowledgments for helping in manuscript preparation and providing photographs

Name of the person/institute	Contribution(s)
Public Health Image Library, Centers for Disease Control and Prevention (CDC), Atlanta	For giving permission to reproduce photographs of public domain
Dr Veeraraghavan, Faculty, Department of Gastroenterology, PIMS, Puducherry	For giving inputs during manuscript preparation of treatment of hepatitis B and C
Dr Karen C Carroll, Professor of Pathology, Johns Hopkins University, USA and Editor of Jawetz, Melnick & Adelberg's Medical Microbiology, 27th edition. Mr Michael Weitz, Editorial Director, McGraw-Hill Professional Publishing, USA	For giving permission from McGraw-Hill Publishers for reproducing photograph from Jawetz, Melnick & Adelberg's Medical Microbiology, 27th edition
Authors of Kuby's Immunology Dr Judith A Owen, Jenni Punt, Dr Sharon Stranford, Dr Patricia P Jones and Ms Lauren Schultz, Senior Acquisitions Editor, WH Freeman and Company	For allowing us to modify the images from Kuby's Immunology, fifth edition
Dr Sherly Antony, Faculty, Pushpagiri Medical College, Kerala	For providing photographs and giving inputs in the correction of content error of first edition
Dr Isabella Princess, Consultant, Apollo Hospitals, Chennai	For providing photographs
Dr Suchitra Mahapatra, Hitech Medical College, Rourkela, Odisha	For providing photographs
Dr Anand B Janagond, Associate Professor, S Nijalingappa Medical College, Bagalkot, Karnataka	For editing the manuscript of first edition, giving inputs in second edition and preparation of instruction manual
Dr Deepashree R, Senior Resident, Department of Microbiology, JIPMER	For giving inputs in updating the book and preparation of instruction manual
Dr Vivekandan, Additional Professor, Medicine, JIPMER, Puducherry	For giving inputs in the treatment aspects of various infectious diseases
Dr Rakesh Singh, Additional Professor, Department of Microbiology, JIPMER	For giving inputs during manuscript preparation of HIV and mycology chapters
Dr Rahul Dhodapkar, Additional Professor, Department of Microbiology, JIPMER	For giving inputs during manuscript preparation of hepatitis, influenza and other viral diseases
Dr Noyal M Joseph, Associate Professor, Department of Microbiology, JIPMER	For giving inputs during manuscript preparation of Mycobacteria chapter
Dr Ramya Raghavan, an outstanding resident from Department of Microbiology, JIPMER	For helping during manuscript preparation of vaccines and provide information on gene and antigen targets for viral diagnosis
Dr Subhadra, Associate Professor, Radiology, JIPMER, Puducherry	For providing radiology photographs
Ms Kiranmayi Lakshmi, Mr Haribabu, bioMerieux India	For providing inputs in writing of MALDITOF and VITEK
Ms Kavitha Kulkarni and Mr Aravindh S, 3M India	For providing inputs in writing of sterilization indicators
Mr Sandeep Pahwa and Mr Liju Verghese, bioMerieux India	For providing photographs
Dr Anand Sastry, Associate Professor, Medicine, Teerthanker Mahaveer University, Moradabad, UP	For giving inputs in the treatment aspects of various infectious diseases
Dr Padmaja Shenoy, Associate Professor, Microbiology, Kasturba Medical College, Manipal	For providing photographs related to anaerobic bacteriology
Ex-residents from JIPMER: Dr Keerthivasan, Dr Raja S, Dr Deepika, Dr Udhaya Sankar and Dr Meenatchi	For giving inputs in the correction of content errors of first edition
Each and every reader (faculty and students) from various parts of the country	For communicating to us by email and other electronic media about the content correction and updates from time to time

Special acknowledgments to my publishers

Jaypee Brothers Medical Publishers (P) Ltd, New Delhi, India.

- Shri Jitendar P Vij (Group Chairman)
- Mr Ankit Vij (Managing Director)
- Mr MS Mani (Group President)
- Ms Ritu Sharma (Director-Content Strategy): She is extremely sweet, and dynamic. She has been the backbone behind publishing this book. We are glad to work with you mam.
- Ms Pooja Bhandari (Production Head)
- Ms Seema Dogra (Cover Visualizer)
- Ms Nidhi Sinha (Development Editor)
- **The Development Team:** Mr Vakil Khan, Mr Deepak Saxena, Mr Rajesh Ghurkundi, Mr Deep Kumar, Mr Laxmidhar Padhiary, and Ms Geeta Barik. These guys are simply outstanding in their work. A special mention for Mr Deepak Saxena, we must say that he is the best operator of India in medical publishing. The way Rajesh Ghurkundi does the designing of photographs is extraordinary. It is a treat for us to work with all of you. You guys are extremely workaholic and have a very good team spirit. We salute them, for their professionalism.
- **Staff from other branch office:** Mr Venugopal, Mr Palani A and Mr Venkatesh E (Bengaluru Branch), Mr Parimal, Mr Sharma and Mr Rajesh Malothu (Hyderabad Branch), Mr Senthil Kumar, Mr TM Bhaskar, Mr Dharani Kumar P and Mr R K Dharani (Chennai Branch), Mr Muralidharan (Puducherry Branch), Mr CS Gawde (Mumbai Branch), Mr Sandip Gupta and Mr Sanjoy Chakraborty (Kolkata Branch), Mr Vasudev H (Mangaluru Branch), Mr Arun Kumar (SSPO Thiruvananthapuram Branch), Mr Sujeesh (Kochi Branch) and Mr Dinesh Waghade (Ahmedabad Branch).

Lastly, we would like to keep in record that without the support of our son, parents (of both Dr Sandhya and Dr Apurba) and other family members, it would have been impossible to continue the spirit on, during the journey of the current edition. A special mention to our son (Master Adarsh), who really helped us in giving cooperation. In fact, he was encouraging us to work for the book. We deeply apologize to you, as we could not give enough time and care to you during the manuscript preparation.

Apurba S Sastry
Sandhya Bhat

Contents

Further Reading

1. Abbas' Cellular and Molecular Immunology, 8th Edition.
2. Bailey & Scott's Diagnostic Microbiology, 14th Edition.
3. Centers for Disease Control and Prevention, Atlanta, USA.
4. Gillespie's Medical Microbiology and Infection at a Glance, 4th Edition.
5. Harrison's Principles of Internal Medicine, 19th Edition.
6. Jawetz Melnick and Adelbergs' Medical Microbiology, 27th Edition.
7. Koneman's Color Atlas and Textbook of Diagnostic Microbiology, 7th Edition.
8. Kuby's Immunology, 7th Edition.
9. Mackie and McCartney's Practical Medical Microbiology, 14th Edition.
10. Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases, 8th Edition.
11. Manson's Tropical Diseases, 23rd Edition.
12. National AIDS Control Organisation (NACO), India.
13. National Center for Disease Control (NCDC) Guidelines, India.
14. National Vector Borne Disease Control Program (NVBDCP), India.
15. Patrick R Murray's Medical Microbiology, 7th Edition.
16. Plotkin's Vaccines, 7th Edition.
17. Prescott's Microbiology, 9th Edition.
18. Revised National Tuberculosis Control Program (RNTCP), India.
19. Robbins & Cotran Pathologic Basis of Disease, 9th Edition.
20. Stites' Medical Immunology, 10th Edition.
21. Topley and Wilson's Microbiology and Microbial Infections, 10th Edition.
22. Various national and international journals and other internet sources.
23. World Health Organization (WHO).

General Microbiology

SECTION 1

Section Outline

Introduction and Bacterial Taxonomy

1 CHAPTER

Chapter Preview

- History
- Bacterial taxonomy

Medical microbiology is a branch of medicine that deals with the study of microorganisms and their role in human health and diseases. It also concerns with the diagnosis, treatment and prevention of various infectious diseases. There are four kinds of microorganisms that cause infectious diseases: bacteria, fungi, parasites and viruses. The branches of medical microbiology are as follows:

- **General microbiology:** Study of general properties of microorganisms, such as bacterial morphology, sterilization and disinfection, culture identification methods (conventional, automated and molecular methods), bacterial genetics, etc.
- **Immunology:** The study of the immune system
- **Bacteriology:** The study of bacteria
- **Virology:** The study of viruses
- **Mycology:** The study of fungi
- **Parasitology:** The study of parasites; it has two arms
  - Protozoology: The study of protozoa
  - Helminthology: The study of helminths.

HISTORY

The existence of microorganisms was hypothesized for many centuries before their actual discovery. The teaching of Mahavira (Jainism, 6th century BC) and the postulation of Varo and Columelia (who named the invisible organisms as '*Animalia minuta*') were some of those attempts.

CONTRIBUTORS IN MICROBIOLOGY

Antonie Philips van Leeuwenhoek (1676)

He was the first scientist who observed bacteria and other microorganisms, using a single-lens microscope constructed by him and he named those small organisms as '*Little animalcules*' (Fig. 1.1A).

Edward Jenner

Edward Jenner 1796, developed the first vaccine of the world, the smallpox vaccine. He used the cowpox virus (*Variolae vaccinae*) to immunize children against smallpox from which the term **'vaccine'** has been derived. The same principles are even used today for developing vaccines.

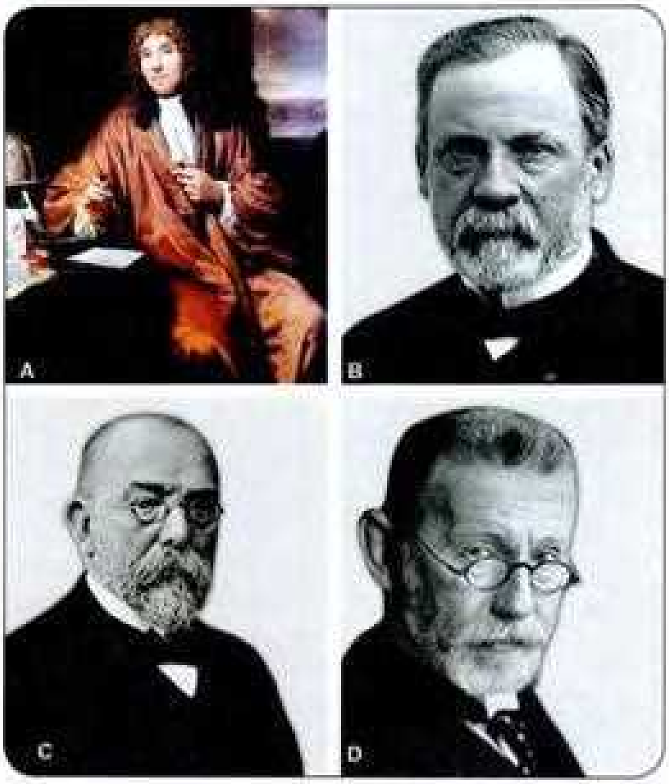
Louis Pasteur

Microbiology developed as a scientific discipline from the era of Louis Pasteur (1822–1895). He is also known as **father of microbiology**. He was a professor of chemistry in France. His studies on fermentation led him to take interest to work in microbiology (Fig. 1.1B). His contributions to microbiology are as follows:

- He had proposed the **principles of fermentation** for preservation of food
- He introduced the **sterilization techniques** and developed steam sterilizer, hot air oven and autoclave
- He described the method of **pasteurization of milk**
- He had also contributed for the vaccine development against several diseases, such as anthrax, fowl cholera and rabies
- He disproved the theory of spontaneous generation of disease and postulated the '**germ theory of disease**'. He stated that disease cannot be caused by bad air or vapor, but it is produced by the microorganisms present in air
- Liquid media concept: He used nutrient broth to grow microorganisms
- He was the founder of the Pasteur Institute, Paris.

Joseph Lister

Joseph Lister (1867) is considered to be the **father of antiseptic surgery**. He had observed that postoperative



Figs 1.1A to D: Eminent microbiologists. **A.** Antonie van Leeuwenhoek; **B.** Louis Pasteur; **C.** Robert Koch; **D.** Paul Ehrlich

Source: Wikipedia (with permission).

infections were greatly reduced by using disinfectants such as diluted carbolic acid during surgery to sterilize the instruments and to clean the wounds.

Robert Koch

Robert Koch provided remarkable contributions to the field of microbiology. He was a German general practitioner (1843–1910) (Fig. 1.1C). His contributions are as follows:

- He introduced **solid media** for culture of bacteria. Eilshemius Hesse, the wife of, one of the Koch's assistants had suggested the use of **agar** as solidifying agents
- He also introduced methods for isolation of bacteria in **pure culture**
- He described hanging drop method for testing motility
- He discovered bacteria such as the anthrax bacilli, tubercle bacilli and cholera bacilli
- He introduced staining techniques by using aniline dye
- **Koch's phenomenon:** Robert Koch observed that guinea pigs already infected with tubercle bacillus developed a hypersensitivity reaction when injected with tubercle bacilli or its protein. Since then, this observation is called as Koch's phenomenon
- **Koch's postulates:** Robert Koch had postulated that a microorganism can be accepted as the causative agent of an infectious disease only if four criteria are fulfilled. These criteria are as follows:
  1. The microorganism should be constantly associated with the lesions of the disease.
  2. It should be possible to isolate the organism in pure culture from the lesions of the disease.
  3. The same disease must result when the isolated microorganism is inoculated into a suitable laboratory animal.
  4. It should be possible to re-isolate the organism in pure culture from the lesions produced in the experimental animals.

An additional **fifth** criterion was introduced subsequently which states that antibody to the causative organism should be demonstrable in the patient's serum.

Exceptions to Koch's postulates: It is observed that it is not always possible to apply these postulates to study all the human infectious diseases. There are some bacteria that do not satisfy one or more of the four criteria of Koch's postulates. Those organisms are:

- *Mycobacterium leprae* and *Treponema pallidum:* They cannot be grown *in vitro*; however, they can be maintained in experimental animals
- *Neisseria gonorrhoeae:* There is no animal model; however, it can be grown *in vitro.*

Molecular Koch's Postulates

It was a modification of Koch's postulates formulated by the microbiologist Stanley Falkow (1988). He stated that gene (coding for virulence) of a pathogenic microorganism that contributes to the disease should satisfy all the criteria of Koch's postulates rather than the microorganism itself.

- The virulence trait under study should be associated much more with pathogenic strains of the species than with nonpathogenic strains
- Inactivation of the gene associated with the suspected virulence trait should substantially decrease pathogenicity.
- Replacement of the mutated gene with the normal wild-type gene should fully restore pathogenicity
- The gene should be expressed at some point during the infection and disease process
- Antibodies or immune system cells directed against the gene products should protect the host.

Paul Ehrlich

Paul Ehrlich (1854–1915) was a German scientist and is also known as **father of chemotherapy** (Fig. 1.1D). His contributions are as follows:

- He was the first to report the acid-fast nature of tubercle bacillus
- He developed techniques to stain tissues and blood cells
- He proposed a toxin-antitoxin interaction called **Ehrlich phenomenon** and also introduced methods of standardising toxin and antitoxin

- He proposed the '**side chain theory** for antibody production'
- **Chemotherapy:** He discovered **salvarsan**, an arsenical compound (also called as the 'magic bullet') as the first effective medicinal treatment for syphilis, thereby initiating and also naming the concept of chemotherapy
- The bacteria '*Ehrlichia*' was named after him
- In 1908, he received the Nobel prize in Physiology or Medicine for his contributions to immunology
- He was the founder and first director of what is known now as the Paul Ehrlich Institute, Germany.

Other Important Contributors

- **Hans Christian Gram (in 1884):** He developed a method of staining bacteria which was named as 'Gram stain' to make them more visible and differentiable under a microscope
- **Charles Chamberland:** He is one of Pasteur's associates, constructed a porcelain bacterial filter in 1884 by which the discovery of viruses and their role in disease was made possible. The first viral pathogen to be studied was the tobacco mosaic virus
- **Ernst Ruska:** He was the founder of electron microscope (1931)
- **Alexander Fleming (in 1929):** He discovered the most commonly used antibiotic substance of the last century, i.e. penicillin
- **Goodpasture:** He described the viral culture technique in chick embryo
- **Lady Mary Wortley Montagu:** Introduced variolation for immunizing against smallpox
- **Elie Metchnikoff:** He described phagocytosis and termed *phagocytes*
- **Emmy Klieneberger (1941):** She described the existence of L forms of bacteria
- **Barbara McClintock:** She described the mobile genetic elements in bacteria called transposons
- **Walter Gilbert and Frederick Sanger** were the first to develop (1977) the method of DNA sequencing
- **Karry B Mullis:** Discovered polymerase chain reaction (PCR) and was awarded Noble prize in 1993.

Discovery of Microorganisms

Several microorganisms were discovered by scientists (Table 1.1). The names of some of the bacteria are coined in the honor of the scientists who discovered them (Table 1.2).

Nobel Laureates

A number of scientists in medicine or physiology have been awarded Nobel prizes for their contributions in microbiology (Table 1.3).

Table 1.1: Discovery of important microorganisms

Discoverer	Organism
Ogston	*Staphylococcus aureus*
Neisser	*Neisseria gonorrhoeae*
Weichselbaum	*Neisseria meningitidis*
Loeffler	*Corynebacterium diphtheriae*
Frenkel	*Streptococcus pneumoniae*
Bruce	*Brucella melitensis*
Kitasato	*Clostridium tetani*
Hansen	*Mycobacterium leprae*
Yersin and Kitasato	*Yersinia pestis*
Schaudinn and Hoffman	*Treponema pallidum*
Daniel Carrion	*Bartonella bacilliformis*
d'Herelle	Bacteriophages
W.H. Welch	*Clostridium perfringens*
Anthony Epstein and Yvonne Barr	Epstein-Barr virus

Table 1.2: Bacteria named after the discoverers

Common name	Scientific name
Kleb-Loeffler bacillus	*Corynebacterium diphtheriae*
Preisz Nocard bacillus	*Corynebacterium pseudotuberculosis*
Koch Week bacillus	*Haemophilus aegyptius*
Pfeiffer's bacillus	*Haemophilus influenzae*
Whitmore's bacillus	*Burkholderia pseudomallei*
Battey bacillus	*Mycobacterium intracellulare*
Johne's bacillus	*Mycobacterium paratuberculosis*
Eaton's agent	*Mycoplasma pneumoniae*
Gaffky-Eberth bacillus	*Salmonella* Typhi

BACTERIAL TAXONOMY

Bacterial taxonomy comprises of three separate but interrelated important areas.

1. **Classification:** It refers to hierarchy based arrangement of bacteria into taxonomic groups or taxa (singular, taxon) on the basis of similarities or differences in their biochemical, physiological, genetic, and morphological properties.
2. **Nomenclature:** It refers to the naming of taxa according to their characteristics, by following the international rules.
3. **Identification:** It refers to the practical use of a classification scheme such as: (1) Identification of an unknown taxon by comparing with a defined and named taxon, (2) To isolate and identify the causative agent of a disease.

Table 1.3: Nobel laureates in medicine or physiology for their contributions in microbiology

Nobel laureate	Year	Research done
Emil A von Behring	1901	Development of antitoxin against diphtheria
Sir Ronald Ross	1902	Life cycle of malarial parasite in mosquitoes
Robert Koch	1905	Discovery of the causative agent of tuberculosis
Charles LA Laveran	1907	Discovery of malarial parasite in unstained preparation of blood
Paul Ehrlich and Elie Metchnikoff	1908	Discovered selective theory of antibody formation
Charles Richet	1913	Discovered anaphylaxis
Jules Bordet	1919	Discovered complement and developed complement fixation test
Karl Landsteiner	1930	Described ABO blood group
Sir Alexander Fleming	1945	Discovery of penicillin
F Enders, FC Robbins, TH Weller	1954	Cultivation of polio viruses in tissue culture
J Lederberg and EL Tatum	1958	Discovery of conjugation in bacteria
Sir M Burnet and Sir PB Medawar	1960	Postulated immunological tolerance
Watson and Crick	1962	Discovered double helix structure of DNA
Peyton Rous	1966	Discovered viral oncogenesis
Holley, Khurana and Nirenberg	1968	Discovered genetic code
BS Blumberg	1976	Discovered Australia antigen (HBsAg)
Rosalyn Yallow	1977	Developed radioimmunoassay
B Benacerraf, F Dausset and G Snell	1980	Discovered HLA antigen
Barbara McClintoch	1983	Discovered mobile genetic elements (transposon)
Georges Kohler	1984	Developed hybridoma technology for monoclonal antibodies
Niels Jerne		Postulated idiotype network hypothesis (Jerne hypothesis)
S Tonegawa	1987	Elucidated the nature of antibody diversity
Kary B Mullis	1993	Invented polymerase chain reaction
Stanley B Prusiner	1997	Described Prions
J Robin Warren and Barry J Marshal	2005	Discovery of *Helicobacter pylori* and its role in peptic ulcer disease
Luc Montagnier and F Barre-Sinoussi	2008	Discovery of human immunodeficiency virus (HIV)
Harald zur Hausen		Human papilloma viruses causing cervical cancer
Bruce A Beutler and Jules A Hoffmann	2011	For their discoveries concerning the activation of innate immunity
Ralph M Steinman		For his discovery of dendritic cell and its role in adaptive immunity
Sir John B Gurdon and S Yamanaka	2012	For the discovery that 'mature cells can be reprogrammed to become pluripotent
William C Campbell	2015	For discovering anti-parasitic effect of ivermectin in filariasis
Youyou Tu	2015	For discovering artemisinin, a novel drug used for malaria

BACTERIAL CLASSIFICATION

The most recent taxonomic classification of bacteria is based on Cavalier and Smith's six kingdoms classification (1998). It is the most accepted classification at present, surpassed the previous five kingdom classification (Whittaker, 1969) and three domain classification (Woese,1990) (Table 1.4).

Cavalier and Smith's Classification

It is a molecular classification, which divides all living structures of the earth into six kingdoms—Bacteria, Protozoa, Chromista, Plantae, Fungi and Animalia.

Kingdom Bacteria is divided successively in decreasing order of hierarchy into phylum/division, class, order, suborder, family, tribe, genus and species. For example, the full taxonomical position of *Escherichia coli* is given in Table 1.5.

Principle Used to Classify Bacteria

There is no universally accepted principle to classify bacteria. There are mainly three approaches: phylogenetic, Adansonian and molecular.

Phylogenetic Classification

This is a hierarchical classification representing a branching tree-like arrangement; one characteristic (or trait) is being employed for division at each node of the tree (Fig. 1.2).

- This system is called phylogenetic because it implies an evolutionary arrangement of species

Table 1.4: Taxonomic classification of living beings

Linnaeus 1735	Haeckel 1866	Chatton 1925	Copeland 1938	Whittaker 1969	Woese et al. 1990	Cavalier-Smith 1998
2 Kingdoms	3 Kingdoms	2 Empires	4 Kingdoms	5 Kingdoms	3 Domains	6 Kingdoms
		Prokaryota	Monera	Monera	Bacteria Archaea	Bacteria
(not treated)	Protista		Protista	Protista		Protozoa Chromista
Vegetabilia	Plantae	Eukaryota	Plantae	Plantae Fungi	Eucarya	Plantae Fungi
Animalia	Animalia		Animalia	Animalia		Animalia

Table 1.5: Taxonomical position of *Escherichia coli*

Taxa	Ends with suffix	Taxonomic name
Kingdom	-om	Bacteria
Phylum		Proteobacteria
Class	-ia	Gammaproteobacteria
Subclass	-idae	
Order	-ales	Enterobacteriales
Suborder	-ineae	
Family	-aceae	Enterobacteriaceae
Subfamily	-oideae	
Tribe	-eae	Escherichieae
Subtribe	-inae	
Genus		*Escherichia*
Species		*E. coli*

- Here, the characteristics are arbitrarily given special weightage. Depending on the characteristic so chosen, the classification would give different patterns
- The characteristics which are given importance depend upon various properties of the organisms such as:
  - Morphology of bacteria—cocci or bacilli
  - Staining property such as gram-positive and gram-negative
  - Cultural characteristics such as lactose fermenting and non-lactose fermenting colonies
  - Biochemical reactions, e.g. coagulase positive *Staphylococcus* and coagulase negative *Staphylococcus*
  - Antigenic structure, e.g. antigenic structure of somatic antigen present in bacterial cell wall.
- Though, this classification is a **convenient and user friendly** method, it is not a perfect method. Because the weighted characters used may not be valid all the time for a given bacterium. For example, fermentation of lactose though is an important property to classify family Enterobacteriaceae; is not a permanent trait. In due course of the time, bacteria may lose or gain the property to ferment lactose.

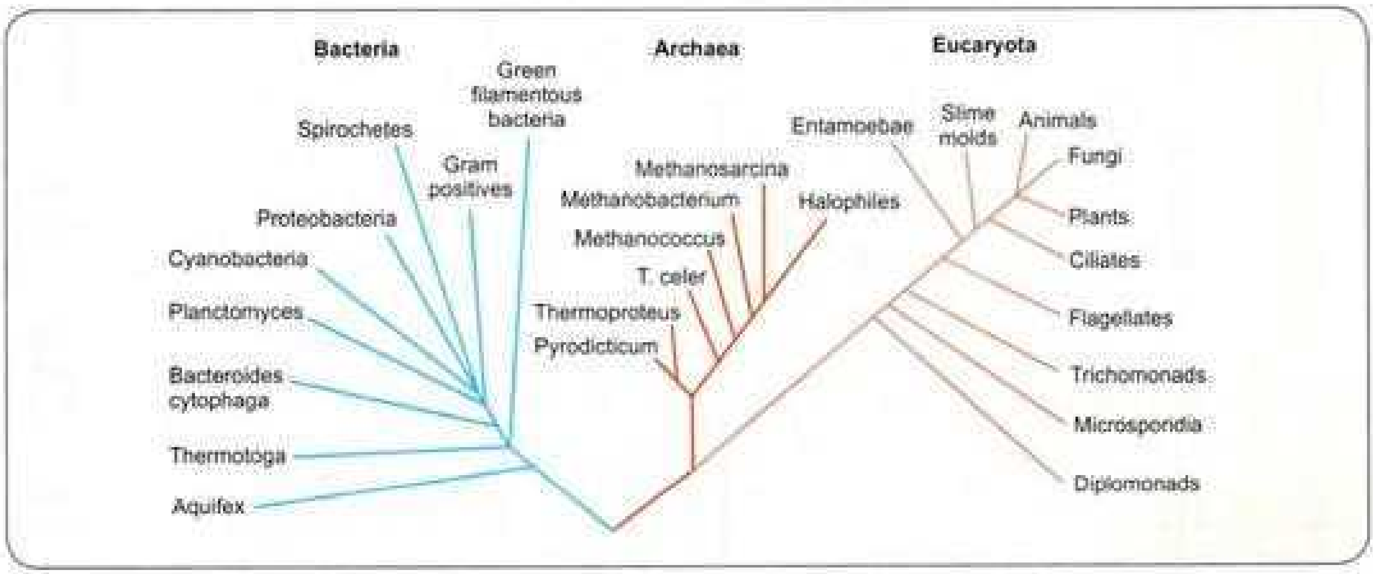


Fig. 1.2: Phylogenetic tree of classifying living structures based on weighted characters
Concept adapted and modified from Jawetz, Melnick, & Adelberg's Medical Microbiology, McGraw-Hill Education (*with permission*).

Adansonian Classification

To avoid the use of weighted characteristics, Michel Adanson proposed another method (1774) that classifies organisms based on giving equal weight to every character of the organism.

- This is also called phenetic classification. It has its greatest application in numeric taxonomy
- Numerical taxonomy: The concept was first developed by Robert R Sokal and Peter HA Sneath in 1963
- With the advent of computer facilities, the principle of phenetic classification has been extended further so that very large numbers of characters of several organisms can be compared at the same time
- They have created a taxonomic system by using numeric algorithms like cluster analysis rather than using subjective evaluation of their properties which are arbitrarily given special weightage.

Molecular Classification

It is based on the degree of genetic relatedness of different organisms. Guanine + cytosine (G + C) content of bacteria is estimated after extracting DNA from pure bacterial culture. The nucleotide base composition and the base ratio vary widely among different groups of microorganisms, but for any one particular species, it is constant.

The recent change in taxonomic name of medically-important microorganisms has been depicted in Table 1.6.

NOMENCLATURE

Nomenclature is the branch of taxonomy, that is concerned with designating scientific names to taxa, based on a particular classification scheme and in accordance with agreed international rules and conventions.

- Bacterial nomenclature also follows the same rules as proposed by Swedish botanist **Carolus Linnaeus** who invented the modern system of binomial nomenclature
- Scientific names for taxonomic levels above genus are always capitalized but not italicized; for example, Phylum Proteobacteria
- In binomial nomenclature system, the scientific name of bacteria comprises of a genus name (starts with a capital letter) and species name. Both genus and species should be written in *italic* or are underlined; e.g. *Staphylococcus aureus* or Staphylococcus aureus
- The genus (plural: genera) is usually a Latin noun whereas the species refers to a defined taxon of organisms within a particular genus.
- The genus and species are coined based on some property of the bacteria; for example,
  - *Staphylococcus aureus* is named after their arrangement in cluster (*Staphyle* means bunch of grapes) and type of pigmentation they produce (*aureus* meaning golden yellow)
  - *Neisseria meningitidis* is named after—the discoverer (U. Neisser) and the disease it causes (meningitis)
  - *Brucella suis* and *Brucella melitensis* (named after the discoverer (*Brucella* from David Bruce) and the animal host (*suis* meaning pig) and the place of discovery (*melitensis* from Malta, Europe).
- **Typing:** The species can also be classified further by various typing methods as described in Chapter 5.

Type Cultures

There are many international reference laboratories which are designated as type culture reference centers.

- They maintain the representative cultures of the established species, which show all the standard characteristics of the original strain
- The strains isolated in the laboratories are compared using the standard strains supplied by these type culture centers
- The original cultures of any new species described are deposited in type collection centers
- The two most important type collection centers of the world are:
  - **ATCC** (American Type Culture Collection), USA
  - **NCTC** (National Collection of Type Cultures), UK.

Table 1.6: Taxonomic name changes of medically-important microorganisms

Old name	New name
Bacteria	
Clostridium difficile	*Clostridioides difficile*
Haemophilus aphrophilus	*Aggregatibacter aphrophilus*
Haemophilus paraphrophilus	*Aggregatibacter paraphrophilus*
Enterobacter aerogenes	*Klebsiella aerogenes*
Propionibacterium acnes	*Cutibacterium acnes*
Fungi	
Absidia corymbifera	*Lichtheimia corymbifera*
Madurella grisea	*Trematosphaeria grisea*
Penicillium marneffei	*Talaromyces marneffei*
Geotrichum candidum	*Dipodascus geotrichum*
Rhizopus oryzae	*Rhizopus arrhizus*
Parasites	
Isospora belli	*Cystoisospora belli*
Balantidium coli	*Neobalantidium coli*

EXPECTED QUESTIONS

I. **Write short notes on:**
   1. Bacterial taxonomy.
   2. Contributions of Louis Pasteur in the field of microbiology.
   3. Contributions of Robert Koch in the field of microbiology.

Morphology and Physiology of Bacteria

2 CHAPTER

Chapter Preview

- Classification of microorganisms
- Microscopy
- Staining techniques
- Morphology of bacteria
    - Bacterial cell wall
    - Cell wall appendages and other structures
- Physiology of bacteria
    - Bacterial growth and nutrition
    - Bacterial metabolism

CLASSIFICATION OF MICROORGANISMS

Microorganisms are grouped under both prokaryotes and eukaryotes.

- Bacteria and blue green algae are placed under prokaryotes. They have a primitive nucleus, and other properties of a prokaryotic cell (Table 2.1)
- Whereas other algae, fungi and parasites (protozoa and helminths) belong to eukaryotes; having a well-defined nucleus and various eukaryotic cellular organelles
- Viruses are considered neither prokaryotes nor eukaryotes because they lack the characteristics of living things, except the ability to replicate.

Size of the microorganisms

Microorganisms are extremely small. The size of the bacteria is expressed in micrometers (1μm = 10^{-3} mm) whereas viruses are measured in nanometers (1nm = 10^{-3}μm).

Most of the bacteria of medical importance generally measure 0.2-1.5 μm in diameter and about 3-5 μm in length, while the majority of the human pathogenic viruses range 20-300 nm in diameter.

Because of the small size, microorganisms cannot be seen distinctly with the unaided eye but need a microscope for their visualization. Most bacteria can be observed by light microscope, whereas viruses need an electron microscope. Hence, it is important to understand how the microscope works and the method of specimen preparation (staining techniques) for examination.

Table 2.1: Characteristics of prokaryotes and eukaryotes

Characteristics	Prokaryotes	Eukaryotes
Major groups	Bacteria, blue green algae	Fungi, parasites, other algae, plants and animals
Nucleus	Diffuse	Well defined
Nuclear membrane	Absent	Present
Nucleolus	Absent	Present
Ribonucleoprotein	Absent	Present
Cell division	Binary fission	Mitosis, meiosis
Chromosome	One, circular	Many, linear
Extrachromosomal DNA	Found in plasmid	Found in mitochondria
Cell membrane	Does not contain sterols except in *Mycoplasma*	Contain sterols
Cellular organelles like mitochondria, etc.	Absent (except ribosome)	Present
Ribosome	70S	80S
Site of respiration	Mesosome	Mitochondria
Pinocytosis	Absent	Present

Abbreviation: S, Svedberg unit.

MICROSCOPY

The following types of microscopes are in use now.

- Bright-field or light microscope
- Dark field (or dark ground) microscope
- Phase contrast microscope
- Fluorescence microscope
- Electron microscope.

Properties of a microscope

A good microscope should have at least three properties:

1. **Good resolution:** Resolution power refers to the ability to produce separate images of closely placed objects so that they can be distinguished as two separate entities. The resolution power of:
    - Unaided human eye is about 0.2 mm (200 μm)

- Light microscope is about 0.2 µm
- Electron microscope is about 0.5 nm.

Resolution depends on refractive index of the medium. Oil has a higher refractive index than air; hence, use of oil enhances the resolution power of a microscope.

2. **Good contrast:** This can further be improved by staining the specimen. When the stains bind to the cells, the contrast is increased.
3. **Good magnification:** This is achieved by use of lenses. There are two types of convex lenses used:
   - Ocular lens with a magnification power of 10x
   - Objective lens-scanning (4x), low power (10x), high power (40x) and oil immersion (100x).

   Total magnification of a field is the product of the magnification of objective lens and ocular lens:
   - Scanning field (40x)
   - Low power field (100x)
   - High power field (400x) and
   - Oil immersion field (1000x).

BRIGHT-FIELD OR LIGHT MICROSCOPE

The bright-field or light microscope forms a dark image against a brighter background, hence the name.

Structure

The parts of a bright-field microscope are divided into three groups (Fig. 2.1):

Mechanical Parts

- **Base:** It holds various parts of the microscope, such as the light source, the fine and coarse adjustment knobs
- **C-shaped arm:** It holds the microscope, and it connects the ocular lens to the objective lens
- **Mechanical stage:** The arm bears a stage with stage clips to hold the slides and the stage control knobs to move the slide during viewing. It has an aperture at the center that permits light to reach the object from the bottom.

Magnifying Parts

- **Ocular lens:** The arm contains an eyepiece that bears an ocular lens of 10x magnification power. Microscopes with two eye pieces are called as binocular microscopes
- **Objective lens:** The arm also contains a revolving nose piece that bears three to four objectives with lenses of differing magnifying power (4x,10x,40x and 100x).

Illuminating Parts

- **Condenser:** It is mounted beneath the stage which focuses a cone of light on the slide
- **Iris diaphragm:** It controls the light that passes through the condenser
- **Light source:** It may be a mirror or an electric bulb

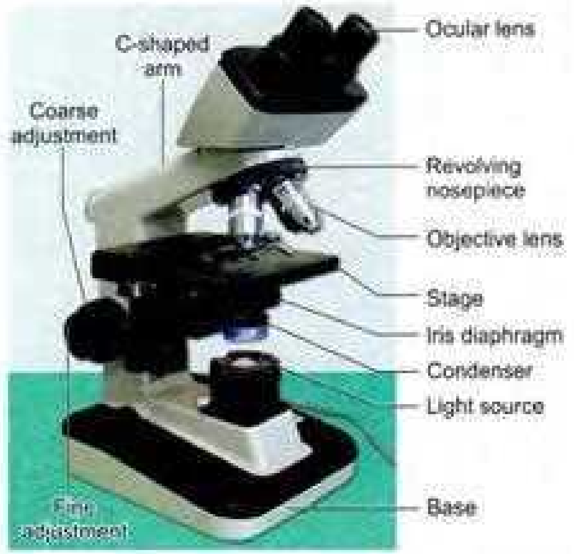


Fig. 2.1: Bright-field microscope
Source: Nikon Alphaphot (with permission).

- **Fine and coarse adjustment knobs:** They sharpen the image.

Working Principle

The rays emitted from the light source pass through the iris diaphragm and fall on the specimen. The light rays passing through the specimen are gathered by the objective and a magnified image is formed. This image is further magnified by the ocular lens to produce the final magnified virtual image (Fig. 2.2).

DARK FIELD MICROSCOPE

Principle

In dark field (or dark ground) microscope, the object appears bright against a dark background. This is made possible by use of a special dark field condenser (Fig. 2.2).

- The dark field condenser has a central opaque area that blocks light from entering the objective lens directly and has a peripheral annular hollow area which allows the light to pass through and focus on the specimen obliquely
- Only the light which is reflected by the specimen enters the objective lens whereas the unreflected light does not enter the objective. As a result, the specimen is brightly illuminated; but the background appears dark.

Applications

Dark field microscope is used to identify the living, unstained cells and thin bacteria like spirochetes which cannot be visualized by light microscopy.

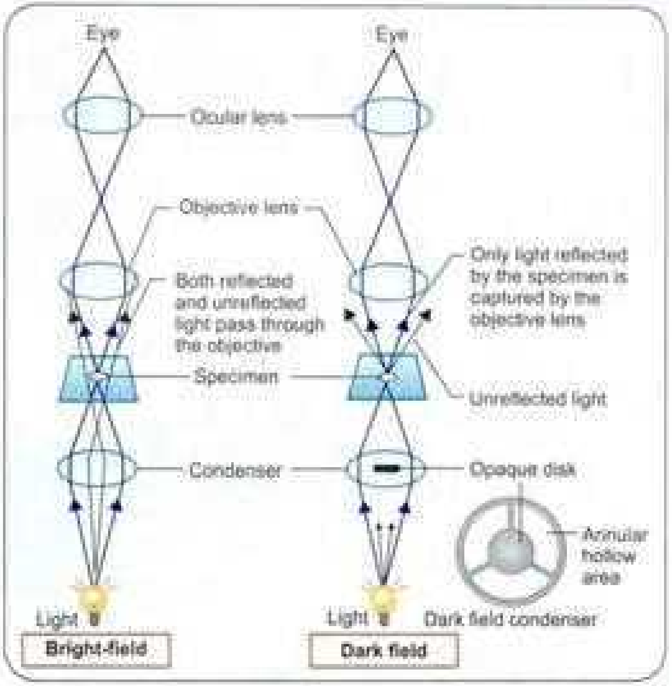


Fig. 2.2: Light pathways of bright-field and dark field microscopes

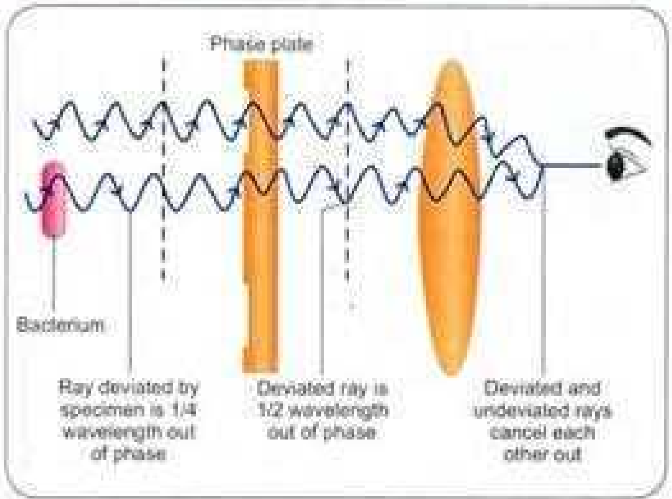


Fig. 2.3: Principle of phase contrast microscope

PHASE CONTRAST MICROSCOPE

As per its name, in phase contrast microscope the contrast is enhanced. This microscope visualizes the unstained living cells by creating difference in contrast between the cells and water. It converts slight differences in refractive index and cell density into easily detectable variations in light intensity. Contrast can also be enhanced by staining the specimen, but as staining kills the microbes, the properties of living cells cannot be studied.

Principle

The condenser is similar to that of dark field microscope, consists of an opaque central area with a thin transparent ring, which produces a hollow cone of light.

- As this cone of light passes through a cell, some light rays are bent due to variations in density and refractive index within the specimen and are retarded by about one-fourth of a wavelength (Fig. 2.3)
- The undeviated light rays strike a **phase ring** in the phase plate, (a special optical disk located in the objective), while the deviated rays miss the ring and pass through the rest of the plate
- The phase ring is constructed in such a way that the undeviated light passing through it is advanced by one-fourth of a wavelength, the deviated and undeviated waves will be about half wavelength out of the phase and will cancel each other when they come together to form an image (Fig. 2.3)
- The background, formed by undeviated light, is bright, while the unstained object appears dark and well-defined.

The light rays go through → condenser → specimen (e.g. bacteria) → phase ring → objective lens → ocular lens.

Applications

Phase contrast microscopy is especially useful for studying:

- Microbial motility
- Determining the shape of living cells, and
- Detecting bacterial components, such as endospores and inclusion bodies which become clearly visible because they have refractive indices markedly different from that of water.

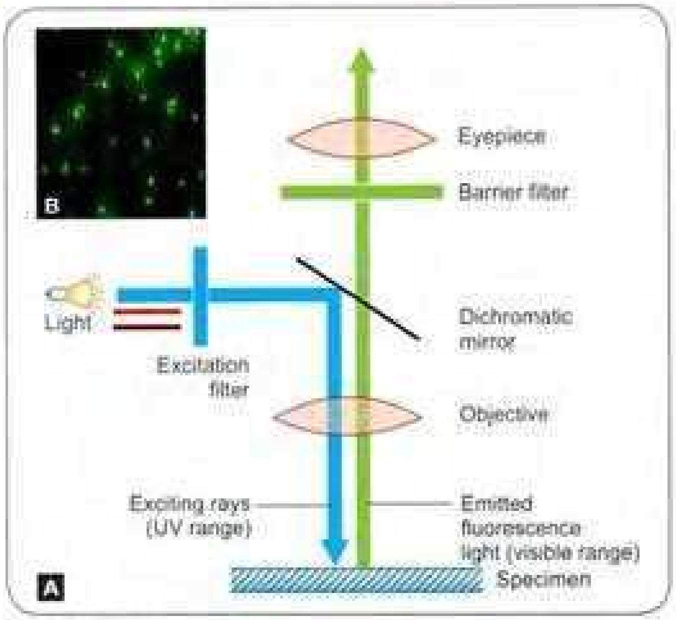
FLUORESCENCE MICROSCOPE

The "fluorescence microscope" refers to any microscope that uses fluorescence property to generate an image.

Principle

When fluorescent dyes are exposed to ultraviolet rays (UV) rays, they become excited and are said to fluoresce, i.e. they convert this invisible, short wavelength rays into light of longer wavelengths (i.e. visible light) (Fig. 2.4).

- The source of light may be a mercury lamp which emits rays that pass through an excitation filter
- The excitation filter is so designed that it allows only short wavelength UV light (about 400 nm, called as the exciting wavelength of light) to pass through; blocking all other long wavelength rays
- The exciting rays then get reflected by a dichromatic mirror in such a way that they fall on the specimen which is previously stained by fluorescent dye and focused under the microscope



Figs 2.4A and B: A. Principle of fluorescence microscope; **B.** Tubercle bacilli seen under fluorescence microscope

Source: Department of Microbiology, JIPMER, Puducherry (with permission).

- The fluorescent dye absorbs the exciting rays of short wavelength, gets activated and in turn emits rays of higher wavelength
- A barrier filter positioned after the objective lenses removes any remaining ultraviolet light, which could otherwise damage the viewer's eyes, or blue and violet light, which would reduce the image's contrast.

Applications

Epifluorescence microscope: It is the simplest form of fluorescence microscope, which has the following applications.

- **Auto fluorescence:** Certain microbes directly fluoresce when placed under UV lamp, e.g. *Cyclospora* (a protozoan parasite)
- **Microbes coated with fluorescent dye:** Certain microbes fluoresce when they are stained non-specifically by fluorochrome dyes
  - Acridine orange dye is used for the detection of malaria parasites by a method called as quantitative buffy coat (QBC) examination
  - Auramine phenol is used for the detection of tubercle bacilli (Fig. 2.4B).
- **Immunofluorescence:** It uses florescent dye tagged immunoglobulins to detect cell surface antigens or antibodies bound to cell surface antigens. There are two types- direct and indirect immunofluorescence test (described in detail in Chapter 12).

Table 2.2: Differences between light microscope and electron microscope

Features	Light microscope	Electron microscope
Highest practical magnification	About 1,000–1,500	Over 100,000
Best resolution	0.2 µm	0.5 nm
Radiation source	Visible light	Electron beam
Medium of travel	Air	High vacuum
Specimen mount	Glass slide	Metal grid (usually copper)
Type of lens	Glass	Electromagnet

Confocal microscope: It is an advanced design of fluorescence microscope, which uses optical sectioning to get better resolution of the fluorescent image.

ELECTRON MICROSCOPE

An electron microscope (EM) uses accelerated electrons as a source of illumination. Because the wavelength of electrons can be up to 100,000 times shorter than that of visible light photons, the EM has a much better resolving power than a light microscope; hence, it can reveal the details of flagella, fimbriae and intracellular structures of a cell. It was invented by German physicist **Ernst Ruska** in 1931. Differences between light microscope and EM are listed in Table 2.2. Electron microscopes are of two types:

1. Transmission electron microscope (TEM, most common type) (Fig. 2.5).
2. Scanning electron microscope (SEM).

Transmission Electron Microscope

Specimen Preparation

The specimen to be viewed under EM should be able to maintain its structure when it is bombarded with electrons. Hence, only very thin specimens (20–100 nm thickness) are suitable for EM. However, bacterial cells are thicker than this; hence, they need to be sliced into thin layers. To prepare the thin specimen, the following steps are needed:

- **Fixation:** Cells are fixed by using glutaraldehyde or osmium tetroxide for stabilization
- **Dehydration:** Specimen is then dehydrated with organic solvents (e.g. acetone or ethanol)
- **Embedding:** Specimen is embedded in plastic polymer and then, is hardened to form a solid block. Most plastic polymers are water insoluble; hence, complete dehydration of specimen is must before embedding
- **Slicing:** Specimen is then cut into thin slices by an ultramicrotome knife. Such thin slices of the specimen are mounted on a metal slide (copper).

Fig. 2.5: Transmission electron microscope (parts)

Source: David J Morgan/Wikipedia (with permission).

Electron Pathway

Electrons are generated by electron gun, which travel in high speed. The medium of travel in EM should be a fully vacuum path because in air path, electrons can get deflected by collisions with air molecules.

- Electron pass through a magnetic condenser and then bombard on the thin sliced specimen mounted on the copper slide
- The specimen scatters electrons passing through it, and then the electron beam is focused by magnetic lenses (objective lens followed by projector lenses) to form an enlarged, visible image of the specimen on a fluorescent screen (Fig. 2.6)

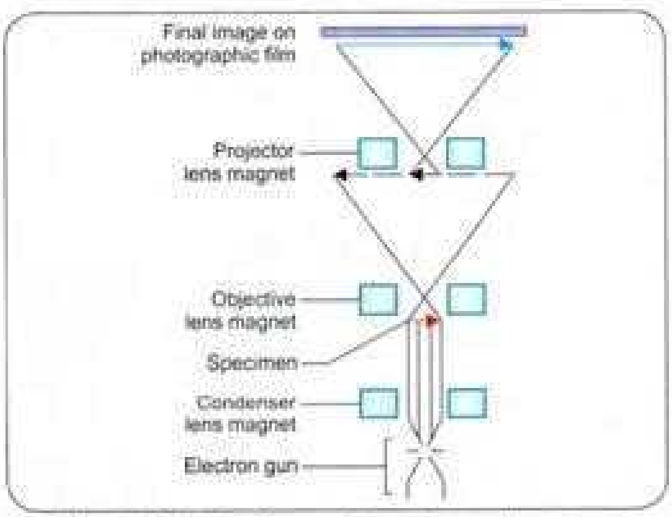


Fig. 2.6: Principle of transmission electron microscope

- A denser region in the specimen scatters more electrons and therefore appears darker in the image since fewer electrons strike that area of the screen. In contrast, electron-transparent regions are brighter.

Measures to Increase the Contrast of EM

- **Staining:** Like light microscopy, in EM also, the specimens can be stained. Here, the stains used are solutions of heavy metal salts like lead citrate and uranyl acetate which bind to the cell structures and make them more electron opaque, thus increasing the contrast in the material
- **Negative staining:** The specimen is spread out in a thin film with heavy metals like phosphotungstic acid or uranyl acetate
  - Heavy metals do not penetrate the specimen but render the background dark, whereas the specimen appears bright in photographs
  - Negative staining is an excellent method to study the structure of viruses, bacterial gas vacuoles, and other similar material.
- **Shadowing:** Specimen is coated with a thin film of platinum or other heavy metal at 45° angle so that the metal strikes the microorganism on only one side
  - The area coated with metal scatters electrons and appears light in photographs, whereas the uncoated side and the shadow region created by the object appears dark
  - This technique is particularly useful in studying virus morphology, bacterial flagella, and plasmids.

Freeze-etching Technique

It is an alternate method for specimen preparation to disclose the shape of organelles within the microorganisms.

- Cells are rapidly frozen in liquid nitrogen and then warmed to –100°C in a vacuum chamber. This makes the cells weaker so that the cells can be fractured by a knife exposing the internal organelles. The knife should be precooled with liquid nitrogen (–196°C)
- **Sublimation:** The specimen is left in high vacuum for a minute or more so that some of the ice can sublimate away and uncover more structural details
- Finally, the exposed surfaces are shadowed and coated with layers of **platinum and carbon** to form a replica of the surface
- After the specimen has been removed chemically, this replica is studied in the TEM, which provides a detailed, **three-dimensional** view of intracellular structure
- In freeze-etching, the danger of artefacts is minimal as the cells are frozen quickly rather than subjected to chemical fixation as done in conventional specimen preparation.

Scanning Electron Microscope

Scanning electron microscope (SEM) has been used to examine the surfaces of microorganisms in great detail. It has a resolution of 7 nm or less. The SEM differs from TEM, in producing an image from electrons emitted by an object's surface rather than from transmitted electrons.

STAINING TECHNIQUES

Structural details of bacteria cannot be seen under light microscope due to lack of contrast. Hence, it is necessary to use staining methods to produce color contrast and thereby increase the visibility. Before staining, the fixation of the smear to the slide is done.

Fixation

Fixation is the process by which the internal and external structures of cells are preserved and fixed in position. It also inactivates the enzymes that might disrupt cell morphology. It toughens cell structure so that they do not change during staining. It kills and fixes the cells on to the slide.

There are two types of fixation as follows:

- **Heat fixation:** It is usually done for **bacterial smears** by gently flame heating an air-dried film of bacteria. This adequately preserves overall morphology but not structures within the cells
- **Chemical fixation:** It can be done using ethanol, acetic acid, mercuric chloride, formaldehyde, methanol and glutaraldehyde. They are used to protect the fine internal structure of the cells. This is useful for examination of **blood smears**.

The fixed smear is stained by appropriate staining technique.

COMMON STAINING TECHNIQUES USED IN MICROBIOLOGY

- **Simple stain:** Basic dyes, such as methylene blue or basic fuchsin are used as simple stains. They provide the color contrast, but impart the same color to all the bacteria in a smear
- **Negative staining:** A drop of bacterial suspension is mixed with dyes, such as India ink or nigrosin. The background gets stained black where as unstained bacterial/yeast capsule stand out in contrast. This is very useful in the demonstration of bacterial/yeast capsules which do not take up simple stains
- **Impregnation methods:** Bacterial cells and structures that are too thin to be seen under the light microscope, are thickened by impregnation of silver salts on their surface to make them visible, e.g. for demonstration of bacterial flagella and spirochetes
- **Differential stain:** Here, two stains are used which impart different colors to different bacteria or bacterial structures, which help in differentiating bacteria. The most commonly employed differential stains are:
  - **Gram stain:** It differentiates bacteria into gram-positive and gram-negative groups
  - **Acid-fast stain:** It differentiates bacteria into acid-fast and non acid-fast groups
  - **Albert stain:** It differentiates bacteria having meta-chromatic granules from other bacteria that do not have them.

GRAM STAIN

This staining technique was originally developed by Hans Christian Gram (1884). Even after more than 130 years of its discovery and even in the presence of newer modern diagnostic facilities, still Gram stain remains the most widely used test in diagnostic bacteriology.

Procedure (Fig. 2.7)

- **Fixation:** The smear made on a slide from bacterial culture or specimen, is air dried and then heat fixed
- **Step 1 (Primary stain):** The smear is stained with pararosaniline dyes such as crystal violet (or gentian violet or methyl violet) for one minute. Then the slide is rinsed with water. Crystal violet stains all the bacteria violet in color (irrespective of whether they are gram-positive or gram-negative)
- **Step 2 (Mordant):** Gram's iodine (dilute solution of iodine) is poured over the slide for one minute. Then the slide is rinsed with water. Gram's iodine acts as a mordant

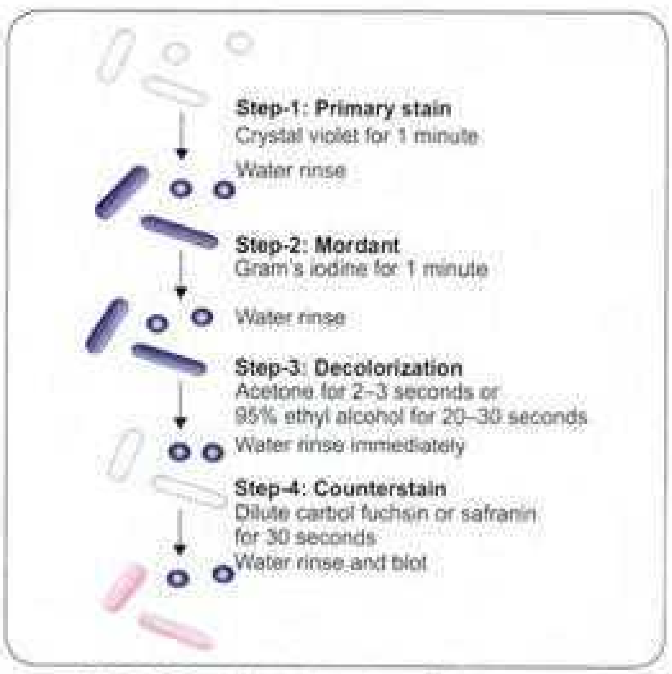


Fig. 2.7: Principle and procedure of Gram staining

- **Step 3 (Decolorization):** Next step is pouring of few drops of decolorizer to the smear: e.g. acetone (for 1-2 sec) or ethyl alcohol (20-30 sec) or acetone alcohol (for 10 sec) or iodine acetone. Slide is immediately rinsed with water. Decolorizer removes the primary stain from gram-negative bacteria while the gram-positive bacteria retain the primary stain

Note: Decolorization is the most crucial step of Gram stain. If the decolorizer is poured for more time, even gram-positive bacteria loose color **(over decolorization)** and if poured for less time, the gram-negative bacteria do not lose the color of primary stain properly **(under decolorization)**.

- **Step 4 (Counter stain):** Secondary stains such as safranin or dilute carbol fuchsin is added for 30 seconds. It imparts pink or red color to the gram-negative bacteria. Alternatively, neutral red may also be used as counter stain, especially for gonococci. The slide is rinsed in tap water, dried, and then examined under oil immersion objective.

Interpretation of Gram Stain

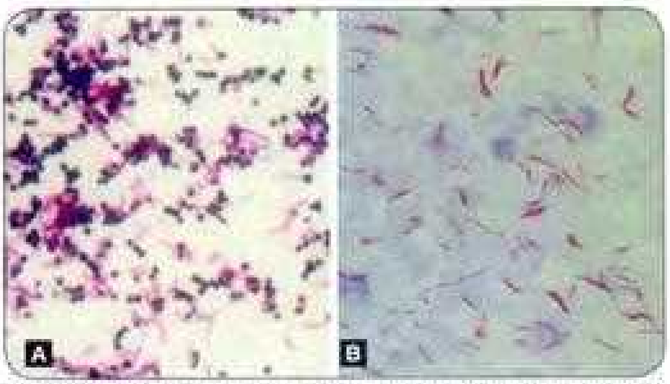
Smear is examined under oil immersion objective (Fig. 2.8A):

- Gram-positive bacteria resist decolorization and retain the color of primary stain i.e. violet
- Gram-negative bacteria are decolorized and, therefore, take counterstain and appear pink.

Principle of Gram Staining

Though the exact mechanism is not understood, the following theories have been put forward.

- **pH theory:** Cytoplasm of gram-positive bacteria is more acidic, hence, can retain the basic dye (e.g. crystal violet) for longer time. **Iodine** serves as mordant, i.e. it combines with the primary stain to form a dye-iodine complex which gets retained inside the cell



Figs 2.8A and B: A. Gram staining demonstrating violet-colored gram-positive cocci in clusters and pink colored gram-negative bacilli in scattered arrangement; **B.** Acid-fast staining shows long slender straight or slightly curved beaded red acid-fast bacilli.

Source: A. Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry; B. Department of Microbiology, JIPMER, Puducherry (with permission).

- **Cell wall theory:** This is believed to be the most important postulate to describe the mechanism of Gram stain
  - Gram-positive cell wall has a thick peptidoglycan layer (50-100 layers thick), with tight cross linkages
  - The peptidoglycan itself is not stained; instead, it seems to act as a permeability barrier preventing loss of crystal violet. More so, alcohol is thought to shrink the pores of the thick peptidoglycan
  - Gram-negative cell wall is more permeable thus allowing the out flow of crystal violet easily. This is attributed to:
    - The thin peptidoglycan layer in gram-negative cell wall which is not tightly cross linked
    - Presence of lipopolysaccharide layer in the cell wall of gram-negative bacteria, which gets disrupted easily by the action of acetone or alcohol; thus allowing the primary stain to come out of the cytoplasm
    - After mordanting with Gram's iodine, bigger dye-iodine complexes are formed in the cytoplasm. Following decolorization, as more lipid content in gram-negative bacterial cell wall gets dissolved leading to formation of larger pores through which the dye-iodine complexes escape. Due to less lipid in gram-positive bacterial cell wall smaller pores are formed which do not allow the dye-iodine complexes to escape.

Modifications of Gram Staining

There are few minor modifications of Gram stain which vary slightly from the method described earlier.

- **Kopeloff and Beerman's modification:** Primary stain and counter stain used are methyl violet and basic fuchsin respectively
- **Jensen's modification:** This method involves use of absolute alcohol as decolorizer and neutral red as counter stain. It is useful for meningococci and gonococci
- **Weigert's modification:** This modification is useful for staining tissue sections. Here, aniline-xylol is used as a decolorizer
- **Preston and Morrell's modification:** Here, iodine-acetone is used as decolorizer.

Uses of Gram Stain

- **To differentiate bacteria into gram-positive and gram-negative:** It is the first step towards identification of bacteria
- **For identification:** Gram staining from bacterial culture gives an idea to put the corresponding biochemical tests for further identification of bacteria
- **To start empirical treatment:** Gram stain from specimen gives a preliminary clue about the bacteria present (based

Contd...

Contd...

on the shape and Gram staining property of the bacteria) so that the empirical treatment with broad spectrum antibiotics can be started early before the culture report is available

- **For fastidious organisms**, such as *Haemophilus* which takes time to grow in culture; Gram stain helps in early presumptive identification
- **Anaerobic organisms**, such as *Clostridium*, which do not grow in routine culture. Hence, Gram stain gives a preliminary clue to put anaerobic culture
- **Yeasts:** In addition to stain the bacteria, Gram stain is useful for staining certain fungi such as *Candida* and *Cryptococcus* (appear gram-positive).

ACID-FAST STAIN

The acid-fast stain was discovered by Paul Ehrlich and subsequently modified by Ziehl and Neelsen. This staining is done to identify acid-fast organisms, such as *Mycobacterium tuberculosis* and others (Table 2.3). Acid-fastness is due to presence of mycolic acid in the cell wall.

Ziehl-Neelsen Technique (Hot Method)

Smear preparation

Smear measuring 2 × 3 cm in size is prepared in a new clean grease free scratch free slide from the yellow purulent portion of the sputum.

- The smear should neither be too thick nor too thin. When placed over a printed matter, the print should be readable through the smear
- Smear preparation should be done near a flame, as six inches around the flame is considered sterile zone (as heat coagulates the aerosols raised during smear preparation).

Heat Fixation

The smear is air dried for 15-30 minutes and then heat fixed by passing over the flame 3-5 times for 3-4 seconds each time. Coagulation of the proteineceous material in the sputum will facilitate fixing of the smear.

Table 2.3: Acid-fast organisms/structures and percentage of sulfuric acid suitable for staining

Acid-fast organisms /structures	Sulfuric acid (%) needed for decolorization
Mycobacterium tuberculosis	25%
Mycobacterium leprae	5%
Nocardia	1%
Acid-fast parasites such as *Cryptosporidium, Cyclospora, Cystoisospora*, Microsporidia, *Taenia saginata* (segments and eggs), hooklets of hydatid cyst and Eggs of *Schistosoma mansoni*	1%
Bacterial spore	0.25–0.5%
Sperm head	0.5–1%
Legionella micdadei	0.5–1%

Procedure

Step 1 (Primary stain)

Smear is poured with strong carbol fuchsin (1%) for 5 minutes. Intermittent heating is done by flaming the underneath of the slide until the vapours rise. Heating helps in better penetration of the stain.

- Care must be taken to ensure that the smear does not dry out, to counteract drying more solution of stain is added to the slide and the slide reheated
- Rinse the slide with tap water, until all free carbol fuchsin stain is washed away. At this point, the smear on the slide looks red in color.

Step 2 (Decolorization)

It is done with pouring 25% sulfuric acid over the slide and allowed it to stand for 2-4 minutes. The slide is gently rinsed with tap water and tilted to drain off the water.

- A properly decolorized slide appears light pink. If the slide is still red, sulfuric acid is reapplied for 1-3 minutes and then rinsed gently with tap water
- The back of the slide is wiped clean with a swab dipped in sulfuric acid.

Step 3 (Counter staining)

0.1% methylene blue is poured onto the slide and left for 30 seconds. Then the slide is rinsed gently with tap water and allowed to dry.

- The slide is examined under the binocular microscope using 40× lens to select the suitable area and then examined under oil immersion field (100×)
- Contaminated materials/slide should be discarded in jar containing 5% phenol.

Interpretation

Mycobacterium tuberculosis appears as long slender, straight or slightly curved and beaded, red colored acid-fast bacillus. Other non-acid fast organisms present in the smear and the background take up the counter stain and appear blue (Fig. 2.8B).

Modifications of Acid-Fast Staining

Hot method (Ziehl-Neelsen technique) is the most commonly done acid-fast staining technique. Other modifications are as follows:

- **Cold method (Kinyoun's method):** It is modification, where the intermittent heating is not required (described in Chapter 27)
- Acid-alcohol can be used as decolorizer alternatively
- Malachite green can be used as counter stain
- Concentration of sulfuric acid may vary depending on the acid-fastness of the structure to be demonstrated.

More the content of mycolic acid in the cell wall, more is the acid-fastness, hence more is the percentage of sulfuric acid required for decolorization (Table 2.3).

ALBERT STAIN

Albert stain is used to demonstrate the metachromatic granules of *Corynebacterium diphtheriae*.

Procedure

- Fixation: The smear is heat fixed
- Smear is covered with Albert I (Albert's stain) for 5 minutes, then is washed in water, and blotted dry
- Albert II (iodine solution) is added for 1 minute
- Slide is washed in water, blotted dry and examined under oil immersion field.

Interpretation

Corynebacterium diphtheriae appears as green colored bacilli arranged in Chinese letter or cuneiform pattern, with bluish black metachromatic granules at polar ends. (Refer Fig. no. 24.1C of Chapter 24). These can be differentiated from diphtheroids which do not show granules and are arranged in palisade pattern. However, certain bacteria, such as *Corynebacterium xerosis* and *Gardnerella vaginalis* also possess metachromatic granules.

MICROSCOPY OF BACTERIA IN LIVING STATE

Unstained (Wet) Preparations

Unstained preparations are examined mainly for checking bacterial motility (e.g. hanging drop and wet mount preparations) and for demonstration of spirochetes (e.g. dark field or phase contrast microscopy).

Vital Stains

Vital stains are capable of differentiating the living cells from dead cells. The live cells are capable of excluding specific dyes (due to their intact plasma membrane); whereas the dead cells take up the stain. The viability can be assessed by counting the percentage of total cells that do not take up the stain. Vital stains have greater applications in some diagnostic and surgical techniques.

- In **supravital staining**, living tissues that have been removed from an organism are stained (in vitro), whereas the **intravital staining** is done by injecting stain into the body (in vivo) and then the stained tissue is removed and examined
- Examples of vital stains are eosin, propidium iodide, trypan blue, erythrosine and neutral red.

MORPHOLOGY OF BACTERIA

SHAPE OF BACTERIA

Depending on their shape, bacteria are classified into:

- Cocci (singular coccus, from: kokkos, meaning berry)- are oval or spherical cells and
- Bacilli or rods (singular bacillus, meaning rod shaped).

Cocci are arranged in groups (clusters), pair or chains. Similarly, bacilli can be arranged in chain, pair, and some bacilli are curved, comma shaped, or cuneiform shaped (Table 2.4 and Fig. 2.9).

Both cocci and bacilli are further classified based on Gram staining property into (Table 2.4 and Fig. 2.9):

- Gram-positive cocci
- Gram-negative cocci
- Gram-positive bacilli
- Gram-negative bacilli.

However, there are some bacteria that are weakly Gram stained and hence need special stains for their demonstration, such as:

- Spirochetes (*Treponema* and *Leptospira*)—thin spirally coiled bacilli
- *Mycoplasma* (cell wall deficient free living bacteria)
- Rickettsiae and chlamydiae are obligate intracellular bacteria.

Bacterial cell anatomy comprises of the following structures (Fig. 2.10):

Table 2.4: Classification of bacteria depending on their morphology and Gram staining property

Bacteria	Example
Gram-positive cocci arranged in	
Cluster	*Staphylococcus*
Chain	*Streptococcus*
Pairs, lanceolate shaped	*Pneumococcus*
Pair or in short chain, spectacle shaped	*Enterococcus*
Tetrads	*Micrococcus*
Octate	*Sarcina*
Gram-negative cocci arranged in	
Pairs, lens shaped	Meningococcus
Pairs, kidney shaped	Gonococcus
Gram-positive bacilli arranged in	
Chain (bamboo stick appearance)	*Bacillus anthracis*
Chinese letter or cuneiform pattern	*Corynebacterium diphtheriae*
Palisade pattern	Diphtheroids
Branched and filamentous form	*Actinomyces* and *Nocardia*
Gram-negative bacilli arranged in	
Pleomorphic (various shapes)	*Haemophilus; Proteus*
Thumb print appearance	*Bordetella pertussis*
Comma shaped (fish in stream appearance)	*Vibrio cholerae*
Curved	*Campylobacter* (Gull-wing shaped) and *Helicobacter*
Chain	*Streptobacillus*
Spirally coiled, flexible	Spirochetes
Rigid spiral forms	*Spirillum*
Bacteria that lack cell wall	*Mycoplasma*

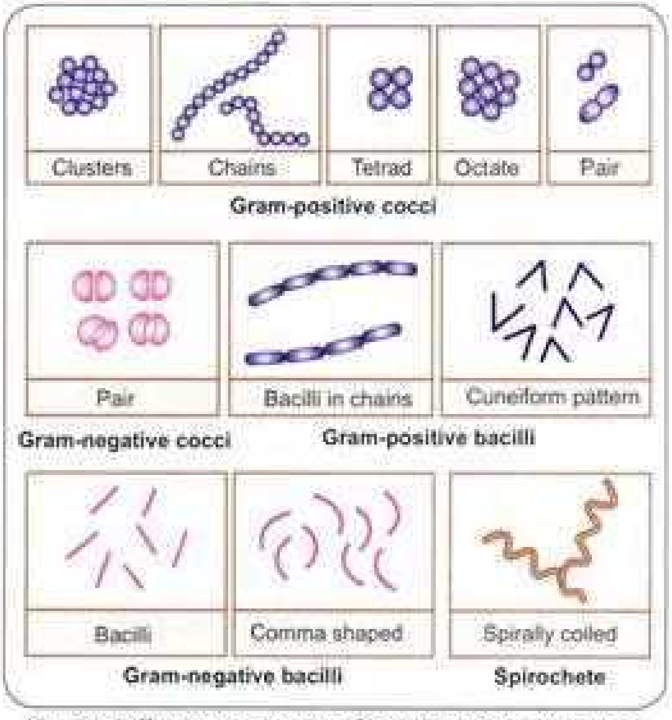


Fig. 2.9: Different morphology of bacteria and Gram-staining property

- The outer layer or the **envelope** of a bacterial cell consists of—(1) a rigid cell wall and (2) underlying plasma membrane
- The cytoplasm contains cytoplasmic inclusions (mesosomes, ribosomes, inclusion granules, vacuoles) and a diffuse nucleoid containing single circular chromosome
- Some bacteria may possess additional cell wall appendages such as capsule, flagella and fimbriae.

BACTERIAL CELL WALL

The cell wall is a tough and rigid structure, surrounding the bacterium. It is 10-25 nm in thickness and weighs about 20-25% of the dry weight of the cell.

The cell wall has following functions:

- It provides protection to the cell against osmotic lysis
- It confers rigidity upon bacteria due to presence of peptidoglycan layer in the cell wall
- It accounts for the shape of the cell
- It takes part in cell division
- The cell wall can protect a cell from toxic substances and is the site of action of several antibiotics
- Virulence factors-Bacterial cell wall contains certain virulence factors (e.g. endotoxin), which contribute to their pathogenicity
- Immunity: Antibody raised against specific cell wall antigens (e.g. antibody to LPS) may provide immunity against some bacterial infection.

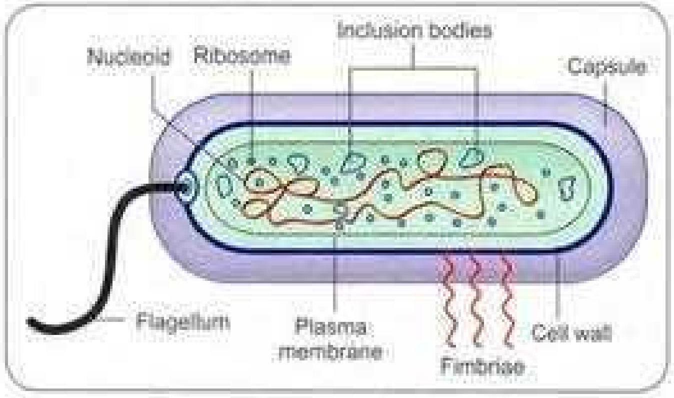


Fig. 2.10: Structure of bacterial cell

Gram-positive Cell Wall

Cell wall of gram-positive bacteria is simpler than that of gram-negative bacteria (Table 2.5).

Peptidoglycan

In gram-positive bacteria, the peptidoglycan layer is much thicker (50-100 layers thick, 16-80 nm) than gram-negative cell wall (Fig. 2.11).

- Each layer is a mucopeptide (murein) chain, composed of alternate units of N-acetyl muramic acid (NAM) and N-acetyl glucosamine (NAG) molecules; cross linked to each other via tetrapeptide side chains and pentaglycine bridges
- A tetrapeptide side chain ascended from NAM molecule is composed of L-alanine—D-glutamine—L-lysine—D-alanine
- The L-lysine of one tetrapeptide chain is covalently linked to the terminal D-alanine of the adjacent chain via a pentaglycine bridge (Fig. 2.12).

Teichoic Acid

Gram-positive cell wall contains significant amount of teichoic acid which is absent in gram-negative bacteria. They are polymers of glycerol or ribitol joined by phosphate

Table 2.5: Differences between gram-positive and gram-negative cell wall

Characters	Gram-positive cell wall	Gram-negative cell wall
Peptidoglycan layer	Thicker (15-80 nm)	Thinner (2 nm)
At third position of tetrapeptide side chain	L-Lysine present	Meso-diaminopimelic acid present
Pentaglycine bridge	Present	Absent
Lipid content	Nil or scanty (2-5%)	Present (15-20%)
Lipopolysaccharide	Absent	Present (**endotoxin**)
Teichoic acid	Present	Absent
Variety of amino acids	Few	Several
Aromatic amino acids	Absent	Present

groups. The functions of these molecules are still unclear, but they may be important in maintaining the structure of the cell wall. Teichoic acids are of two types:

1. **Cell wall teichoic acid:** It is covalently linked to NAM molecules of peptidoglycan.
2. **Lipoteichoic acid:** It is attached to lipid groups of cell membrane.

Gram-negative Cell Wall

Gram-negative cell wall is thinner and more complex than the gram-positive cell wall, comprises of the following components (Fig. 2.13 and Table 2.5).

Peptidoglycan Layer

It is very thin (1-2 layer, 2 nm thick), composed of a mucopeptide chain similar to that of gram-positive cell wall, and consists of alternate NAM and NAG molecules. However, it differs from the latter by (Fig. 2.14):

- Meso-diaminopimelic acid is present at third position of the tetrapeptide side chain ascended form NAM molecule (side chain is composed of L-alanine—D-glutamine-D—meso-diaminopimelic acid—D-alanine)
- The pentaglycine bridge is absent
- The tetrapeptide side chains are directly linked to each other by the covalent linkage between D-alanine of one chain with meso-diaminopimelic acid of the adjacent chain.

Outer Membrane

This is a phospholipid layer which lies outside the thin peptidoglycan layer; firmly attached to the latter by covalent linkage of membrane protein called Braun's lipoprotein.

- It serves as a protective barrier to the cell
- Outer membrane proteins (OMP) or porin proteins. They are the specialized proteins present in outer membrane. Three porin molecules cluster together and span the outer membrane to form a narrow channel through which molecules smaller than about 600-700 daltons can pass
- Larger molecules such as vitamin B must be transported across the outer membrane by specific carriers
- The outer membrane also prevents the loss of constituents such as periplasmic enzymes.

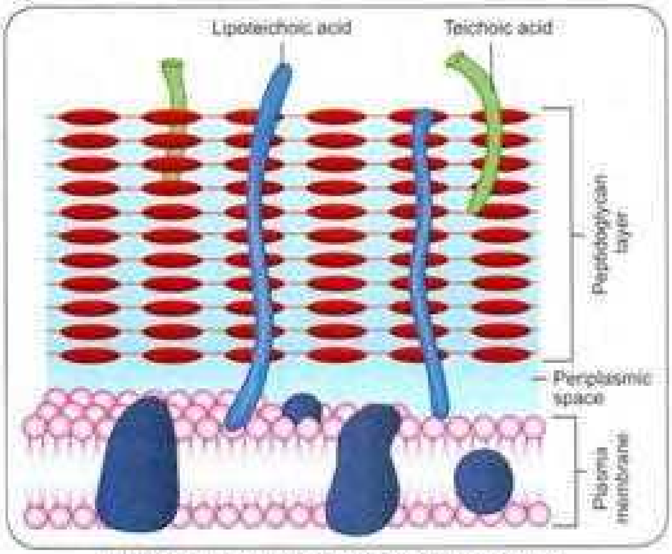


Fig. 2.11: Structure of gram-positive cell wall

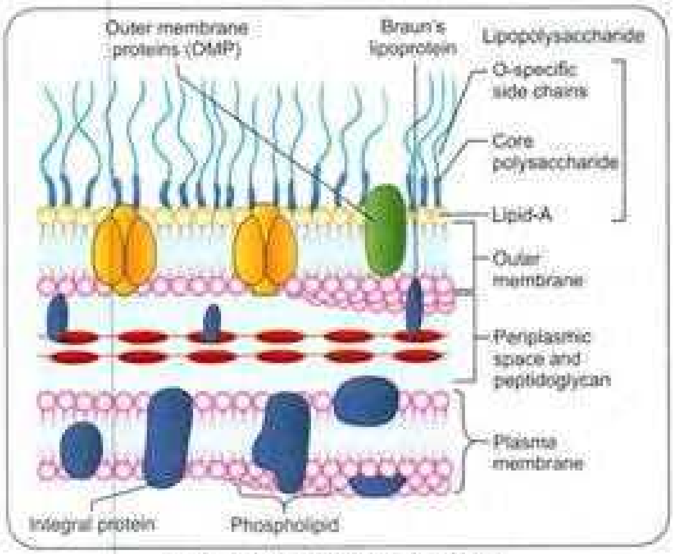


Fig. 2.13: Gram-negative cell wall

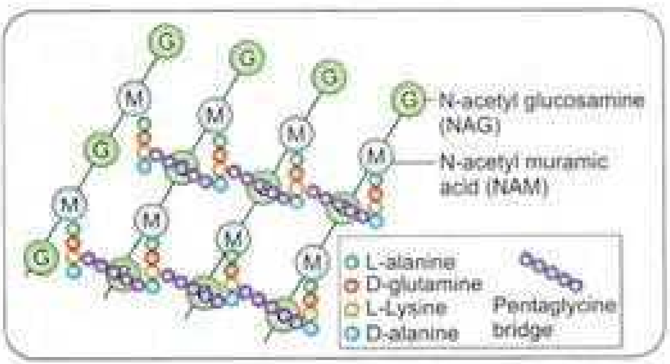


Fig. 2.12: Peptidoglycan layer of gram-positive cell wall

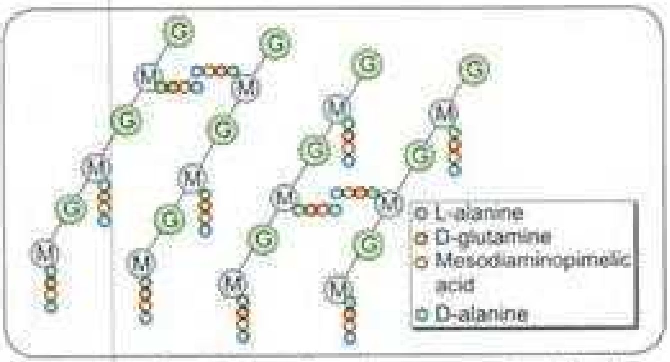


Fig. 2.14: Peptidoglycan layer of gram-negative cell wall

Lipopolysaccharide (LPS)

This layer is unique to gram-negative bacteria which is absent in gram-positives. It consists of three parts:
- **Lipid A or the endotoxin:** It has endotoxic activities, such as pyrogenicity, lethal effect, tissue necrosis, anti-complementary activity, B cell mitogenicity, adjuvant property and antitumor activity
  - It consists of two glucosamine sugar derivatives, each with three fatty acids and phosphate attached
  - It is buried in the outer membrane and the remainder of the LPS molecule projects from the surface.
- **Core polysaccharide:** It is projected from lipid A region. It is composed of 10–12 sugar moieties
- **O side chain (or O antigen):** It is a polysaccharide chain extending outwards from the core polysaccharide region. It is made up of several sugar moieties and it greatly varies in composition between bacterial strains. O antigen is a major surface antigen (called somatic antigen), induces antibody formation. It is also used for serotyping.

Periplasmic Space

It is the space between the inner cell membrane and outer membrane. It encompasses the peptidoglycan layer.

Demonstration of the Cell Wall

The cell wall cannot be seen by light microscope and does not stain with simple dyes. Demonstration of cell wall can be done by methods such as:
- Plasmolysis: When bacteria are placed in a hypertonic saline, shrinkage of the cytoplasm occurs, while cell wall retains original shape and size
- Microdissection
- Differential staining
- Reaction with specific antibody
- Electron microscopy.

CELL MEMBRANE

The plasma membrane is essential for the survival of the bacteria.
- **Fluid mosaic model** is the most widely accepted current model to describe the membrane structure
- It is 5–10 nm thick, composed of bilayered phospholipid in which several proteins are embedded, such as integral proteins and peripheral proteins (Fig. 2.15)
- It differs from eukaryotic membranes in **lacking sterols**, such as cholesterol (except in *Mycoplasma*). However, many bacterial membranes do contain pentacyclic sterol-like molecules called **hopanoids**
- **Carbohydrate:** Some carbohydrates are often attached to the outer surface of plasma membrane proteins.

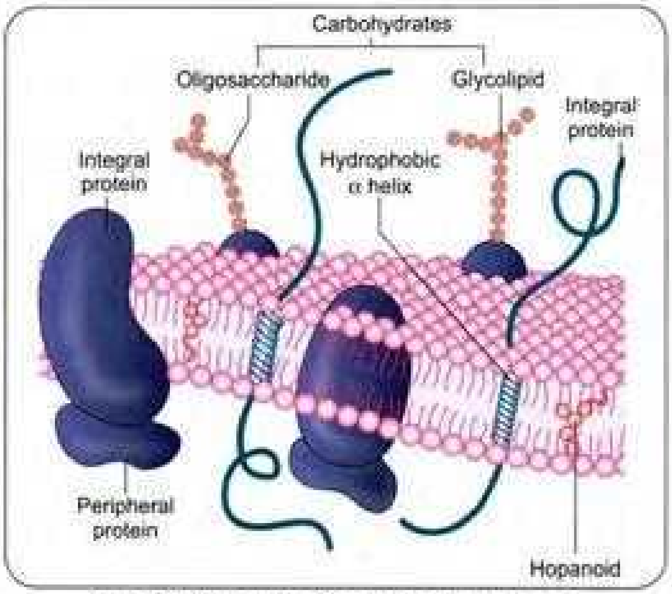


Fig. 2.15: Structure of bacterial cell membrane

Concept adapted and modified from Jawetz, Melnick, & Adelberg's Medical Microbiology; McGraw-Hill Education (with permission).

Functions

- It is a **semi permeable membrane** acting as an osmotic barrier; selectively allows particular ions and molecules to pass, either into or out of the cell, while preventing the movement of others
- **Transport system:** Proteins and enzymes present in cell membrane are involved in nutrient uptake, and waste excretion
- **Site for metabolic processes:** Bacterial cell membrane is the site of a variety of crucial metabolic processes such as: Respiration, the synthesis of lipids and cell wall, and probably chromosome segregation
- Special receptor molecules located in the membrane help the bacteria to detect and respond to chemicals in their surroundings.

CYTOPLASMIC MATRIX

Bacterial cytoplasm, unlike that of eukaryotes, lacks membrane-bound organelles. The cytoplasmic matrix is mainly composed of water (about 70% of bacterial mass is water) and is packed with ribosomes, storage granules called **inclusions** and cell membrane invaginations called **mesosomes**. They lack true cytoskeleton, but do have a cytoskeleton-like system of proteins. The plasma membrane and everything within it is called as **protoplast**.

Ribosomes

Ribosomes are the sites for protein synthesis. These are composed of rRNA and ribosomal proteins.

- Ribosomes are integrated with the mRNA to form the polysomes
- At this site, the genetic codons of the mRNA are translated into peptide sequences
- They are 10-20 nm size, with a sedimentation constant of 70 S (S for Svedberg units)
- Each 70 S unit consists of a 30 S and a 50 S subunits.

Intracytoplasmic Inclusions

They are the storage sites of nutrients/energy present in some bacteria. They are formed by the bacteria under nutritional deficiency conditions and disappear when the deficient nutrients are supplied. There are two types of inclusions:

1. **Organic inclusion bodies:** Examples include glycogen granules and polyhydroxyl butyrate granules.
2. **Inorganic inclusion bodies:** Examples include polymetaphosphate or volutin or metachromatic granules: They are found in certain bacteria, such as *Corynebacterium diphtheriae.*

Mesosomes

Mesosomes are invaginations of the plasma membrane in the shape of vesicles, tubules, or lamellae. They are generally more prominent in gram-positive bacteria.

- **Location:** Mesosomes often are found next to septa in dividing bacteria or sometimes seen attached to the bacterial chromosome
- **Function:** They are believed to be involved in:
  - Site of bacterial respiration: They possess respiratory enzyme and are analogous to mitochondria of eukaryotes
  - They may be involved in cell wall formation during division
  - They also probably play a role in chromosome replication and distribution to daughter cells.

Nucleoid

Bacteria do not have a true nucleus, but the genetic material is located in an irregularly shaped region called the nucleoid. There is no nuclear membrane or nucleolus.

- Bacteria possess a single haploid chromosome, comprising of super coiled circular double stranded DNA of 1 mm length. The bacterial DNA lacks basic proteins
- However, some bacteria have a linear chromosome and some have two chromosomes (e.g. *Vibrio cholerae*)
- Bacterial DNA divides by simple binary fission (described later in this Chapter)
- The nucleoid can be seen by electron microscopy or on staining with stain such as the Feulgen stain
- Bacteria also possess extrachromosomal DNA called **plasmids** (described in detail in Chapter 6).

CELL WALL APPENDAGES

Capsule and Slime Layer

Some bacteria possess a layer of amorphous viscid material lying outside the cell wall called **glycocalyx.**

- When the glycocalyx layer is well organized and not easily washed off, it is called **capsule** (Fig. 2.16)
- When the glycocalyx layer is in the form of diffuse, unorganized loose material that can be removed easily, it is called **slime layer** (Fig. 2.16).
  (Some bacteria may possess both capsule and slime layer, as in *Streptococcus salivarius).*

Examples

Most of the bacterial capsules are polysaccharide in nature (Table 2.6), except in *Bacillus anthracis* where it is polypeptide in nature. Capsule is also seen in fungi, e.g. *Cryptococcus neoformans.*

Function/Uses

The capsule has various functions as follows:

- **Contributes to bacterial virulence:**
  - Capsule protects the bacterium from phagocytosis
  - It can also prevent complement-mediated bacterial cell lysis
  - Prevent cell from drying out (desiccation)
  - It protects the bacterium from the action of lysozyme and bacteriophages

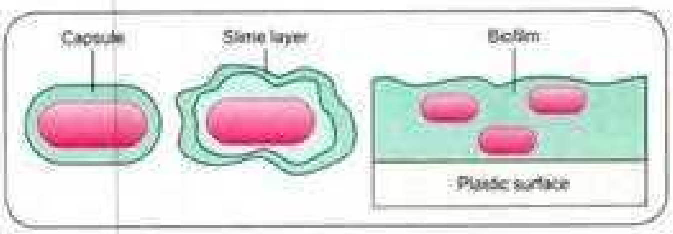


Fig. 2.16: Capsule, slime layer and biofilm

Table 2.6: Capsulated bacteria

Capsulated bacteria	Composition
Pneumococcus	Polysaccharide
Meningococcus	Polysaccharide
Haemophilus influenzae	Polysaccharide
Klebsiella pneumoniae	Polysaccharide
Pseudomonas aeruginosa	Polysaccharide
Bacteriodes fragilis	Polysaccharide
Bacillus anthracis	Polypeptide (glutamate)
Streptococcus pyogenes (some strains)	Hyaluronic acid
Capsulated fungus	
Cryptococcus neoformans	Polysaccharide

- Capsule of certain bacteria (e.g. *Bacteroides fragilis*) may be toxic to the host cells and induces abscess formation
- Biofilm formation and adhesion (see below):

Biofilm Formation

A biofilm is a living ecosystem made of millions of adherent bacterial cells embedded within a self-produced matrix of extracellular polymeric substance (i.e. the polysaccharide slime layer).

- Persistent biofilms containing pathogenic bacteria are capable of adherence to damaged tissues and plastic surfaces (e.g. medical devices, such as catheters and pacemakers)
- This is the first step in bacterial colonization and sometimes it leads to disease, e.g. prosthetic valve endocarditis and catheter related urinary tract infection (Fig. 2.16).

- **Source of nutrients and energy:** Capsules can be a source of nutrients and energy to microbes. *Streptococcus mutans*, which colonizes teeth, ferments the sugar in the capsule and so formed acid by-products contribute to the tooth decay
- **Capsules as vaccine:** Capsules of few bacteria are antigenic and anticapsular antibodies are protective in nature. Hence, capsular antigens of many bacteria are used as potential vaccine candidates. Capsular vaccines are available for bacteria, such as pneumococcus, meningococcus and *Haemophilus influenzae* serotype-b.

Demonstration of Capsule

Capsule can be detected by various methods as follows:

- **Negative staining** by India ink and nigrosin stain: Capsule appears as a clear refractile halo around the bacteria; where as both the bacteria and the background appear black (Fig. 52.21A)
- **M'Faydean capsule stain:** It is used for demonstration of capsule of *Bacillus anthracis* by using polychrome methylene blue stain (Fig. 25.3A)
- **Serological test:** Capsular material is antigenic and can be demonstrated by mixing it with a specific anti-capsular serum
  - Quellung reaction: Capsular serotypes of *Streptococcus pneumoniae* can be detected by adding antisera mixed with methylene blue. Capsule becomes swollen, refractile and delineated (Fig. 22.10E)
  - Capsular antigen: It can be detected in the sample (e.g. CSF) by latex agglutination test by using specific anticapsular antibodies coated on latex particles. This is available for pneumococcus, *Cryptococcus*, *Haemophilus influenzae* and meningococcus.

Flagella

Flagella are thread-like appendages, protruding from the cell wall, that confer motility to the bacteria (organs of locomotion). They measure 5–20 µm in length and 0.01–0.02 µm in thickness.

Arrangement of Flagella

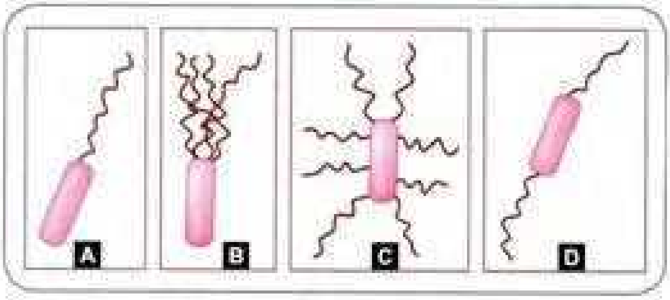
There are various patterns of arrangement of flagella with respect to the bacterial surface (Figs 2.17A to D):

- Monotrichous (single polar flagellum), e.g. *Vibrio cholerae*, *Pseudomonas* and *Campylobacter*
- Lophotrichous (multiple polar flagella), e.g. *Spirillum*
- Peritrichous (flagella distributed over the entire cell surface), e.g. *Salmonella* Typhi, *Escherichia coli*
- Amphitrichous (single flagellum at both the ends)—e.g. *Alcaligenes faecalis*.

Ultrastructure of Flagella

Electron microscope reveals that the bacterial flagellum is composed of three parts (Fig. 2.18).

1. **Filament:** It is the longest portion of the flagellum that extends from the cell surface to the tip. It is a hollow, rigid cylinder, made up of a single protein **flagellin**
2. The **basal body:** This is the portion of flagellum which is embedded in the cell. It is the most complex part of a flagellum, made up of 2–4 rings connected to a central rod
   - In most gram-negative bacteria, there are four rings named as:



Figs 2.17A to D: Types of bacterial flagellar arrangement. **A.** Monotrichous; **B.** Lophotrichous; **C.** Peritrichous; **D.** Amphitrichous

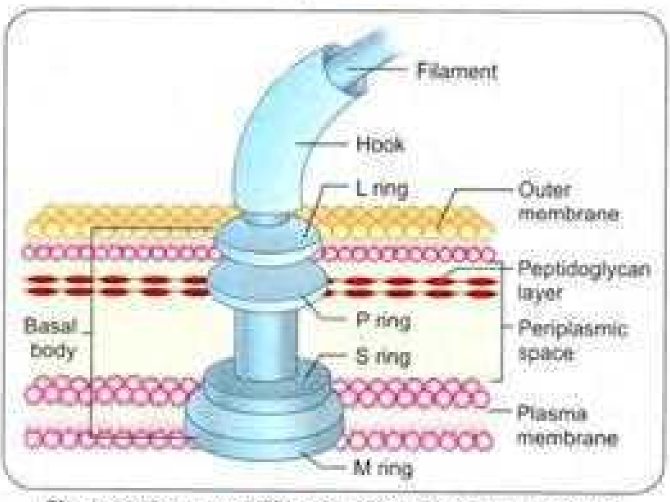


Fig. 2.18: Structure of flagella (gram-negative bacterium)

- The outer L and P rings associated with the LPS and peptidoglycan layers, respectively
- The inner S ring lies in periplasmic space and M ring is in contact with the plasma membrane.
- Gram-positive bacteria have only two basal body rings, an inner ring connected to the plasma membrane and an outer one probably attached to the peptidoglycan.

3. **Hook:** It is a short, curved flexible segment that links the filament to its basal body.

Detection of Flagella

Flagella can be demonstrated by:

- Direct demonstration of flagella
  - Tannic acid staining (Leifson's method and Ryu's method)
  - Electron microscopy.
- Indirect means by demonstrating the motility:
  - Craigie tube method
  - Hanging drop method
  - Semisolid medium, e.g. mannitol motility medium
  - Dark ground or phase contrast microscopy.

Bacterial Motility

Bacteria can produce characteristic type of motility which helps in their identification (Table 2.7).

Fimbriae or Pili

Pili (singular pilus) are short, fine, hair-like appendages that help in bacterial adhesion; hence called as the organ of adhesion. A special type of pili (called sex pilus) also exists which helps in conjugation.

- Though pili and fimbriae (singular fimbria) are interchangeably used, but in true sense fimbriae represent the type of pli that help in adhesion
- Pili are made up of protein called pilin
- They are antigenic; however, the antibodies against pilin antigens are not protective
- They are not related to motility and can be found both in motile as well as in nonmotile organisms.

Type of Pili

According to the functions; pili are of two types.

Table 2.7: Types of motility shown by different bacteria

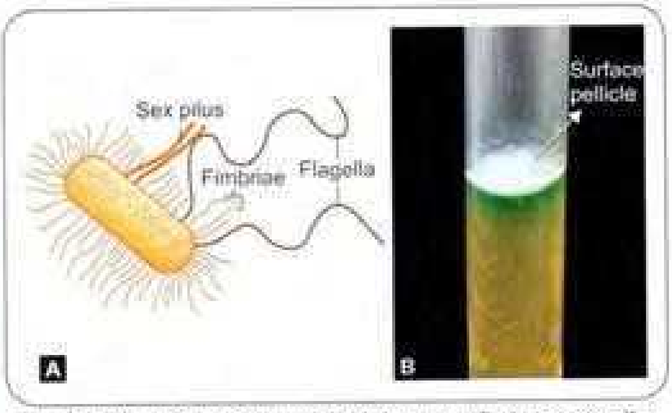
Types of motility	Bacteria
Tumbling motility	*Listeria*
Gliding motility	*Mycoplasma*
Stately motility	*Clostridium*
Darting motility	*Vibrio cholerae, Campylobacter*
Swarming on agar plate	*Proteus, Clostridium tetani*
Corkscrew, lashing, flexion extension motility	Spirochete

1. **Common pili or fimbriae:** They help in bacterial adhesion to epithelial surface helping in colonization (hence called as colonization factor), to red blood cells (causing hemagglutination), and to surfaces of yeast and fungal cells
   - There are six types of common pili; classified based on their morphology, number per cell, adhesive properties and antigenic nature
   - They are very small, measuring 0.5µm long and 10 nm in thickness
   - A single bacterium can have as many as 1,000 fimbriae (Fig. 2.19A)
   - They are present in gram-negative and some gram-positive bacteria.
2. **Sex pili:** They help in bacterial conjugation by forming conjugation tube through which bacterial gene transfer takes place
   - They are long thick tubular structures
   - The number of sex pili are less; 1-10 per bacterial cell
   - They are only found in gram-negative bacteria.

Detection of Fimbriae

Electron microscopy is the only method for direct demonstration of fimbriae. However, there are some indirect methods to know the presence of fimbriae such as:

- **Hemagglutination:** Many fimbriated bacteria (e.g. *Escherichia coli, Klebsiella*, gonococci) strongly agglutinate with red blood cells of guinea pigs, fowl, horses and pigs. This property of hemagglutination is a simple method for detecting the presence of fimbriae. In some bacteria, the hemagglutination may be specifically inhibited by D-mannose
- **Surface pellicle:** Some aerobic fimbriated bacteria form a thin layer at the surface of a broth culture called as **pellicle.** The pellicle consists of many aerobic bacteria that adhere to the surface by their fimbriae (Fig. 2.19B).



Figs 2.19A and B: A. Differentiation between fimbriae, sex pilus and flagella; **B.** Surface pellicle (arrow showing)
Source: Department of Microbiology, JIPMER (with permission)

Atypical Forms of Bacteria

- **Involution forms:** They are swollen and aberrant forms of bacteria (e.g. gonococci and *Yersinia pestis*) formed in ageing cultures in high salt concentration
- **Pleomorphic bacteria:** Some bacteria exhibit great variation in the shape and size of individual cells, e.g. *Proteus* and *Haemophilus*. This is known pleomorphism
- **Cell wall deficient forms:** *See below.*

Pleomorphism and involution forms are often caused by defective cell wall synthesis. Involution forms may also be formed due to the activity of autolytic enzymes.

L Form (Cell Wall Deficient Forms)

L forms are the cell wall deficient bacteria, discovered by E. Klieneberger, while studying *Streptobacillus moniliformis*.

- She named it as L form after its place of discovery, i.e. Lister Institute, London (1935)
- When bacteria loose cell wall, they become spherical irrespective of original shape. This may occur spontaneously or after exposure to penicillin or lysozyme
- L forms play a role in the persistence of pyelonephritis and other chronic infections.

Types of L forms

Two types of L forms are distinguished:

- **Unstable L forms:** Bacteria lose the cell wall in presence of penicillin, a mechanism of resistance shown by the bacteria against penicillin. Such L forms are maintained only in presence of penicillin. They are capable of dividing, but can revert back to the original morphology once penicillin is removed
  - Protoplasts: They are gram-positive bacteria whose cell wall is entirely removed
  - Spheroplasts: They are derived from gram-negative bacteria whose cell wall is partially removed
- **Stable L forms:** L forms that are unable to revert to the original bacteria are called stable L forms
  - *Mycoplasma* do not have a true cell wall; the peptidoglycan layer is replaced by sterol
  - It is postulated that *Mycoplasma* may represent stable L forms of a yet to be identified parent bacteria
  - But many researchers do not consider *Mycoplasma* as L forms, since they are not derived from bacteria that normally have cell walls.

BACTERIAL SPORES

Spores are highly resistant resting (or dormant) stage of the bacteria formed in unfavorable environmental conditions as a result of the depletion of exogenous nutrients. Bacterial spores formed within the parent cell, are called endospores and the remaining part of the bacterium is called the sporangium.

Structure: Bacterial spore comprises of several layers. From innermost towards the outermost, the layers are: core → cortex → coat → exosporium (Fig. 2.20A).

- The **core** is the inner most part containing the DNA material and is walled off from the cortex by an inner membrane and the germ cell wall
- Cortex and the coat layers lie external to the core, and are separated from each other by an outer membrane
- The outermost layer is called as the exosporium.

Sporulation

Sporulation (or sporogenesis) refers to the process of formation of spores from vegetative stage of bacteria. It is not a method of reproduction because the bacteria do not divide during sporulation. Sporulation commences when growth ceases due to lack of nutrients. It is a complex process, takes about 10 hours which may be divided into seven stages (Fig. 2.20B).

1. **Stage I (axial filament formation):** Bacterial cell division occurs and an axial filament of nuclear material is formed.
2. **Stage II (septum formation):** Cell membrane is folded inwards to enclose part of the DNA and to produce the forespore septum.
3. **Stage III (engulfment of forespore):** The membrane continues to grow and engulfs the immature spore in a second membrane.
4. **Stage IV:** The cortex synthesis occurs in the space between the two membranes with deposition of calcium and dipicolinic acid.
5. **Stage V:** Formation of protein coat and exosporium around the cortex
6. **Stage VI:** Maturation of the spore occurs; acquiring properties of heat resistance and refractility.
7. **Stage VII (release):** Finally, lytic enzymes destroy the sporangium releasing the spore.

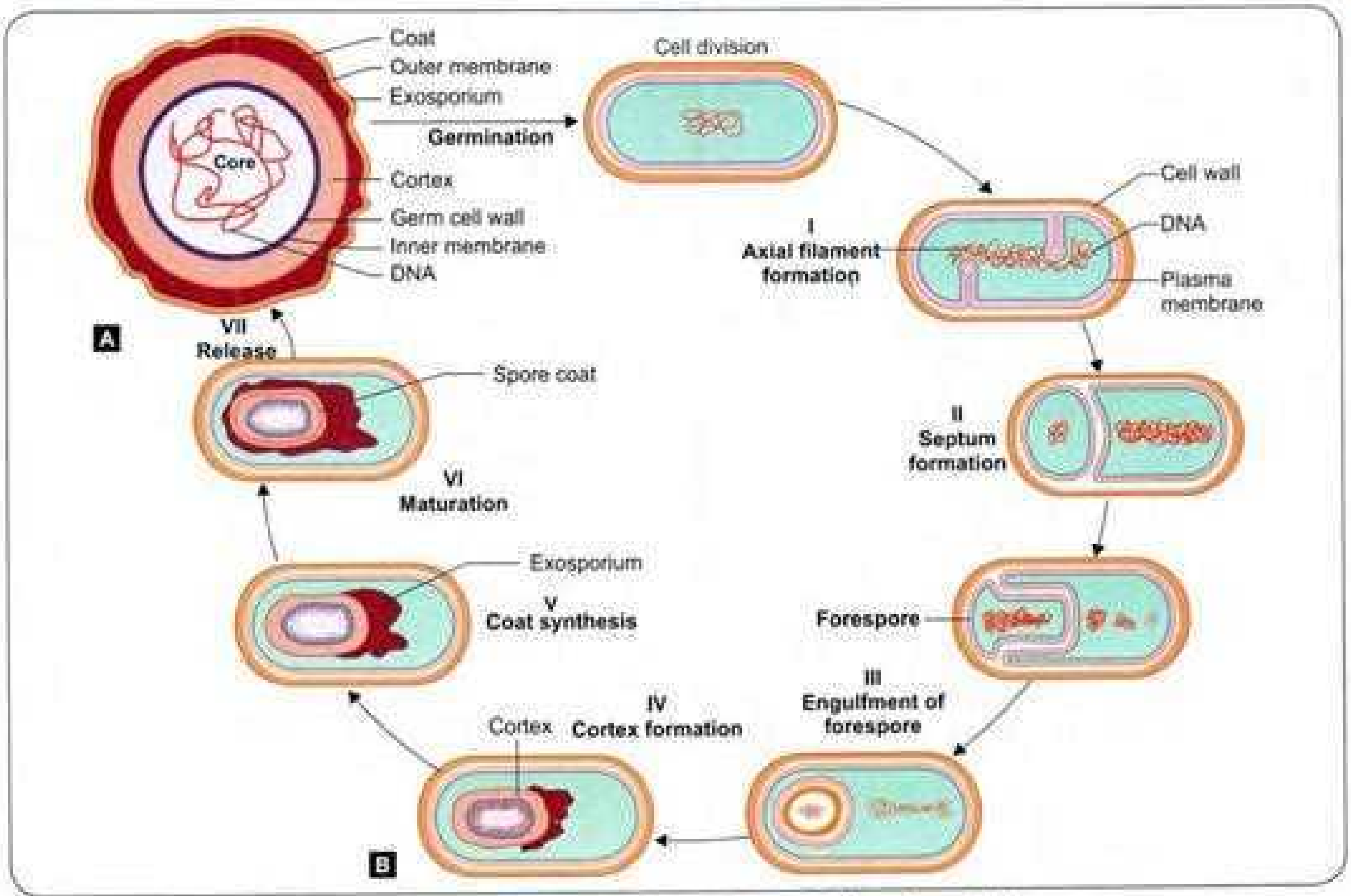
Germination

It is the transformation of dormant spores into active vegetative cells when grown in a nutrient-rich medium. It comprises of three stages:

1. **Activation:** It is reversible process that prepares spores for germination and usually results from treatments like heating.
2. **Germination:** It is the process of breaking of the spore's dormant state, characterized by spore swelling, rupture or absorption of the spore coat, loss of resistance to heat and other stresses, loss of refractility, release of spore components, and increase in metabolic activity.
3. **Outgrowth:** The spore protoplast emerges from the remains of the spore coat, and develops into an active bacterium.

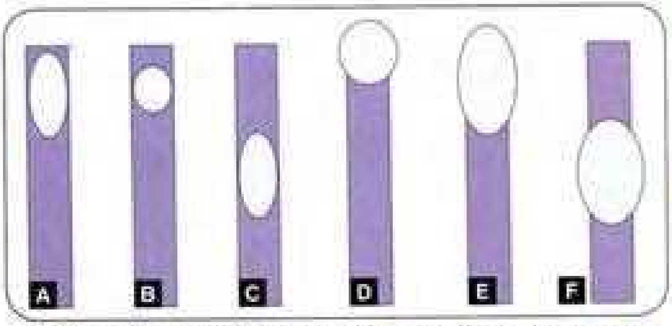
Shape and Position of Spores

For a given species, the precise position, shape and relative size of the spore are constant.



Figs 2.20A and B: A. Structure of bacterial spore; **B.** Steps of sporulation

Concept adapted and modified from Jawetz, Melnick, & Adelberg's Medical Microbiology; McGraw-Hill Education (with permission).



Figs 2.21A to F: Position and shape of spores. **A.** Non-bulging, oval and terminal; **B.** Non-bulging, round, and subterminal; **C.** Non-bulging, oval and central; **D.** Bulging, round and terminal; **E.** Bulging, oval and terminal; **F.** Bulging, oval, and central

- **Position:** Spores may be central, subterminal or terminal (Figs 2.21A to F)
- **Shape:** They may be oval or spherical in shape
- **Width:** The diameter of spore may be same or less than the width of bacteria (non-bulging spore—e.g. as in *Bacillus*), or may be wider than the bacillary body producing a distension or bulge in the cell (bulging spore, e.g. as in *Clostridium*).

Sporicidal Agents

Spores are resistant to most of the routinely used disinfectants. Only limited sterilization methods are available to kill the spores (refer Chapter 3).

Demonstration of Spores

- **Gram staining:** Spores appear as unstained refractile bodies within the cells
- **Modified Ziehl-Neelsen staining:** Spores are weakly acid-fast and appear red color when ZN staining is performed using 0.25% sulfuric acid as decolorizer
- **Special techniques** for endospore staining include the Schaeffer-Fulton stain and the Moeller stain.

Applications

- Spores of certain bacteria are employed as indicators for proper sterilization. Absence of the spores after autoclaving or processing in hot air oven indicates proper sterilization
  - Spores of *Geobacillus stearothermophilus* are used as sterilization control for autoclave
  - Spores of *Bacillus atrophaeus* are used as sterilization control for hot air oven.

- Spores have also been used as agents of bioterrorism, e.g. endospores of *Bacillus anthracis* were used in the 2001 anthrax bioterrorism attack.

PHYSIOLOGY OF BACTERIA

BACTERIAL GROWTH AND NUTRITION

Bacterial Growth Requirement

Water constitutes about 80% of total bacterial cell. The minimum nutritional requirements that are essential for growth and multiplication of bacteria include sources of carbon, nitrogen, hydrogen, oxygen and some inorganic salts (such as small amounts of sulfur, phosphorus and other elements like sodium, potassium, magnesium, iron and manganese).

Bacterial Vitamin

Some fastidious bacteria do not grow in the routine culture medium unless certain organic compounds (that are essential to those bacteria) are added to the medium. These are known as growth factors or bacterial vitamins. In most instances, bacterial vitamins are same as the vitamins necessary for mammalian nutrition, particularly those belonging to the vitamin B group-thiamine, nicotinic acid, riboflavin, pyridoxine, folic acid and vitamin B12 (Table 2.8).

Table 2.8: Bacterial vitamins

Vitamins	Bacteria requiring vitamin
Biotin	*Leuconostoc* species
Cyanocobalamin (B12)	*Lactobacillus* species
Folic acid	*Enterococcus faecalis*
Pantothenic acid	*Morganella morganii*
Pyridoxine (B6)	*Lactobacillus* species
Niacin (nicotinic acid)	*Brucella abortus, Haemophilus influenzae*
Riboflavin (B2)	*Bacillus anthracis*

Bacterial Cell Division

Bacteria divide by a relatively simple form of cell division, i.e. by binary fission. The cell division commences when a bacterial cell reaches a critical mass in its cellular constituents. The nuclear division precedes cytoplasmic division.

- **Nuclear division:** The two strands of bacterial DNA are separated and then they replicate to form new complementary strands. Thus two identical molecules of ds DNA are formed
- **Cytoplasmic division:** A transverse septum grows across the cell from the cell membrane, following which the cell wall materials are deposited and then the two daughter cells get separated
- In few bacteria, the daughter cells may remain partially attached even after cell division; so that the bacterial cells are arranged in pair or in chain (e.g. streptococci) or in clusters (e.g. staphylococci).

Rate of Multiplication in Bacteria

Generation time is the time required for a bacterium to give rise to two daughter cells under optimum condition. The generation time for different bacteria is as follows:

- *Escherichia coli* and most of the other pathogenic bacteria: 20 minutes
- *Mycobacterium tuberculosis:* 10-15 hours
- *Mycobacterium leprae:* 12-13 days.

As bacteria grow so rapidly and by geometric progression, a single bacterium can theoretically give rise to 10^{21} daughter cells in 24 hours. Fortunately, it does not happen in reality, because the bacterial multiplication is arrested after a few cell divisions due to exhaustion of nutrients and accumulation of toxic products.

Bacterial Count

Bacterial count may be expressed in terms of total count and viable count.

- **Total count:** It indicates total number of bacteria (live or dead) in the specimen. This is done by counting the bacteria under microscope using counting chamber
- **Viable count:** It measures the number of living (viable) cells in the given specimen. Viable count may be obtained by:
  - Pour plate method (described in Chapter 4)
  - Surface viable count by spreading method
  - Surface viable count by Miles and Misra method.

Bacterial Growth Curve

When a bacterium is inoculated into a suitable liquid culture medium and incubated, its growth follows a definite course. When bacterial count of such culture is determined at different intervals and plotted in relation to time, a **bacterial growth curve** is obtained comprising of four phases (Fig. 2.22 and Table 2.9).

1. **Lag phase:** It is the period between inoculation and beginning of multiplication of bacteria. After inoculating into a culture medium, bacteria do not start multiplying immediately, but take some time to build-up enzymes and metabolites.
   - Bacteria increase in size due to accumulation of enzymes and metabolites
   - Bacteria reach their maximum size at the end of lag phase.
2. **Log phase:** In this phase bacteria divide exponentially so that the growth curve takes a shape of straight line. At this stage, the bacterium is:
   - Smaller in size

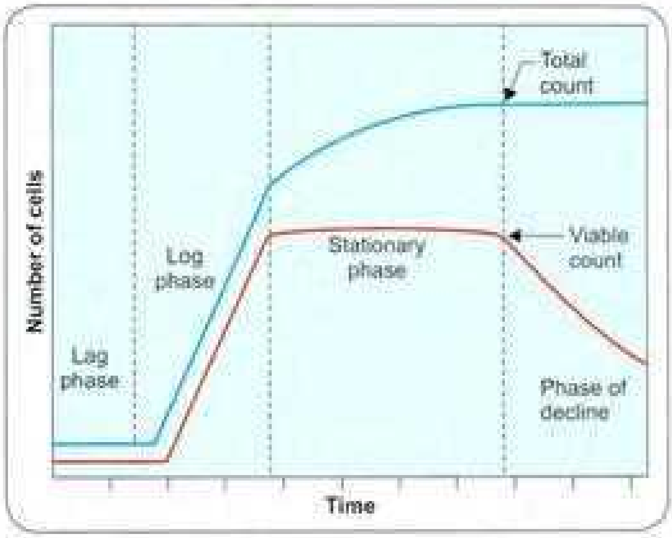


Fig. 2.22: Bacterial growth curve

Table 2.9: Various phases of bacterial growth curve

	Lag	Log	Stationary	Decline
Bacteria divide	No	Yes	Yes	No
Bacterial death	No	No	Yes	Yes
Total count	Flat	Raises	Raises	Flat
Viable count	Flat	Raises	Flat	Falls
Special features	Accumulation of enzymes and metabolites Attains maximum size	Uniformly stained Metabolically active Small size	Gram variable **Produce:** Granules spores, exotoxin, antibiotics, bacteriocin	**Produce:** Involution forms

- Biochemically active: It is the best stage to perform the biochemical reactions
- Uniformly stained: It is the best time to perform the Gram stain.

3. **Stationary phase:** After the log phase, the bacterial growth ceases almost completely due to exhaustion of nutrients, accumulation of toxic products and autolytic enzymes.
   - The number of progeny cells formed is just enough to replace the number of cells that die
   - Hence, the number of viable cells remain stationary as there is almost a balance between the dying cells and the newly formed cells. But the total count keeps raising. In this phase:
     - Bacterium becomes gram-variable
     - More storage granules are formed
     - Sporulation occurs in this phase
     - Bacteria produce exotoxins, antibiotics and bacteriocins.
4. **Decline phase:** Gradually, the bacteria stop dividing completely; while the cell death continues due to exhaustion of nutrients, and accumulation of toxic products.
   - There is decline in viable count and not in total count
   - Involution forms are seen.

Continuous Culture

It is a special type of culture, which supports the bacterial growth in a particular phase (e.g. log phase) for longer period continuously without depletion of nutrients or without accumulation of toxic products.

- Special devices are used which supply the replenishing nutrients and remove the dying bacterial cells and their toxic products
- Continuous cultures can be maintained mainly by two methods
  - Turbidostat culture
  - Chemostat culture.
- Continuous culture of bacteria is required sometimes for industrial and research purposes.

Bacterial Growth *in Vivo*

When bacteria multiply in host tissues, the situation may be intermediate between a routine culture (batch culture) and a continuous culture. The source of nutrients may be plenty, but the defence mechanisms of the body influence the bacterial growth *in vivo*.

FACTORS AFFECTING GROWTH OF BACTERIA

There are several environmental factors that affect the growth of the bacteria.

Oxygen

On the basis of their oxygen requirements bacteria are classified as:

- **Obligate aerobes:** They can grow only in the presence of oxygen (e.g. *Pseudomonas, Mycobacterium tuberculosis, Bacillus, Brucella* and *Nocardia)*
- **Facultative anaerobes:** They are aerobes that can also grow anaerobically (e.g. most of the pathogenic bacteria, e.g. *E. coli, S. aureus*, etc.)
- **Facultative aerobes:** They are anaerobes that can also grow aerobically (e.g. *Lactobacillus*)
- **Microaerophilic bacteria:** They can grow in the presence of low oxygen tension, i.e. 5-10% of oxygen (e.g. *Campylobacter* and *Helicobacter*)
- **Obligate anaerobes:** These bacteria can grow only in absence of oxygen, as oxygen is lethal to them (e.g. *Clostridium)*
- **Aerotolerant anaerobe:** They can tolerate oxygen for some time, but do not use it (*Clostridium histolyticum)*.

Carbon Dioxide

Organisms that require higher amounts of carbon dioxide (5-10%) for growth are called **capnophilic bacteria**. Examples include *Brucella abortus*, *Streptococcus pneumoniae*, etc.

Temperature

Most of the pathogenic bacteria grow optimally at 37°C (i.e. human body temperature). However, the optimal temperature range varies with different bacterial species. Accordingly bacteria can be grouped into:

- **Psychrophiles:** These grow best at temperatures below 20°C; example, most of the saprophytes, e.g. *Pseudomonas*
- **Mesophiles:** These grow within a temperature range 25°C and 40°C; example, most of the pathogenic bacteria
- **Thermophiles:** These bacteria grow at a high temperature range of 55°C-80°C, e.g. *Geobacillus stearothermophilus*.

pH

Most pathogenic bacteria grow between pH 7.2-pH 7.6. Very few bacteria (e.g. lactobacilli) can grow at acidic pH below pH 4, while bacteria such as *Vibrio cholerae* are capable of growing at alkaline pH (8.2-8.9).

Light

Bacteria (except phototrophs) grow well in darkness. They are sensitive to ultraviolet rays and other radiations in light. Photochromogenic mycobacteria produce pigments only on exposure to light.

Osmotic Effect

Bacteria are able to withstand a wide range of external osmotic variation because of the mechanical strength of the cell wall.

- Sudden exposure of bacteria to hypertonic solutions may cause plasmolysis—osmotic withdrawal of water leading to shrinkage of protoplasm. This occurs more readily in gram-negative than in gram-positive bacteria
- Sudden transfer of bacteria from concentrated solution to distilled water may cause plasmoptysis—excessive osmotic imbibition of water, leading to swelling and rupture of the cell.

Mechanical and Sonic Stresses

Though bacteria have tough cell walls, they may be ruptured and disintegrated by vigorous shaking with glass beads and by exposure to ultrasonic vibrations.

Moisture and Desiccation

Moisture is an essential requirement for the growth of bacteria because 80% of the bacterial cell consists of water. However, the drying has varying effects on different organisms.

- Some organisms like *Treponema pallidum* and *N. gonorrhoeae* die quickly after drying, while *M. tuberculosis* and *Staphylococcus aureus* may survive drying for several weeks
- Drying in cold and vacuum (lyophilization) is used for preservation of microorganisms.

BACTERIAL METABOLISM

Bacterial metabolism is the process by which a microbe obtains the energy and nutrients (e.g. carbon) for its survival and reproduction. Bacterial metabolism can be based on three principles:

1. How the bacteria obtain carbon for synthesizing cell mass?
   - **Autotrophs:** These bacteria can synthesize all their organic compounds by using atmospheric CO_2 as their sole source of carbon
   - **Heterotrophs:** They use reduced, preformed organic molecules as carbon sources.
2. How the bacteria obtain reducing equivalents (electrons) used either in energy conservation or in biosynthetic reactions?
   - **Lithotrophs:** These bacteria obtain reducing equivalents (electrons) from inorganic compounds
   - **Organotrophs:** They obtain reducing equivalents from organic compounds.
3. How bacteria obtain energy for living and growing?
   - **Chemotrophs:** These bacteria obtain energy from external chemical compounds
   - **Phototrophs:** They obtain energy from light.

Bacteria usually possess combination of these properties. Most of the pathogenic bacteria fall into **chemoorganoheterotrophs** group. These bacteria obtain energy, carbon, and reducing equivalents for biosynthetic reactions from organic compounds, e.g. *Escherichia coli*.

Metabolism of Pathogenic Bacteria

The bacterial metabolism is dependent on whether they are aerobic or anaerobic.

- Aerobic bacteria utilize glucose by oxidation
- Whereas the anaerobes utilize glucose by fermentation.

Fermentation

Utilization of glucose under anaerobic condition is called fermentation. Bacterial fermentation occurs via three pathways:

1. **Glycolysis** (also called EMP or Embden-Meyerhof-Parnas pathway): Glucose is converted to pyruvate; seen in most of the bacteria.

2. **Entner-Doudoroff (ED) pathway:** It is rarely seen in few bacteria such as *Pseudomonas*. Here, the glucose is converted to KDPG (keto-deoxy-phosphogluconate) which is then further converted into pyruvate.
3. **Pentose phosphate pathway:** It seen in most of the bacteria; glucose is converted to pentose sugars, such as xylulose phosphate and ribulose phosphate which are used in the biosynthesis of aromatic amino acids, vitamin B6 and ribose 5-phosphate, which is a major component of nucleic acids.

Utilization of Pyruvate

Pyruvate produced at the end of EMP and ED pathways are further utilized in many ways (as given below) to produce acids (lactic acid, formic acid, pyruvic acid), gas (hydrogen, carbon dioxide) and alcohols.

- **Homolactic fermentation** produces lactic acid. This is seen in enterococci, streptococci and lactobacilli
- **Heterolactic fermentation** produces ethanol. This is seen in *Leuconostoc*, lactobacilli and yeast
- **Mixed acid fermentation:** Pyruvate is metabolized to produce a number of different products, such as acetic acid, ethanol, succinic acid and formic acid. The nature and amount of acid production depends on the organism
- **Butanediol fermentation:** Pyruvate is metabolized to acetoin, which is further converted to butanediol. This is produced by *Klebsiella* and few other bacteria. Production of acetoin is detected by an important biochemical test called Voges-Proskauer reaction
- **Butanol formation:** It is seen in anaerobic bacteria *Clostridium*
- **Propionic acid formation:** This is observed in anaerobic bacteria, such as *Bacteroides*, and *Propionibacterium*.

During fermentation, energy rich phosphate molecules are produced as by-products which convert adenosine diphosphate (ADP) to adenosine triphosphate (ATP). This process is known as **substrate-level phosphorylation**.

- During glycolysis, net of 2 ATP molecules per mole of glucose are produced
- Fermentation is carried out by both obligate and facultative anaerobes.

Oxidation

Oxidation refers to oxidative utilization of glucose (by Krebs cycle) followed by production of ATP via oxidative phosphorylation and transfer of electrons in electron transport system.

- **Krebs cycle:** The pyruvic acid produced at the end of glycolysis and ED pathway undergoes a series of oxidative decarboxylation steps to produce a number of high energy compounds, such as nicotinamide adenine dinucleotide phosphate (NADPH) and flavin adenine dinucleotide (FADH) which finally enter the electron transport system
- **Electron transport system:** It is located in cell membrane of bacteria, in contrast to mitochondria of eukaryotes
  - It consists of alternating hydrogen and electron carriers located in a sequence across the cell membrane
  - Transfer of protons down the electron transport system creates a membrane potential. The energy of this potential is harnessed by ATP synthase which catalyses the formation of ATP from ADP
  - Krebs cycle results in a net gain of 38 ATP molecules per mole of glucose.

EXPECTED QUESTIONS

I. Essay:

1. Describe in detail the structure and function of the cell wall and cell membrane of a gram-negative rod with the help of a diagram.

II. Write short notes on:

1. Bacterial capsule.
2. Bacterial spore.
3. Principle and uses of dark field microscope.
4. Principle and uses of fluorescence microscope.
5. Principle and uses of electron microscope.
6. Bacterial growth curve.
7. Bacterial flagella.

III. Multiple Choice Questions (MCQs):

1. **Cuneiform arrangement is characteristic of:**
   a. *Staphylococcus* b. *Streptococcus*
   c. *C. diphtheriae* d. *Bacillus anthracis*
2. **Electrons are used as a source of illumination in:**
   a. Light microscope
   b. Dark field microscope
   c. Phase contrast microscope
   d. Electron microscope
3. **All of the following are acid-fast, *except*:**
   a. *Mycobacterium* b. *Nocardia*
   c. *Cystoisospora belli* d. *Staphylococcus*
4. **Bacterial capsule can be best demonstrated by:**
   a. Gram staining b. Acid-fast staining
   c. Negative staining d. Albert staining
5. **Lipopolysaccharide is a component of cell wall of:**
   a. Gram-positive bacteria
   b. Gram-negative bacteria
   c. Virus d. Fungi
6. **Bacterial structure involved in respiration is:**
   a. Ribosome b. Pili
   c. Mesosome d. Flagella
7. **Resolution of a microscope improves by using:**
   a. oil b. stain
   c. lenses d. condenser

8. Unaided human eye has a resolution power of:
a. 0.2 mm b. 0.2 µm
c. 0.2 nm d. 0.5 nm

9. Phase contrast microscopy is useful for studying:
a. Microbial motility
b. Determining the shape of living cells
c. Detecting bacterial components, such as endospores and inclusion bodies
d. All of the above

10. Which of the following microscopes, the object appears bright against a dark background?
a. Simple light microscope
b. Dark ground microscope
c. Compound light microscope
d. Electron microscope

11. Electron microscope differs from light microscope in all, *except*:
a. Highest practical magnification
b. Medium of travel
c. Resolution
d. No need for specimen preparation

12. Regarding Gram staining- which is wrong?
a. Primary stain-crystal violet
b. Mordant-iodine
c. Decolorizer- sulphuric acid
d. Counterstain- safranin

13. Uses of Gram staining are all, *except*:
a. Helps to start empirical treatment
b. Helps to detect yeast
c. Helps for identification
d. Can differentiate motile and non-motile bacteria

14. Modifications of Gram staining include all, *except*:
a. Kopeloff and Beerman's modification
b. Kinyoun's modification
c. Jensen's modification
d. Preston and Morrell's modification

15. Acid-fast organisms are all, *except*:
a. *Mycobacterium tuberculosis*
b. *Legionella pneumophila*
c. *Cryptosporidium*
d. *Nocardia*

16. Concentration of sulfuric acid used for acid-fast staining for *Mycobacterium tuberculosis* according to RNTCP?
a. 20% b. 25%
c. 5% d. 0.5–1%

17. Concentration of sulfuric acid used for acid-fast staining for *Mycobacterium leprae*?
a. 20% b. 25%
c. 5% d. 0.5–1%

18. Which of the following cocci–arrangement is wrong?
a. Chain-*Streptococcus* b. Pair-Pneumococcus
c. Tetrad-Gonococcus d. Cluster-*Staphylococcus*

19. Which of the following cocci–arrangement is wrong?
a. Pairs, lens shaped-*Enterococcus*
b. Pairs, kidney shaped-Gonococcus
c. Tetrads-*Micrococcus*
d. Octate-*Sarcina*

20. Which of the following gram-positive bacilli – arrangement is wrong?
a. Chain (bamboo stick appearance)-*Bacillus anthracis*
b. Chinese letter pattern-*Corynebacterium diphtheriae*
c. Palisade pattern-*Diphtheroids*
d. Filamentous form-*Mycobacterium tuberculosis*

21. Which of the following gram-negative bacilli – arrangement is wrong?
a. *Gull-wing shaped*-Spirochetes
b. Pleomorphic shaped-*Haemophilus*
c. Thumb print appearance-*Bordetella pertussis*
d. Comma shaped-*Vibrio cholerae*

22. Gram-positive cell wall differs from gram-negative cell wall by all, *except*:
a. Mesodiaminopimelic acid-present
b. Pentaglycine bridge-present
c. Lipopolysaccharide-absent
d. Teichoic acid-present

23. All the following are the components of gram-negative cell wall, *except*:
a. Lipopolysaccharide b. Outer Membrane
c. Peptidoglycan d. Teichoic acid

24. All the following are the components of lipopolysaccharide (LPS), *except*:
a. O side chain b. H antigen
c. Core polysaccharide d. Lipid A

25. Which of the following bacteria is commonly capsulated?
a. *Escherichia coli* b. *Staphylococcus*
c. *Salmonella* d. *Klebsiella*

26. Which of the following bacteria possess polypeptide capsule?
a. Pneumococcus b. Meningococcus
c. *Haemophilus* d. *Bacillus anthracis*

27. Which of the following is not a function of capsule?
a. Source of nutrients and energy
b. Helps in bacterial motility
c. Protects the bacterium from phagocytosis
d. Prevent cell from drying out

28. Bacterial motility can be demonstrated by all, *except*:
a. Craigie tube b. Hanging drop
c. Semisolid medium d. India ink staining

29. Flagella can be demonstrated by:
a. India ink staining b. Tannic acid staining
c. Albert staining d. GMS staining

30. Which of the following is wrong?
a. Tumbling motility-*Listeria*
b. Gliding motility-*Mycoplasma*
c. Corkscrew motility-*Campylobacter*
d. Stately motility-*Clostridium*

Answers

1. c	**2.** d	**3.** d	**4.** c	**5.** b	**6.** c	**7.** a	**8.** a	**9.** d	**10.** b	**11.** d	**12.** c	**13.** d	**14.** b	**15.** b	**16.** b
17. c	**18.** c	**19.** a	**20.** d	**21.** a	**22.** a	**23.** d	**24.** b	**25.** d	**26.** d	**27.** b	**28.** d	**29.** b	**30.** c		

Sterilization and Disinfection

3
CHAPTER

Chapter Preview

- Introduction
- Agents used for sterilization/ disinfection
  - Physical agents
  - Chemical agents
- Testing of disinfectants
- Spaulding's classification of medical devices
- Central Sterile Services Department (CSSD)

INTRODUCTION

Sterilization, disinfection, asepsis and decontamination are the four separate but interrelated terminologies, all aiming at removing or destroying the microorganisms from materials or from body surfaces.

Sterilization

Sterilization is a process by which all living microorganisms, including viable spores, are either destroyed or removed from an article, body surface or medium.

- It results in reduction of at least 10^6 log colony-forming units of microorganisms and their spores
- It can be achieved by a physical agent or a chemical agent (called chemical sterilant).

Disinfection

It refers to a process that destroys or removes most if not all pathogenic organisms but not bacterial spores.

- It results in reduction of at least 10^3 log colony-forming units of microorganism, but not spores
- The primary goal in disinfection is to destroy potential pathogens, but it also substantially reduces the total microbial population
- **Agents:** Disinfection can also be achieved by a physical agent or a chemical agent (called disinfectant) and they are normally used only on inanimate objects, not on body surfaces.

Asepsis

It is a process where the chemical agents (called antiseptics) are applied on to the body surfaces (skin), which kill or inhibit the microorganisms present on the skin.

- Antiseptics prevent the entry of pathogens into sterile tissues and thus prevent infection or sepsis
- However, they are generally not as toxic as disinfectants as they must not harm the host tissue.

Decontamination (or Sanitization)

It refers to the reduction of pathogenic microbial population to a level at which items are considered as safe to handle without protective attire.

- It results in reduction of at least 1 log colony-forming unit of most of the microorganisms, but not spores
- Examples include manual or mechanical cleaning by soap and detergents to eliminate debris or organic matter from the medical devices.

Agents used for sterilization/disinfection can also be named with suffix based on whether they kill or inhibit the microorganisms.

- Suffix 'cide' is used for the agents that can kill micro-organisms. Examples include bactericide, virucide and fungicide
- Suffix 'static' is used for the agents that do not kill; but inhibit the microbial growth. If these agents are removed, growth will resume, for example, bacteriostatic and fungistatic.

Although these agents have been described in terms of their effects on pathogens, it should be noted that they also kill or inhibit the growth of nonpathogens as well.

Factors Influencing Efficacy of Sterilant/Disinfectant

The efficiency of a sterilant/disinfectant is affected by at least seven factors.

1. **Organism load:** Larger microbial population requires a longer time to die than a smaller one.

2. **Nature of organisms:** It greatly influences the efficacy of the disinfectants or sterilizing agents.

> **The decreasing order of resistance** of microorganisms to disinfectant or sterilizing agents is as follows:
> Prions (highest resistance) > *Cryptosporidium* oocysts > Bacterial spores > Mycobacteria > Other parasite cysts (e.g. *Giardia*) > Small non-enveloped viruses > Trophozoites > Gram-negative bacteria > Fungi > Large non-enveloped viruses > Gram-positive bacteria > Enveloped viruses.

3. **Concentration** of the chemical agent or the temperature of heat sterilization.
4. **Nature of the sterilant/disinfectant such as its:**
   - Microbicidal ability
   - Rapidity of action
   - Ability to act in presence of organic matter
   - Residual activity.
5. **Duration of exposure:** More is the exposure time to sterilant/disinfectant, better is the efficacy.
6. **Temperature:** An increase in the temperature at which a chemical acts often enhances its activity. A lower concentration of disinfectant or sterilizing agent can be used at a higher temperature.
7. **Local environment:** The microorganisms to be controlled are not isolated but surrounded by environmental factors that may either offer protection or aid in its destruction, e.g.
   - **pH:** Heat kills more readily at an acidic pH
   - **Organic matter:** Presence of organic matter, such as pus, blood, and stool can protect the organisms against heating and chemical agents
     - It is necessary to mechanically clean an object (e.g. syringes and medical equipment) before it is disinfected or sterilized
     - Materials containing organic substances require more time or volume or concentration of chemical agent for sterilization/disinfection. More chlorine must be added to disinfect drinking water if a city's water supply has a high content of organic material.
   - **Biofilm:** Formation of biofilm is another mechanism which prevents the entry of disinfectants to act on the microorganisms which are embedded inside the biofilm.

Both physical and chemical methods are used to achieve control of microorganisms (Table 3.1).

Table 3.1: Classification of sterilization/disinfection methods

Physical methods	
Drying	
Heat	
Dry heat: • Flaming • Incineration • Hot air oven	Moist heat: • Temperature below 100°C, e.g. pasteurization, water bath and inspissation • Temperature at 100°C, e.g. boiling, steaming and tyndallization • Temperature above 100°C, e.g. autoclave
Filtration: Depth filters and membrane filters	
Radiation • Ionizing radiation: γ rays, X-rays and cosmic rays • Nonionizing radiation: Ultraviolet (UV) and infrared rays	
Ultrasonic vibration	
Chemical methods	
Alcohols: Ethyl alcohol, isopropyl alcohol	
Aldehydes: Formaldehyde, glutaraldehyde, Ortho-phthalaldehyde	
Phenolic compounds: Cresol, lysol, chlorhexidine, chloroxylenol, hexachlorophene	
Biguanide: Chlorhexidine gluconate	
Halogens: Chlorine, iodine, iodophors	
Oxidizing agents: Hydrogen peroxide, peracetic acid	
Heavy metal salts: Mercuric chloride, copper salts	
Surface active agents: Quaternary ammonium compounds and soaps	
Dyes: Aniline dyes and acridine dyes	
Gas sterilization: • Low temperature steam formaldehyde • Ethylene oxide (ETO) • Betapropiolactone (BPL) • Plasma sterilization	

Note: Sometimes the words sterilization and disinfection are loosely used to describe physical and chemical agents respectively. However, this practice is incorrect, because there are some physical agents that do not produce complete sterilization; similarly many chemical agents (called as chemical sterilants) can kill the spores and achieve sterilization.

PHYSICAL AGENTS OF STERILIZATION/ DISINFECTION

Drying

Moisture is essential for the growth of bacteria. 70–80% of the weight of the bacterial cell is due to water. Drying, therefore has a deleterious effect on many bacteria. Both drying and sunlight are not reliable. They do not affect many microbes, including spores.

Heat

Heat is the most reliable and commonly employed method of sterilization/disinfection. It should be considered as the method of choice unless contraindicated. Two types of heat are used, dry heat and moist heat.

Mechanism of action of heat:
- Dry heat kills the organisms by charring, denaturation of bacterial protein, oxidative damage and by the toxic effect of elevated levels of electrolytes
- Moist heat kills the microorganisms by denaturation and coagulation of proteins
- Materials containing organic substances require more time for sterilization/disinfection.

Dry Heat

Flaming

Items are held in the flame of a Bunsen burner either for long time or short time.

- **Longer time exposure in flame till they become red hot:** This is done for inoculating wires or loops, tips of forceps, etc.
- **For shorter period of time without allowing the items to become red hot:** This is done for fragile items, e.g. mouth of test tubes.

Incineration

Incineration is used for the disposal of biomedical waste materials. It burns (sterilizes) the anatomical waste and microbiology waste by providing a very high temperature 870–1,200°C and thereby converting the waste into ash, flue gas and heat (Chapter 55).

Hot Air Oven (Dry Heat Sterilizer)

Hot air oven is the most widely used method of sterilization by dry heat. It is electrically heated and is fitted with a fan to ensure adequate and even distribution of hot air in the chamber (Fig. 3.1). It is also fitted with a thermostat which maintains the chamber air at a chosen temperature.

- **Temperature:** A holding temperature and time of 160°C for 2 hours is required for sterilization in hot air oven
- **Materials sterilized:** Hot air oven is the best method for sterilization of:
  - Glassware like glass syringes, petri dishes, flasks, pipettes and test tubes
  - Surgical instruments like scalpels, forceps, etc.
  - Chemicals such as liquid paraffin, fats, glycerol, oil, and glove powder, etc.

Fig. 3.1: Hot air oven

- **Precautions:** The following precautions should be taken while using hot air oven
  - Overloading of hot air oven should be avoided
  - The material should be arranged in a manner so that free circulation of air is maintained
  - Material to be sterilized should be dried completely
  - Cotton plugs should be used to close the mouths of test tubes, flasks, etc.
  - Paper wrapping of the items should be done
  - Any inflammable material like rubber (except silicone rubber) should not be kept inside the oven
  - The oven must be allowed to cool for two hours before opening the doors, since the glassware may crack by sudden cooling.
- **Sterilization control:** The effectiveness of the sterilization done by hot air oven can be monitored by:
  - Biological indicator: Spores (10^6) of *Bacillus atrophaeus* are used to check the effectiveness of sterilization by dry heat. Earlier, non-toxigenic strain of *Clostridium tetani* was used. These spores should be destroyed if the sterilization is done properly
  - Physical control: For example, digital displays on the equipment displaying temperature and time.

Moist Heat

Moist heat kills the microorganisms at a lower temperature than dry heat. Moist heat may be used at different temperatures as follows:

Moist Heat at a Temperature Below 100°C

- **Pasteurization:** It is a method used for control of microorganisms from beverages like fruit and vegetable juices, beer, and dairy products, such as milk
  - Two methods are available—Holder method (63°C for 30 minutes) and Flash method (72°C for 20 seconds followed by rapid cooling to 13° or lower)
  - All nonsporing pathogens, including mycobacteria, brucellae and salmonellae are killed except *Coxiella burnetii* which being relatively heat resistant may survive in holder method.
- **Water bath:** It is used for disinfection of serum, body fluids and vaccines (Fig. 3.2)
  - Bacterial vaccines are disinfected at 60°C for 1 hour
  - Serum or heat labile body fluids can be disinfected at 56°C for one hour.
- **Inspissation (fractional sterilization):** It is a process of heating an article on 3 successive days at **80–85°C for 30 minutes** by a special instrument called inspissator (Fig. 3.3)
  - Working principle: In inspissator, the first exposure kills all the vegetative forms, and in the intervals between the heatings the remaining spores

Fig. 3.2: Water bath

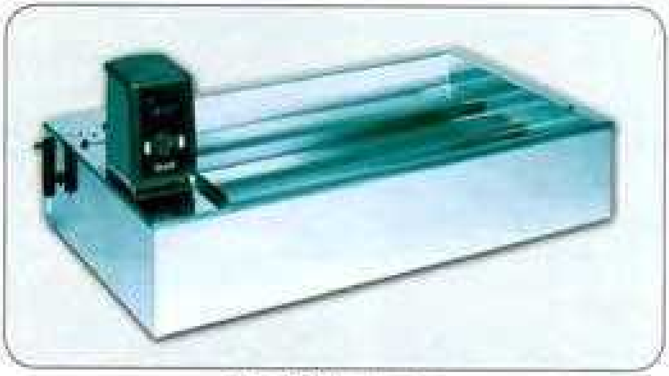


Fig. 3.3: Inspissator

germinate into vegetative forms which are then killed on subsequent heating

- Uses: Inspissation is useful for sterilization of egg and serum-based media which generally get destroyed at higher temperature
    - Egg-based media—For example, Lowenstein-Jensen medium and Dorset's egg medium
    - Serum-based media—For example, Loeffler's serum slope.

Moist Heat at a Temperature of 100°C

- **Boiling:** Boiling of the items in water for 15 minutes may kill most of the vegetative forms but not the spores, hence not suitable for sterilization of surgical instruments. Though boiling is a simple, easily available option to most people; however, boiling can be hazardous and not effective; hence should not be used if better methods are feasible
- **Steaming:** Koch's or Arnold's steam sterilizer are useful for those media which are decomposed at high temperature of autoclave
    - The articles are kept on a perforated tray through which steam can pass
    - They are exposed to steam (100°C) at atmospheric pressure for 90 minutes
    - Most of the vegetative forms are killed by this method except thermophiles and spores.
- **Tyndallization or intermittent sterilization** (named after John Tyndall) involves steaming at 100°C for 20 minutes for 3 consecutive days
    - The principle is similar to that of inspissation, except that here, the temperature provided is 100°C, instead of 80°C
    - It is used for sterilization of gelatin and egg, serum or sugar containing media, which are damaged at higher temperature of autoclave.

Moist Heat at a Temperature above 100°C (Autoclave)

Principle of Autoclave

Autoclave functions similar to a pressure cooker and follows the general laws of gas.

- Water boils when its vapor pressure equals that of the surrounding atmosphere. So, when the atmospheric pressure is raised, the boiling temperature is also raised
- At normal pressure, water boils at 100°C but when pressure inside a closed vessel increases, the temperature at which water boils also increases.

Components of Autoclave

Autoclave comprises of three parts: a pressure chamber, a lid and an electrical heater.

- **Pressure chamber consists of:**
    - It is a large cylinder (vertical or horizontal) in which the materials to be sterilized are placed. It is made up of gunmetal or stainless steel and placed in a supporting iron case
    - A steam jacket (water compartment).
- **The lid is fastened by screw clamps and rendered air tight by an asbestos washer. The lid bears the following:**
    - A discharge tap for air and steam discharge
    - A pressure gauge (sets the pressure at a partcular level)
    - A safety valve (to remove the excess steam).
- An electrical heater is attached to the jacket; that heats the water to produce steam.

Procedure

A cylinder is filled with sufficient water and the material to be sterilized is placed inside the pressure chamber. The lid is closed and the electrical heater is put on. The safety valve is adjusted to the required pressure.

- After the water boils, the steam and air mixture is allowed to escape through the discharge tap till all the air has been displaced
    - This can be tested by passing the steam-air mixture liberated from the discharge tap into a pail of water through a connecting rubber tube

- When the air bubbles stop coming in the pail, it indicates that all the air has been displaced by steam. The discharge tap is then closed.

❖ The steam pressure rises inside and when it reaches the desired set level [e.g. 15 pounds (lbs) per square inch in most cases], the safety valve opens and excess steam escapes out (Fig. 3.4)

❖ The holding period is counted from this point of time, which is about 15 minutes in most cases

❖ After the holding period, the electrical heater is stopped and the autoclave is allowed to cool till the pressure gauge indicates that the pressure inside is equal to the atmospheric pressure

❖ The discharge tap is opened slowly and air is allowed to enter the autoclave. The lid is now opened and the sterilized materials are removed.

Sterilization Conditions

Autoclave can be set to provide higher temperatures by adjusting the pressure provided to the vessel.

❑ 121°C for 15 minutes at pressure of 15 pounds (lbs) per square inch (psi): This is the most commonly used sterilization condition for autoclave

❑ 126°C for 10 minutes at pressure of 20 psi

❑ 133°C for 3 minutes at pressure of 30 psi.

Uses of Autoclave

Autoclave is particularly useful for media containing water that cannot be sterilized by dry heat. It is the method of choice for sterilization of the following:

❖ Surgical instruments

❖ Culture media

❖ Autoclavable plastic containers

❖ Plastic tubes and pipette tips

❖ Solutions and water

❖ Biohazardous waste

❖ Glassware (autoclave resistible).

Precautions

The following precautions should be taken while using an autoclave:

❖ Autoclave should not be used for sterilizing waterproof materials, such as oil and grease or dry materials, such as glove powder

❖ Materials are loaded in such a way that it allows efficient steam penetration (do not overfill the chamber)

❖ Material should not touch the sides or top of the chamber

❖ The clean items and the wastes should be autoclaved separately

❖ Polyethylene trays should not be used as they may melt and cause damage to the autoclave.

Types of Autoclaves

Different types of autoclaves are available.

❖ **Gravity displacement type autoclave:** It is the most common type used in laboratories. They are available in various sizes and dimensions.
  - Vertical type (small volume capacity) (Fig. 3.5)
  - Horizontal autoclave (large volume capacity) (Fig. 3.4).

❖ Positive pressure displacement type autoclave

❖ Negative pressure (vacuum) displacement type.

Sterilization control

The effectiveness of the sterilization done by autoclave can be monitored by:

❖ **Biological indicator:** Spores of *Geobacillus stearothermophilus* (formerly called *Bacillus stearothermophilus*)

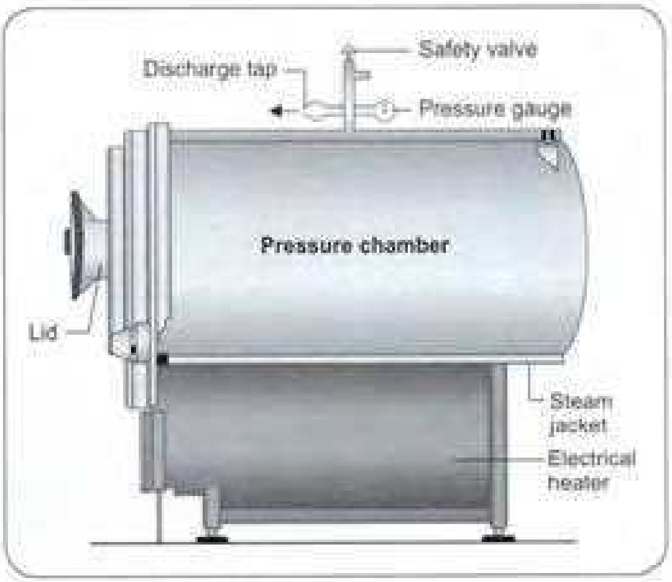


Fig. 3.4: Schematic diagram of horizontal autoclave

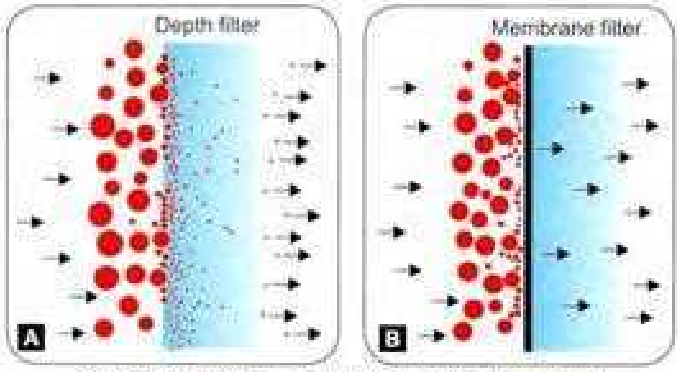
Fig. 3.5: Vertical autoclave

are the best indicator, because they are resistant to steaming. Their spores are killed in 12 minutes at 121°C.

- **Chemical indicators (Table 3.4 for detail):**
  - Class I (external pack control, e.g. autoclave tape)
  - Class II or equipment control (Bowie-Dick test)
  - Class IV/V (internal pack control).
- **Physical control:** For example, digital displays on the equipment displaying temperature, time and pressure.

Filtration

Filtration is an excellent way to remove the microbial population in solutions of heat-labile materials like vaccine, antibiotics, toxin, serum and sugar solution as well as for purification of air.



Figs 3.6A and B: Filtration methods. **A.** Depth filters; **B.** Membrane filters

Types of Filters

There are two types of filters: depth and membrane filters.

Depth Filters

They are porous filters that retain particles throughout the depth of the filter, rather than just on the surface (Fig. 3.6A).

- Depth filters are composed of random mats of metallic, polymeric, or inorganic materials
- These filters rely on the density and thickness of the filter to trap particles rather than the pore size
- The advantages are—(1) they can retain a large mass of particles before becoming clogged, (2) flow rate of the fluid is high, (3) low cost
- Depth filters are commonly used when the fluid to be filtered contains a high load of particles, e.g. industrial applications, such as filtration of food, beverages and chemicals
- **Disadvantages:** As some of the particles still come out in the filtrate; hence, they are not suitable for filtration of solution containing bacteria. Some of the depth filters were used in the past for bacterial purification, such as:
  - Candle filters made up of diatomaceous earth (Berkefeld filters), unglazed porcelain (Chamberland filters)
  - Asbestos filters (Seitz and Sterimat filters)
  - Sintered glass filters.

Membrane Filters

They are the most widely used filters for bacterial filtration. They are porous; retain all the particles on the surface that are larger than their pore size (Fig. 3.6B).

- Membrane filters are made up of cellulose acetate, cellulose nitrate, polycarbonate, polyvinylidene fluoride, or other synthetic materials
- **Pore size:** Most commonly used membrane filters have an average pore diameter of 0.22 μm which removes most of the bacteria; allowing the viruses to pass through. While, filters of 0.45 μm are used to retain coliform bacteria in water microbiology and of 0.8 μm filters are used to remove airborne microorganisms in clean rooms and for the production of bacteria free gases.

Filtration of Liquid

Filtration of the liquid solution is done for the following purposes:

- To sterilize sera, sugar and antibiotic solutions
- Separation of toxins and bacteriophages from bacteria
- To obtain bacteria free filtrates of clinical samples for virus isolation
- Purification of water—when water samples pass through filter disks, retain the organisms which can then be cultured, e.g. testing of water samples for *Vibrio cholerae* or typhoid bacilli.

Filtration of Air

Air filters are used to deliver bacteria-free air. Air can be filtered by various methods:

- Surgical masks (that allow air in but keep microorganisms out) are the simplest examples
- There are two important air filters that are used in biological safety cabinets and laminar airflow systems):
  - **HEPA filters** (High-efficiency particulate air filters): HEPA filter removes 99.97% of particles that have a size of 0.3 μm or more
  - **ULPA filters** (Ultra-low particulate/penetration air): An ULPA filter can remove from the air at least 99.999% of dust, pollen, mold, bacteria and any airborne particles with a size of 0.12 μm or larger.

Sterilization Control

The sterilization control of membrane filters includes *Brevundimonas diminuta* and *Serratia marcescens.*

Radiation

Two types of radiations are available: ionizing and non-ionizing.

Ionizing Radiation

Ionizing radiations include, X-rays, gamma rays (from Cobalt 60 source), and cosmic rays.

- **Mechanism:** It causes breakage of DNA without temperature rise (hence this method is also called as **cold sterilization)**
  - Ionizing radiation is an excellent agent for sterilization/disinfection. It penetrates deep into the object
  - It destroys bacterial endospores and vegetative cells, both eukaryotic and prokaryotic; but not always effective against viruses.
- **Uses:** Gamma radiation is used in the sterilization/disinfection of:
  - Disposable plastic supplies, such as disposable rubber or plastic syringes, infusion sets and catheters
  - Catgut sutures, bone and tissue grafts and adhesive dressings as well as antibiotics and hormones
  - Irradiation of food (permitted in some countries).
- **Advantages** of ionizing radiation—(1) high penetrating power, (2) rapidity of action, and (3) temperature is not raised
- **Sterilization/disinfection control:** Efficacy of ionising radiation is tested by using *Bacillus pumilus*.

Non-ionizing Radiation

Examples of non-ionizing radiation include infrared and ultraviolet radiations.

- They are quite lethal but do not penetrate glass, dirt films, water; hence their use is restricted
- The recommended dose is 250-300 nm wavelength of UV rays, given for 30 minutes
- It is used for disinfection of clean surfaces in operation theaters, laminar flow hoods as well as for water treatment
- Because UV radiation burns the skin and damages eyes, hence the area should be closed and UV lamps must be switched off immediately after use.

Ultrasound (Ultrasonic) Waves

High-frequency ultrasonic and sonic sound waves disrupt bacterial cells; but this method is not reliable, hence is not used nowadays.

CHEMICAL AGENTS OF STERILIZATION/ DISINFECTION

Though commonly called disinfectants, the chemical agents, based on the microbicidal ability are classified into four groups—low level, intermediate level and high level disinfectants and chemical sterilants (Table 3.2).

Antiseptics: They are the low to intermediate level disinfectants that are less toxic to the body surfaces; hence can be applied to skin, open wounds and mucosa.

The various groups of disinfectants are discussed below:

Alcohols

They are among the most widely used disinfectants and antiseptics.

- They are bactericidal and fungicidal but not sporicidal; some enveloped viruses (e.g. HIV) are also destroyed
- **Examples:** The most popular alcohol germicides are ethanol and isopropanol, both are used in 70-80% concentration
- They act by denaturing proteins and possibly by dissolving membrane lipids
- Ethyl alcohol is used as surgical spirit (70%) in hand rubs as antiseptics
- **Isopropyl alcohol:** Clinical thermometers and small instruments are disinfected by soaking in isopropyl alcohol for 10–15 minutes.

Aldehydes

Formaldehyde, glutaraldehyde and ortho-phthalaldehyde are the commonly used disinfectants. They combine with nucleic acids, proteins and inactivate them, probably by cross linking and alkylating the molecules. They are sporicidal and can be used as chemical sterilants.

- **Formaldehyde:** It is usually dissolved in water or alcohol before use. The formalin or formol (40% formaldehyde) is the most widely used preparation available commercially
  - It is best used for—(1) preservation of anatomical specimen, (2) formaldehyde gas is used for fumigation of closed areas, such as operation theaters, (3) preparation of toxoid from toxin
  - Formaldehyde is toxic and irritant when inhaled, as well as it is corrosive to the metals.

Table 3.2: Classification of chemical disinfectants

Level of disinfectant	Bacterial spores	Tubercle bacilli	Nonenveloped viruses	Fungi	Enveloped viruses	Vegetative bacteria
Low-level disinfectant	No	No	No	+/–	Yes	Yes
Intermediate-level disinfectant	No	Yes	Yes	Yes	Yes	Yes
High-level disinfectant	May be	Yes	Yes	Yes	Yes	Yes
Chemical sterilant	Yes	Yes	Yes	Yes	Yes	Yes

- **Glutaraldehyde:** It is less toxic, less irritant and less corrosive; hence is best used to sterilize hospital and laboratory equipment, such as endoscopes and cystoscopes
    - It is used as 2% concentration (2% Cidex). It usually disinfects objects within 20 minutes but may require longer time to kill spores
    - It is available in inactive form; has to be activated by alkalinization before use. Once activated, it remains active only for 14 days.
- **Ortho-phthalaldehyde** (0.55%) solution: This can also be used for sterilization of endoscopes and cystoscopes and has many advantages over glutaraldehyde—(1) it does not require activation, (2) low vapor property, (3) better odor, (4) more stable during storage, (5) ↑mycobactericidal activity.

Phenolic Compounds

Phenol (carbolic acid) was the first widely used antiseptic and disinfectant; was introduced in surgery in 1867 by Joseph Lister (the father of antiseptic surgery).

- The phenol and its derivatives (called phenolics) are produced by distillation of coal tar between temperatures of 170°C and 270°C
- **Mechanisms:** Phenolics act by denaturing proteins and disrupting cell membranes
- **Advantages:** They have tuberculocidal activity, some are effective in the presence of organic materials.

Phenolics as Disinfectants

Cresol, xylenol, lysol and ortho-phenylphenol are the common phenolics used in laboratories and hospitals as disinfectants.

- Lysol is a commercial preparation made of a mixture of phenolics
- All the above phenolics have the ability to retain activity in presence of organic matter
- However they are toxic and irritant to skin, hence they are only used as disinfectants but not as antiseptics.

Phenolics as Antiseptics

Certain phenolics are less irritant to skin, persist in skin for longer period and are widely used as antiseptics. In general, they are more active against gram-positive than gram-negative bacteria.

- **Chloroxylenol:** It is the active ingredient of Dettol
- **Hexachlorophene:** As it can cause brain damage, hence its use as antiseptic is restricted. It is indicated only in response to a staphylococcal outbreak.

Biguanide

Chlorhexidine gluconate (CHG) is probably the most widely used biguanide disinfectant.

- CHG (2-4%) is bactericidal, sporostatic and mycobacteriostatic in action with variable effect on yeasts and protozoa, and viruses. 4% CHG is effective against *C.difficile* spores
- CHG is widely used in antiseptic products, particularly in hand rub, hand wash solutions, mouthwash, body wash solutions (used before surgery), and as skin disinfectant (for dressing). It is also used as preservative
- The wide use of CHG is due to its broad-spectrum efficacy, substantivity for the skin, longer acting action than alcohol hand rub and low irritation
- However, its activity is pH dependent and is greatly reduced in the presence of organic matter.

Halogens

Among the halogens, iodine and chlorine have antimicrobial activity. They exist in free state, and form salt with sodium and most other metals.

Iodine

It is used as a skin antiseptic and kills microorganisms by oxidizing cell constituents and iodinating cell proteins. At higher concentrations, it may even kill some spores.

- **Tincture of iodine:** It is a preparation of iodine (2%) in a water-ethanol solution of potassium iodide. It is an effective antiseptic, but can cause skin allergy and yellowish discoloration
- **Iodophor:** It is prepared by complexing iodine with an organic carrier such as povidone (forming povidone-iodine). It is water soluble, stable, and non-staining, and releases iodine slowly to minimize skin burns and irritation. They are used as preoperative antiseptics as well as disinfectants in laboratories. Some popular brands available are Wescodyne and Betadine.

Chlorine

It is one of the most commonly available disinfectant.

- **Preparations:** It may be available as—(1) chlorine gas, (2) sodium hypochlorite (household bleach, 5.25%), or (3) calcium hypochlorite (bleaching powder)
- **Mechanisms:** All preparations yield hypochlorous acid (HClO), which causes oxidation of cellular materials and destruction of vegetative bacteria and fungi, but not sporess
- **Uses:** It is used as:
    - Chlorine is used for disinfection of municipal water supplies and swimming pool water. It is also employed in the dairy and food industries
    - Sodium hypochlorite (1%) is used as laboratory disinfectant for disinfecting blood and other specimens and is also used to disinfect the blood spillage area

- Calcium hypochlorite (bleaching powder) is used as bleaching agent—to remove the stain from clothes.

❖ **Disadvantages:** (1) Organic matter interferes with its action, hence excess chlorine always is added to water to ensure microbial destruction, (2) Carcinogenic-chlorine reacts with organic compounds to form carcinogenic trihalomethanes, (3) Daily preparation—sodium hypochlorite is unstable, disintegrates and the chlorine evaporates on exposure to sunlight or air. Hence, it has to be prepared daily and should be kept away from sunlight and air, (4) They are not active against *Giardia* and *Cryptosporidium*, (5) Sodium hypochlorite is corrosive and should be handled cautiously.

Oxidizing Agents

Hydrogen Peroxide (H_2O_2)

It is a strong oxidizer; is used as high level disinfectant as well as chemical sterilant.

❖ **Mode of action:** It breaks off H_2O_2, liberates toxic free hydroxyl radicals which are the active ingredients, attack membrane, lipid, DNA, and other cellular components

❖ **Concentration:** H_2O_2 is effective against most organisms at concentration of 3-6%, while catalase producing organisms and spores require higher concentration (10%) of H_2O_2

❖ **Use:** H_2O_2 is used to disinfect ventilator, soft contact lenses, and tonometer biprisms. Vaporized H_2O_2 is used for plasma sterilization (see highlight box below)

❖ **Advantages:** (1) H_2O_2 does not coagulate blood or does not fix tissues to surfaces, and in fact, it may enhance removal of organic matter from equipment, (2) it is less toxic to man, (3) it is environmental safe, (4) it is neither carcinogenic nor mutagenic.

Peracetic Acid

It is another recently described powerful oxidizing agent, even more active than H_2O_2. Concentrations of less than 1% are sporicidal even at low temperature.

❖ **Use:** It is a high level disinfectant and chemical sterilant; often used in conjunction with H_2O_2, to disinfect hemodialyzers and is also used in plasma sterilization (see below). It is also used for sterilizing endoscopes

❖ However, peracetic acid may corrode steel, iron, copper, brass and bronze.

Plasma Sterilization

Plasma sterilization is a recently introduced sterilization method; increasingly used nowadays (Fig. 3.7).

❑ **Principle:** Plasma refers to a gaseous state consisting of ions, photons and free electrons and neutral uncharged particles (such as O and OH). These active agents present in plasma such as photons of ultraviolet rays and radicals (e.g. O and OH) are capable of killing microorganisms and spores efficiently

Contd...

Contd...

❑ **Plasma sterilizer:** It is a special device used to create the plasma state (commercial brands, such as Sterrad and Plazlyte). They maintain a uniform vacuum inside the chamber

❑ Chemical sterilants, such as H_2O_2 alone or a mixture of H_2O_2 and peracetic acid are used which provide O and OH

❑ Low temperature is maintained (37–44°C) throughout the process which preserves the integrity of heat labile items. Cycle duration is around 1 hour, much faster than ethylene oxide sterilizer

❑ **Uses:** Plasma sterilizer is used for sterilization of materials and devices that cannot tolerate high temperatures and humidity of autoclave, such as some plastics, electrical devices, and corrosion-susceptible metal alloys

❑ **Sterilization control:** Efficacy of plasma sterilization is tested by using *Geobacillus stearothermophilus*.

Heavy Metal Salts

Salts of heavy metals, such as **mercury, silver, arsenic, zinc** and **copper** were widely used in the past as germicides, but recently these have been superseded by other less toxic and more effective germicides. However, some of the metallic salts are still in use, for examples:

❖ Silver sulfadiazine is used on burns surfaces

❖ Silver nitrate (1%) eye drops were used for infants to prevent ophthalmia neonatorum. It is now replaced by erythromycin in many hospitals

❖ Copper sulfate is an effective fungicide (algicide) in lakes and swimming pools

❖ Mercury salts, such as mercuric chloride, thiomersal and mercurochrome were known antiseptics and antifungal agents, but are not used nowadays. However, thiomersal

Fig. 3.7: Plasma sterilizer (Sterrad)

Source: Johnson & Johnson Pvt. Ltd (with permission).

(merthiolate) is used as preservative in vaccines, sera and other immunoglobulin preparations
- **Mechanism of action:** Heavy metals combine with bacterial cell proteins, often with their sulfhydryl groups, and inactivate them. They are more bacteriostatic than bactericidal.

Surface Active Agents

Surfactants (or surface active agents) are the compounds that lower the surface tension (or interfacial tension) between two liquids or between a liquid and a solid. Surfactants may act as detergents, wetting agents, and emulsifiers. Because of their amphipathic nature (i.e. they have both polar hydrophilic and nonpolar hydrophobic ends), the detergents solubilize otherwise insoluble residues and are very effective cleansing agents. They are classified into anionic, cationic, nonionic and amphoteric compounds, of which the cationic detergents are effective disinfectants.

- **Cationic surfactants:** Quaternary ammonium compounds are the most popular cationic disinfectants in use
  - Quaternary ammonium compounds have a positively charged quaternary nitrogen and a long hydrophobic aliphatic chain
  - They disrupt microbial membranes and may also denature proteins
  - They kill most bacteria (gram-positives are better killed than gram-negatives) but not *M. tuberculosis* or spores
  - They are stable, and nontoxic to skin, but are inactivated by acidic pH, organic matter, hard water and soap
  - Cationic detergents are often used as disinfectants for food utensils and small instruments and as skin antiseptics
  - Examples of quaternary ammonium compounds include:
    - Alkyl trimethyl ammonium salts
    - Acetyl trimethyl ammonium bromide (cetavlon or cetrimide)
    - Benzalkonium chloride
    - Cetylpyridinium chloride.
- **Anionic surfactants**, e.g. common soaps: They have strong detergent but weak antimicrobial properties. These agents are most active at acidic pH
- **The amphoteric surfactants:** They possess detergent properties of anionic compounds and antimicrobial activity of cationic compounds. They are active over a wide range of pH but the activity is reduced in presence of organic matter. Examples include **Tego compounds** which are the commercial name of a series of disinfectants whose active ingredient is DDAG (dodecyl-di-aminoethyl-glycine). They are used as antiseptics in dental practice, but are known to cause allergic reactions.

Dyes

Aniline and acridine are the two groups of dyes that have been used extensively as skin and wound antiseptics.

Aniline Dyes

These include crystal violet, gentian violet, brilliant green and malachite green.
- They are more active against gram-positive bacteria than gram-negative bacteria and have no activity against *M. tuberculosis*
- They are non-toxic and non-irritant to the tissues
- Their activity is reduced in presence of organic material, such as pus
- They interfere with the synthesis of peptidoglycan component of the cell wall
- These dyes are used in the laboratory as selective agents in culture media (e.g. malachite green in Lowenstein-Jensen medium, which is a selective medium used for isolation of *Mycobacterium tuberculosis*).

Acridine Dyes

These include acriflavine, euflavine, proflavine and aminacrine.
- They are affected very little by the presence of organic material
- They are also more active against gram-positive bacteria than gram-negative bacteria but are not as selective as the aniline dyes
- They interfere with the synthesis of nucleic acids and proteins in bacterial cells.

Gaseous Sterilization

Ethylene oxide (EtO)

Ethylene oxide (EtO) is one of the widely used gaseous chemical sterilants in present days.
- **Mechanism of action:** EtO has both microbicidal and sporicidal activity; acts by combining with cell proteins
- **EtO sterilization cycle:** It comprises of three stages, carried out in a special equipment called ethylene oxide sterilizer (Fig. 3.8):
  - **Preconditioning:** First, the air is removed from the chamber and a leakage test is performed. Then the appropriate conditions (temperature, pressure and humidity) for sterilization will be set in chamber
  - **Sterilization:** The EtO is allowed to enter the chamber. At EtO concentration of 700 mg/liter and 40–80% relative humidity, sterilization is achieved in 4–5 hours at 38°C or 1 hour at 55°C
  - **Aeration (Degassing):** The EtO residues left on surgical instruments and tubing may be toxic to the patients and staff. Therefore, extensive aeration of the sterilized materials for 8–12 hours is necessary to remove residual EtO.

Fig. 3.8: Ethylene oxide sterilizer

Source: 3M India Pvt. Ltd (with permission).

- **Disadvantages:** (i)EtO is highly inflammable, irritant, explosive and carcinogenic. EtO is usually supplied in a 10–20% concentration mixed with inert gases like either CO2 or dichlorodifluoromethane (ii) long duration of cycle (12–14 hours or more), and (iii) high cost of instrument and consumables
- **Advantage:** (i) Large sterilizing volume/chamber capacity, (ii) Items that cannot withstand high temperature of autoclave can be sterilized, (iii) EtO is **highly diffusible**, penetrates areas that cannot be reached by steam, (iv) non corrosive to plastic, metal and rubber materials
- **Sterilization control:** Spores of *Bacillus atrophaeus* is used as biological indicator to check the effectiveness of sterilization
- **Use:** Ethylene oxide is extensively used for sterilization of many heat sensitive items, such as disposable plastic Petri dishes and syringes, heart-lung machine components, sutures, catheters, respirators and dental equipment.

Low Temperature Steam Formaldehyde

This was widely used for fumigation of operation theaters, wards and laboratories. However, this method is no longer preferred, and is being replaced by modern methods of fumigation.

- Formaldehyde gas is generated by adding 150 g of $KMnO_4$ to 280 mL formalin for every 1000 cu. feet of room volume
- The room should be sealed for 48 hours
- The gas is irritant and toxic when inhaled. Hence, after completion of disinfection, the effect of irritant vapors should be nullified by exposure to ammonia vapor.

Betapropiolactone (BPL)

BPL gas (0.2%) is active against all microorganisms including spores, however, it has a low penetrating power and is carcinogenic, hence not used for fumigation. It is used for inactivation of vaccines.

Sporicidal Agents

Sporicidal agents are enlisted in Table 3.3.

Sterilization Indicators

There are three types of indicators used for monitoring the sterilization process of sterilizers such as autoclave (steam sterilizer), ethylene oxide sterilizer and plasma sterilizers: (i) physical indicator, (ii) chemical indicators (six classes) and (iii) biological indicator (Table 3.4).

Common chemical disinfectants used in the hospital and their applications have been enlisted in Table 3.5 and Fig. 3.9.

Comparison of the range of activity against various microorganisms and level of disinfection that can be achieved by common chemical sterilants/disinfectants is given in Table 3.6.

TESTING OF DISINFECTANTS

Phenol Coefficient (Rideal Walker) Test

Phenol coefficient is determined by the dilution of the disinfectant in question which sterilizes/disinfects the suspension of *Salmonella* Typhi in a given time divided by the dilution of phenol which sterilizes/disinfects the suspension in the same time.

- If the phenol coefficient is more than 1, the test disinfectant is said to be more effective than phenol
- The drawbacks of Rideal Walker test are—(1) only the phenolic compounds can be assessed, (2) it does not assess the ability of the disinfectant to act in presence of organic matter.

Phenolic coefficient of a test disinfectant =

$$\frac{\text{Highest dilution of the test disinfectant that kills S. Typhi in a given time}}{\text{Highest dilution of phenol that kills S. Typhi in the same time}}$$

Table 3.3: Sporicidal agents

• Ethylene oxide • Formaldehyde • Glutaraldehyde • Hydrogen peroxide • O-phthalic acid	• Peracetic acid • Plasma sterilization • Heat: Autoclave, hot air oven, incineration

Table 3.4: Indicators used for monitoring the sterilization process of autoclave (steam sterilizer), ethylene oxide sterilizer and plasma sterilizers

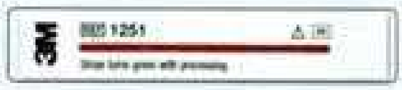
Physical or mechanical indicators: These are the digital displays of the sterilizer equipment which verify, whether the parameters of sterilization cycle are met or not; such as temperature, time and pressure, etc.

Chemical indicators: They are used to monitor the attainment of one or more parameters required for a satisfactory sterilization process. They use heat or chemical sensitive materials which undergo a color change if the sterilization parameter (e.g. time, steam quality and temperature) for which they were used is achieved. They are of 6 types—I to VI

Type I	• Also called as process indicator or exposure indicator or external pack control. • Used on external surface of each pack, to indicate that the pack has been directly exposed to the sterilant. However, it does not assure sterility
Type II	• It is called as *Bowie-Dick* test; used only for pre-vacuum autoclave (steam sterilizer) • It must be used daily in empty cycle before the first load • Used for equipment control, i.e. it checks the efficacy of air removal, air leaks and steam penetration and ensures that the autoclave is functioning well
Type III	It is a single parameter indicator, obsolete now
Type IV	• Internal pack control indicator, used inside each pack • It is designed to measure any two of the critical variables: time, steam quality and temperature
Type V	• It is also an internal pack control indicator, used inside each pack • It measures all three variables: time, steam quality and temperature • It should be used for packs containing critical items • It is considered as equivalent to biological indicators
Type VI	It is also called as emulating indicator. It is cycle specific: the "stated values" correspond to the critical variables that the sterilizer manufacturer has defined for that cycle of sterilization process


Type I


Type II (Bowie-Dick)



Biological indicator (BI)

BI is the best indicator, because here the bacterial spores are used to check the effectiveness of sterilization. The spores are highly resistant and will be destroyed only when the effective condition is achieved

- *Geobacillus stearothermophilus* for autoclave, hydrogen peroxide gas plasma and liquid acetic acid sterilizer
- *Bacillus atrophaeus* for ethylene oxide sterilizer and dry heat

Indication: BI must be used at least weekly (once a day if a sterilizer is used frequently, e.g. several loads per day) and must be used for any load containing implants.

Types: Various types of BI are available which give result in a specific time frame; next generation BI (48 hours), rapid read-out BI (24 minutes to 4 hours) and spore-strips (7 days, obsolete now).

Adapted from Centers for disease control and prevention (CDC) (with permission).

Chick Martin Test

It is a modification of Rideal and Walker test, in which the disinfectants act in the presence of organic matter (e.g. dried yeast, feces, etc.) to simulate the natural conditions.

Capacity (Kelsey-Sykes) Test

It tests the capacity of a disinfectant to retain its activity when repeatedly used microbiologically (i.e. when the microbiological load keeps increasing).

In-use (Kelsey-Maurer) Test

In-use test is used to determine whether an actively used solution of disinfectant in a clinical setting is microbiologically contaminated. It should be routinely performed in the hospital once in every 3 months. For detail of the procedure, refer author's Essentials of Practical Microbiology text book.

SPAULDING'S CLASSIFICATION OF MEDICAL DEVICES

Earle H. Spaulding devised a rational approach to classify the patient-care items and equipment of a hospital into four categories (as critical, semi-critical, non-critical patient care items, and non-critical environmental surfaces) according to the degree of risk for infection involved in use of these items. This classification scheme is so clear and logical that it has been retained, refined, and successfully used by infection control professionals and others (Table 3.7).

Table 3.5: Common chemical disinfectants used in the hospital and their applications

Disinfectants	Uses
Alcohol	• Ethyl alcohol is used as surgical spirit (70%) in hand rubs (Fig. 3.9A) or, as antiseptics • Isopropyl alcohol—used for clinical thermometers and small instrument
Formaldehyde	• It is best used for—i) preservation of anatomical specimen, ii) formaldehyde gas fumigation for operation theaters in the past (not recommended now), iii) preparation of toxoid from toxin • It is toxic and irritant when inhaled, and corrosive to the metals
Glutaraldehyde (2%) (Fig. 3.9B)	• Best used for endoscopes and cystoscopes as it is non-corrosive to metals • It is available in inactive form, has to be activated by alkalinization before use. Once activated, it remains active only for 14 days • Combination of glutaraldehyde and quaternary ammonium compound is used for fogging/ sterilization of operation theater (e.g. Bacillocid-Fig. 3.9C). This has largely replaced formaldehyde fumigation of operation theaters
ortho-Phthalaldehyde (0.55%)	• It can also be used for sterilization of endoscopes and cystoscopes and has the advantage over glutaraldehyde as does not require prior activation
Phenolic compounds	• Phenol, cresol and lysol are used as surface disinfectants in hospitals • Chloroxylenol—It is used as an antiseptics, commercially available as Dettol (Fig. 3.9E)
Biguanide	Chlorhexidine is used as an antiseptics, commercially available as Savlon. It is also used as hand wash (4%) and hand rub solutions (Fig. 3.9D)
Iodophor (Povidone iodine)	It is used as a skin antiseptic for wounds, preoperatively and also before venepuncture; commercially available as Wescodyne or Betadine (Fig. 3.9F)
Chlorine	It is used for disinfection of portable water supplies and also as bleaching agent to remove the stain from clothes
Sodium hypochlorite (Fig. 3.9G)	Known as household bleach, used as laboratory disinfectant in discarding jar (1%), for blood spillages and infected needle or syringe and for disinfection of blood and other specimens before final disposal
Hydrogen peroxide	H_2O_2 is used to disinfect ventilator, soft contact lenses, and tonometer biprisms Vaporized H_2O_2 is used for plasma sterilization
Quaternary ammonium compounds	Example includes benzalkonium chloride. This is used as surface disinfectant alone or in combination with other disinfectants such as glutaraldehyde
Ethylene oxide (Fig. 3.8)	Used in Central Sterile Services Department (CSSD) for sterilization of many heat sensitive items such as disposable plastic petri dishes and syringes, heart-lung machine components, sutures, catheters, respirators and dental equipment

Figs 3.9A to G: Common chemical disinfectants used in the hospital (A to G): **A.** Sterillium (Alcohol hand rub); **B.** Cidex (2% Glutaraldehyde used for disinfection of endoscopes); **C.** Bacillocid (Glutaraldehyde-based combination product used for OT surface cleaning and fogging); **D.** Microshield (4% Chlorhexidine used as hand wash); **E.** Dettol (Chloroxylenol used as skin antiseptic); **F.** Betadine (Povidone Iodine used as skin antiseptic); **G.** Sodium hypochlorite solution (used as laboratory disinfectant)

Table 3.6: Common disinfectants and their spectrum of action

Germicide and their concentrations	Level of disinfectant	Bacteria and enveloped viruses	Fungi	Un-enveloped viruses	*M. tuberculosis*	Spore	Inactivated by organic matter
Glutaraldehyde (2%)	High/CS	+	+	+	+	+	–
Formaldehyde (3–8%)	High/CS	+	+	+	+	+	–
H_2O_2 (3–25%)	High/CS	+	+	+	+	+	+/–
Chlorine (100–1000 ppm of free chlorine)	High	+	+	+	+	+/–	+
Isopropyl alcohol (60–95%)	Intermediate	+	+	+/-	+	–	+/–
Phenol (0.4–5%)	Intermediate	+	+	+/-	+	–	–
Chlorhexidine gluconate (2-4%)	Low	+	+/–	+/–	+/–	+/– (static)	+
Iodophore (30–50 ppm of free iodine)	Intermediate	+	+	+	+/–	–	+
Quaternary ammonium compounds (0.4–1.6%)	Low	+	+/–	–	–	–	+

Abbreviations: CS, chemical sterilant; +, effectively kills; –, unable to kill; +/–, variably kills; ppm, parts per million.

Table 3.7: Spaulding's classification of medical devices

Medical device	Definition	Examples	Recommended sterilization/ disinfection
Critical device	Enter a normally sterile site	Surgical instruments, cardiac and urinary catheters, implants, eye and dental instruments	Heat-based sterilization, chemical sterilant or high-level disinfectant
Semi-critical device	Comes in contact with mucus membranes or minor skin breaches	Respiratory therapy equipment, anesthesia equipment, endoscopes, laryngoscope, rectal/ vaginal/esophageal probes	High-level disinfectant
Non-critical devices	Comes in contact with intact skin	BP cuff, ECG electrodes, bedpans, crutches, stethoscope, thermometer	Intermediate-level or low-level disinfectant
Non-critical environmental surfaces	Less direct contact with patient	Surfaces of medical equipment, examination table, computers	Low-level disinfectant

Abbreviations: BP, blood pressure; ECG, electrocardiography.

CENTRAL STERILE SUPPLY DEPARTMENT

Central Sterile Services Department (CSSD) is an integrated place in hospitals that performs sterilization of medical devices, equipment and consumables; that are used in the operating theater (OT) of the hospital and also for other aseptic procedures.

The processing area of CSSD consists of four unidirectional zones starting from an unsterile area to a sterile area separated by physical barrier (Fig. 3.10).

Decontamination area → Packaging area → Sterilization area → Sterile storage area

1. **Decontamination area:** The items are collected and then decontaminated/cleaned by either manual wash or by automated machines (ultrasonic washer and washer disinfector).
2. **Packaging area:** Here, the items (medical devices) are enclosed in materials or a container designed to allow the penetration and removal of the sterilant during sterilization and then to protect the device from contamination and other damage following sterilization and until the time of use.
3. **Sterilization area:** The packed medical devices received from packaging area are subjected to sterilization process by autoclave, ethylene oxide sterilizer or plasma sterilizer.
4. **Sterile storage area:** After sterilization the serialized items are stored in this area. It has an issue counter to supply the items to OTs and various other areas in the hospital.

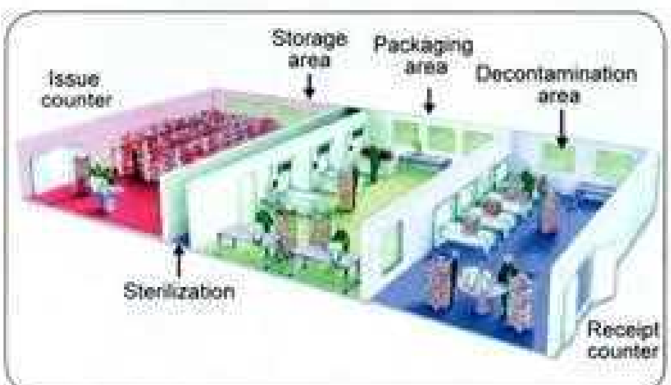


Fig. 3.10: Central Sterile Services Department (CSSD)

EXPECTED QUESTIONS

I. Essay:

1. Define sterilization and disinfection. Describe principle and uses of autoclave. Add a note on chemical sterilants.

II. Write short notes on:

1. Sterilization by dry heat.
2. Pasteurization.
3. Membrane filters.
4. Gaseous sterilization.
5. Testing of disinfectants.
6. Central Sterile Services Department (CSSD)

III. Multiple Choice Questions (MCQs):

1. Tyndallization is a type of:
a. Intermittent sterilization
b. Boiling
c. Pasteurization
d. Dry heat

2. *Geobacillus stearothermophilus* is used as indicator for efficacy of:
a. Hot air oven b. Autoclave
c. Filtration d. Ultraviolet rays

3. Which of the following is most resistant to sterilization?
a. Tubercle bacilli b. Viruses
c. Spores d. Prions

4. Endoscope is sterilized by:
a. Glutaraldehyde b. Formaldehyde
c. Autoclaving d. Hot air oven

5. Which is a form of cold sterilization?
a. Infrared rays b. Steam sterilization
c. Gamma rays d. UV rays

6. Sterilization results in reduction of microorganisms and their spores by:
a. 10^6 log CFU b. 10^4 log CFU
c. 10^3 log CFU d. 10^2 log CFU

7. The decreasing order of resistance of microorganisms to disinfectant or sterilizing agents is:
a. Bacterial spores > Prions > Non-enveloped viruses > Enveloped viruses
b. Prions > Bacterial spores > Non-enveloped viruses > Enveloped viruses
c. Prions > Bacterial spores > Enveloped viruses > Non-enveloped viruses
d. Bacterial spores > Non-enveloped viruses > Prions > Enveloped viruses

8. Moist heat at a temperature Below 100°C include all, *except*:
a. Pasteurization b. Tyndallization
c. Water bath d. Inspissation

9. Which of the following method satisfies the definition of sterilization?
a. Intermittent sterilization
b. Cold sterilization
c. Fractional sterilization
d. None

10. The sterilization control of membrane filters includes:
a. *Serratia marcescens* b. *G. stearothermophilus*
c. *Bacillus atrophaeus* d. *Bacillus subtilis*

11. Which of the following disinfectant is used for hand wash?
a. Ethylene oxide b. Formaldehyde
c. Chlorhexidine d. Povidone iodine

12. Which of the following is used for disinfection of blood spillage area?
a. Phenol b. Hypochlorite
c. Lysol d. Formaldehyde

13. Which of the following disinfectant is used for fogging of operation theatres?
a. Formaldehyde
b. Glutaraldehyde based
c. Hydrogen peroxide based
d. All of the above

14. Which of the following disinfectant is used in plasma sterilization?
a. Formaldehyde b. Glutaraldehyde
c. Hydrogen peroxide d. Ethylene oxide

15. Sporicidal agents are all, *except*:
a. Ethylene oxide b. Formaldehyde
c. Absolute alcohol d. Glutaraldehyde

16. Which test is used to determine whether an actively used solution of disinfectant in a clinical setting is microbiologically contaminated or not?
a. Phenol Coefficient (Rideal-Walker) Test
b. Chick Martin Test
c. Capacity (Kelsey-Sykes) Test
d. In-use (Kelsey-Maurer) Test

17. Which of the following testing method tests the ability of the disinfectant to retain its activity in the presence of organic matter?
a. Phenol Coefficient (Rideal-Walker) Test
b. Chick Martin Test
c. Capacity (Kelsey-Sykes) Test
d. In-use (Kelsey-Maurer) Test

18. Which test is used to determine the capacity of a disinfectant to retain its activity when repeatedly used microbiologically?
a. Phenol Coefficient (Rideal-Walker) Test
b. Chick Martin Test
c. Capacity (Kelsey-Sykes) Test
d. In-use (Kelsey-Maurer) Test

19. Rideal-Walker test— all are true, *except*:
a. It can be used to test phenolic disinfectants only
b. It tests ability of the disinfectant to act in presence of organic matter.
c. Disinfectant is considered to be satisfactory if Phenol coefficient is <1
d. *S. Typhi* is used as a test organism

20. Which of the following chemical indicator is known as exposure indicator or external pack control?
a. Class I b. Class II
c. Class IV d. Class V

Answers

1. a 2. b 3. d 4. a 5. c 6. a 7. b 8. b 9. d 10. a 11. c 12. b 13. d 14. c 15. c 16. d
17. b 18. c 19. b 20. a

Culture Media and Culture Methods

4
CHAPTER

Chapter Preview

- Culture media
  - Constituents of culture media
  - Types of culture media
- Culture methods
  - Methods of culture
  - Anaerobic culture methods
- Preservation of microorganisms
- Methods of isolating bacteria in pure cultures

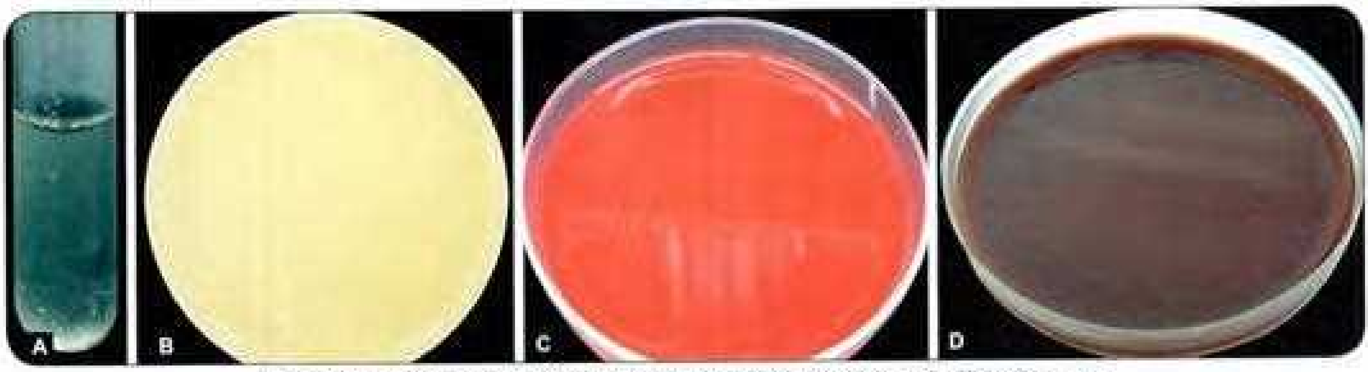
CULTURE MEDIA

Culture media are required to isolate the bacteria from the clinical specimens; following which the appropriate biochemical tests can be performed to identify the causative agent.

CONSTITUENTS OF CULTURE MEDIA

The basic constituents of culture media are:

- **Water:** Distilled water or potable water with low mineral content is suitable for culture media preparation
- **Electrolytes:** Sodium chloride or other electrolytes
- **Peptone:** It is a complex mixture of partially digested proteins
  - Source: It is obtained from lean meat or other protein material, such as heart muscle, casein or fibrin, or soya flour usually by digestion with proteolytic enzymes, such as pepsin
  - Constituents: It contains proteoses, aminoacids, inorganic salts (phosphates, potassium and magnesium), accessory growth factors like nicotinic acid and riboflavin
  - Special brands: Apart from standard grades of bacteriological peptones, some manufacturers supply special grades of peptones which are used for special purposes, e.g. neopeptone, proteose peptone, mycological peptone, etc.
- **Agar:** It is used for solidifying the culture media. It is commercially available in powder form; melts in water after boiling and jellifies after cooling
  - Agar, also called 'agar-agar' is prepared from the cell wall of variety of seaweeds (red algae of species *Gelidium* and *Gracilaria*)
  - **Components:** It contains mainly cell wall derived long-chain polysaccharide (D-galactopyranose units) and a small amount of protein-like material, long chain fatty acids and traces of inorganic salts, such as calcium and magnesium
  - **Agar is preferred over gelatin for solidification:** Agar is bacteriologically inert, it melts at 95°C and usually solidifies at 42°C. Agar does not add any nutritive property to the culture medium. Whereas gelatin is liquefied by a number of bacteria, it melts at 24°C, and remains in liquid state at room temperature
  - **Concentration of agar** used depends on the manufacturer instructions and the purpose for which it is used
    - For solid agar preparation: It is used in concentration of 1-2% (Japanese agar 2% or New Zealand agar 1.2%)
    - For semisolid agar: 0.5%
    - For solid agar to inhibit *Proteus* swarming: 6%.
  - **Preparation of agar media:** The appropriate amount of agar powder is added to water and the mixture is dissolved and then sterilized by placing it in an autoclave. When the temperature of the molten agar comes down to 45°C, it is poured to the Petri dishes and then allowed to set for 20 minutes.
- **Meat extract:** It is a commercial preparation (Lab-Lemco, Oxoid) of highly concentrated meat stock, usually made from beef. It contains protein degradation products, inorganic salts, carbohydrates and growth factors
- **Yeast extract:** It is prepared commercially from washed cells of Baker's yeast. It contains aminoacids, inorganic salts (potassium and phosphates) and carbohydrates



Figs 4.1A to D: A. Peptone water; **B.** Nutrient agar; **C.** Blood agar; **D.** Chocolate agar

Source A to D: Department of Microbiology, JIPMER, Puducherry (with permission).

- **Malt extract:** It consists of maltose (about 50%), starch, dextrin, glucose and 5% protein products
- **Blood and serum:** They are important components of enriched media and provide extra nutrition to fastidious bacteria
  - Usually 5–10% of sheep blood is used. Horse, ox or human blood can also be used
  - Blood should be collected aseptically and rendered noncoagulable by defibrillation (by shaking the blood in a bottle containing sterile glass beads) or adding oxalate or citrate
  - Serum is sterilized by filtration after collection.

TYPES OF CULTURE MEDIA

Bacteriological culture media can be classified in two ways.

A. Based on consistency, culture media are grouped into:

1. Liquid media (or broth)
2. Semisolid media
3. Solid media

B. Based on the growth requirements, culture media are classified as:

1. **Routine laboratory media:** They are prepared from nutrients, such as aqueous extract of meat, peptone, etc. They can further be classified into various types based on functional use or application, as follows—
   - Simple/basal media
   - Enriched media
   - Enrichment broth
   - Selective media
   - Differential media
   - Transport media
   - Anaerobic media.
2. **Defined or synthetic media:** They are prepared from pure chemical substances and the exact composition of the media is known
   - Simple synthetic media
   - Complex synthetic media.

Routine Laboratory Media

Simple/Basal Media

They contain minimum ingredients that support the growth of non-fastidious bacteria. Examples include—

- **Peptone water:** It contains peptone (1%) + NaCl (0.5%) + water (Fig. 4.1A)
- **Nutrient broth:** It is made up of peptone water + meat extract (1%). It is available in three forms: (1) meat extract, (2) meat infusion, (3) meat digest broth
- **Nutrient agar:** It is made up of nutrient broth + 2% agar (Fig. 4.1B)
- **Semisolid medium:** It is prepared by reducing the concentration of agar to 0.2–0.5 %.

Uses of Basal Media

The basal media are used for:

- Testing the non-fastidiousness of bacteria
- They serve as the base for the preparation of many other media
- Nutrient broth is used for studying the bacterial growth curve
- Nutrient agar is the preferred medium for:
  - Performing the biochemical tests, such as oxidase, catalase and slide agglutination test, etc.
  - To study the colony morphology
  - Pigment demonstration.
- Semisolid medium is used for: (1) demonstrating motility of the bacteria; motile bacteria spread throughout the semisolid medium, making the medium hazy, (2) maintaining stock culture.

Enriched Media

When a basal medium is added with additional nutrients, such as blood, serum or egg, it is called enriched medium. In addition to non-fastidious organisms, they also support the growth of fastidious nutritionally exacting bacteria. Examples include:

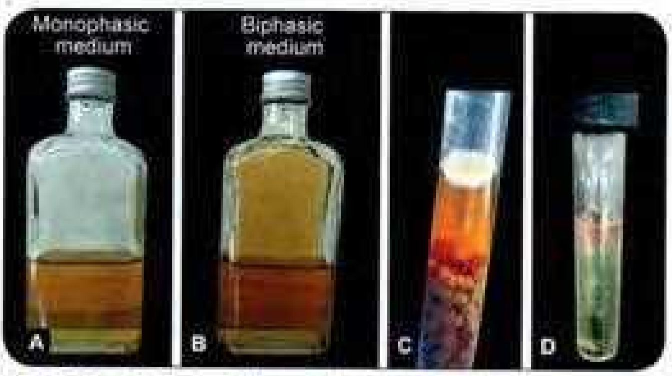
- **Blood agar:** It is prepared by adding 5–10% of sheep blood to the molten nutrient agar at 45°C (Fig. 4.1C). It is the most widely used medium in diagnostic bacteriology. Blood agar also tests the hemolytic property of the bacteria, which may be either: (1) partial or α (green) hemolysis and (2) complete or β hemolysis (described in detail in Chapter 5)

- **Chocolate agar:** It is the heated blood agar, prepared by adding 5–10% of sheep blood to the molten nutrient agar at 70°C, so that the RBCs will be lysed and the content of RBCs will be released, changing the color of the medium to brown (Fig. 4.1D). It is more nutritious than blood agar, and even supports certain highly fastidious bacteria, such as *Haemophilus influenzae* that does not grow on blood agar
- **Loeffler's serum slope:** It contains serum. It is used for isolation of *Corynebacterium diphtheriae*
- **Blood culture media:** They are also enriched media, used for isolating microorganisms from blood. They are either monophasic or biphasic media
  - Monophasic medium: It contains brain-heart infusion (BHI) broth (Fig. 4.2A)
  - Biphasic medium: It has a liquid phase containing BHI broth and a solid agar slope made up of BHI agar (Fig. 4.2B).

Enrichment Broth

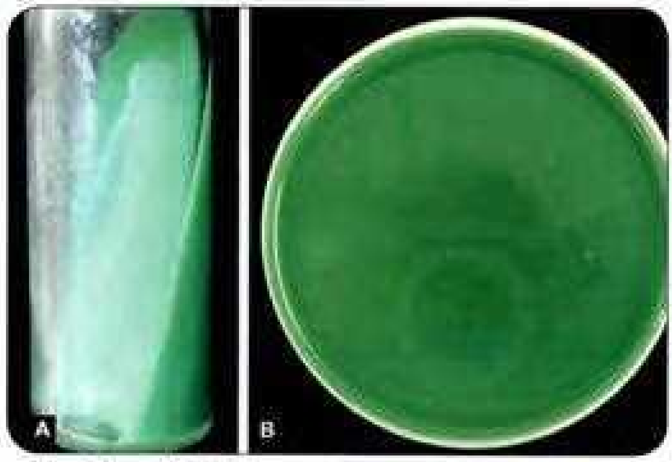
They are the liquid media added with some inhibitory agents which selectively allow certain organism to grow and inhibit others. This is important for isolation of the pathogens from clinical specimens which also contain normal flora (e.g. stool and sputum specimen). Examples for enrichment broth include:

- Tetrathionate broth—Used for *Salmonella* Typhi
- Gram-negative broth—Used for isolation of *Shigella*
- Selenite F broth—Used for isolation of *Shigella*
- Alkaline peptone water (APW)—Used for *Vibrio cholerae*.



Figs 4.2A to D: A. Brain-heart infusion broth; **B.** Biphasic medium (Brain-heart infusion broth/agar); **C.** Robertson's cooked meat medium; **D.** Thioglycollate broth

Source A to C: Department of Microbiology, JIPMER, Puducherry; D. Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (with permission).



Figs 4.3A and B: A. Lowenstein-Jensen medium; **B.** TCBS agar

Source: Department of Microbiology, JIPMER, Puducherry (with permission).

Selective Media

They are solid media containing inhibitory substances that inhibit the normal flora present in the specimen and allow the pathogens to grow.

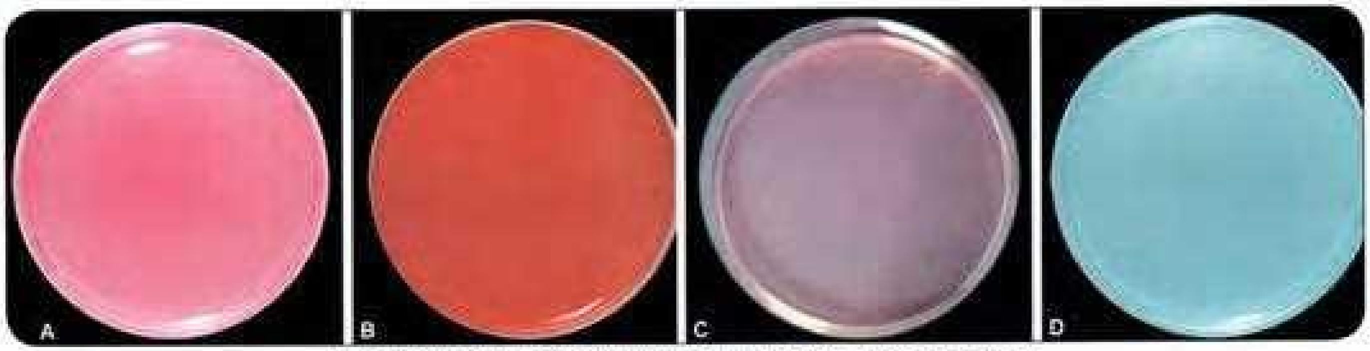
- **Lowenstein-Jensen (LJ) medium:** It is used for isolation of *Mycobacterium tuberculosis* (Fig. 4.3A)
- **Thiosulfate citrate bile salt sucrose (TCBS) agar:** It is used for isolation of *Vibrio* species (Fig. 4.3B)
- **DCA (deoxycholate citrate agar and XLD (xylose lysine deoxycholate) agar:** They are used for the isolation of enteric pathogens, such as *Salmonella* and *Shigella* from stool (Fig. 4.4A and B)
- **Potassium tellurite agar (PTA):** It is is used for isolation of *Corynebacterium diphtheriae*
- **Wilson Blair bismuth sulfite medium:** It is used for isolation of *Salmonella* Typhi.

Transport Media

They are used for the transport of the clinical specimens suspected to contain delicate organism or when delay is expected while transporting the specimens from the site of collection to the laboratory (Table 4.1). Bacteria do not multiply in the transport media, they only remain viable.

Table 4.1: Transport media used for common bacteria

Organism	Transport media
Streptococcus	Pike's medium
Neisseria	Amies medium and Stuart's medium
Vibrio cholerae	• VR (Venkatraman-Ramakrishnan) medium • Autoclaved sea water • Cary Blair medium
Shigella, Salmonella	• Buffered glycerol saline • Cary Blair medium



Figs 4.4A to D: A. DCA; **B.** XLD agar; **C.** MacConkey agar; **D.** CLED agar

Source: Department of Microbiology, JIPMER, Puduchorry (with permission).

Differential Media

These media differentiate between two groups of bacteria by using an indicator, which changes the color of the colonies of a particular group of bacteria but not the other group.

- **MacConkey agar:** It is a differential and low selective medium, commonly used for the isolation of enteric gram-negative bacteria (Fig. 4.4C)
  - It differentiates organisms into LF or lactose fermenters (produce pink colored colonies, e.g. *Escherichia coli*) and NLF or non-lactose fermenters (produce colorless colonies, e.g. *Shigella*)
  - **Composition:** It contains peptone, lactose, agar, neutral red (indicator) and taurocholate
  - Most laboratories use combination of blood agar and MacConkey agar for routine bacterial culture.
- **CLED agar (cysteine lactose electrolyte-deficient agar):** This is another differential medium similar to MacConkey agar, capable of differentiating between LF and NLF. It is used as an alternative to combination of blood agar and MacConkey agar, for the processing of urine specimens (Fig. 4.4D)
  - Advantages over MacConkey agar: It is less inhibitory than MacConkey agar, supports the growth of gram-positive bacteria (except β hemolytic *Streptococcus*) and *Candida*
  - Advantage over blood agar: It can prevent the swarming of *Proteus*.

Anaerobic Culture Media

Anaerobic media contain reducing substances which take-up oxygen and create lower redox potential and thus permit the growth of obligate anaerobes, such as *Clostridium*. Examples are as follows:

- **Robertson's cooked meat (RCM) broth:** It contains chopped meat particles (beef heart), which provide glutathione (a sulfhydryl group containing reducing substance) and unsaturated fatty acids. It is the most widely used anaerobic culture medium (Fig. 4.2C). It is also used for maintenance of stock cultures.
- **Other anaerobic media** include:
  - Thioglycollate broth (Fig. 4.2D)
  - Anaerobic blood agar
  - BHIS agar (Brain-heart infusion agar) with supplements (vitamin K and hemin)
  - Neomycin blood agar
  - Egg yolk agar
  - Phenyl ethyl agar
  - *Bacteroides* bile esculin agar (BBE agar).

Defined or Synthetic Media

Chemically defined media are used for various experimental purposes. They are prepared exclusively from pure chemical substances in such a way that their composition, i.e. exact quantity of each chemical used is known.

Simple Synthetic Media

They contain a carbon and energy source, such as glucose, or lactose, and an inorganic source of nitrogen, usually in the form of ammonium chloride, phosphate or sulfate and various inorganic salts in a buffered aqueous solution. They provide the basic essentials for the growth of many non-fastidious heterotrophs, but they will not support the growth of fastidious bacteria.

Complex Synthetic Media

Here, in addition to the simple synthetic media certain aminoacids, purines, pyrimidines, and other growth factors are incorporated. Hence, they can also support the growth of more exacting bacteria.

CULTURE METHODS

The bacteriological culture is done in a laboratory, for the following purposes:

- Isolating bacteria in pure culture from the clinical specimens
- To perform biochemical tests for the identification of bacteria
- To perform antimicrobial susceptibility testing of the isolated bacteria
- To maintain stock cultures
- To obtain sufficient growth for the preparation of antigens
- For typing of bacterial isolates
- To estimate the viable bacterial count.

METHODS OF CULTURE

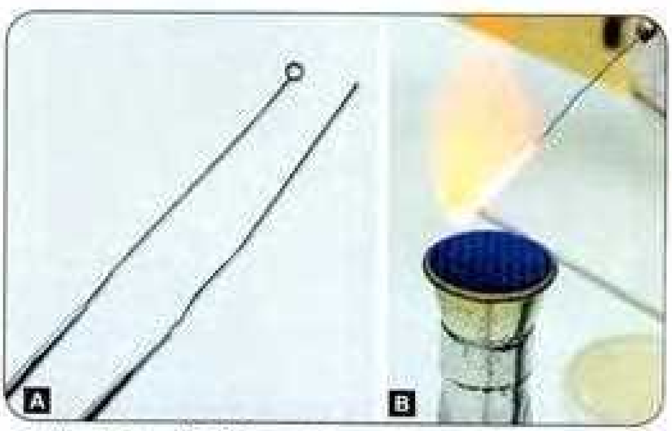
Loops and Straight Wires

Inoculation of specimen onto the culture media is carried out with the help of bacteriological loops and straight wires made up of platinum or nichrome wire (Fig. 4.5A).

- **Bacteriological loops** of 2–4 mm internal diameter are used for streaking the culture plates
- **Bacteriological straight wires:** They are used for making stroke culture and stab culture.
  (However, in lawn culture, wires are not used. Instead, sterile swab soaked in the broth culture of the bacteria is used)
- The inoculating straight wire or loop is first heated in the Bunsen flame by making it red hot (Fig. 4.5B) and then made cool waiting for 10 seconds
- The entire process of bacteriological culture method should be carried out in biological safety cabinet to prevent laboratory acquired infections (Fig. 4.6).

Streak Culture

It is the routine method employed for isolation of bacteria from the clinical specimen or for obtaining individual isolated colonies from a mixed culture of bacteria.



Figs 4.5A and B: A. Bacteriological loop and straight wire; **B.** Flaming the loop (red hot)

Source: A. Department of Microbiology, JIPMER, Puducherry (*with permission*).

Fig. 4.6: Biological safety cabinet

Source: Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (*with permission*).

Method of streaking

A loopful of specimen is smeared onto the surface of a dried solid culture plate near the peripheral area with the help of a sterile bacteriological loop to form the primary inoculum.

- From the primary inoculum, it is spread thinly over the culture plate by streaking with the loop in parallel lines to form the secondary, tertiary inoculum and finally a feathery tail end (Fig. 4.7A)
- Intermittent heating: The loop is flamed and cooled in between the different set of streaks to get isolated colonies
- The culture plate is incubated at 37°C for 12–18 hours (overnight)
- Confluent growth occurs at the primary inoculum and isolated colonies are obtained on the final streaks (Fig. 4.7B)
- Obtaining isolated colonies is the prerequisite to perform various biochemical tests to confirm the identification of bacteria.

Lawn or Carpet Culture

Lawn culture provides uniformly thick surface growth of the bacterium on the solid medium (Fig. 4.7C). There are two methods of obtaining lawn cultures, which are as follows:

- Swabbing: A sterile swab soaked in liquid bacterial culture is inoculated on to the culture plate and then incubated at 37°C overnight to obtain uniform lawn of bacterial growth on the surface of the culture plate
- Flooding: The surface of the culture plate is flooded with a liquid culture or suspension of the bacterium and then excess material is drained out.

Figs 4.7A to C: A. Streak culture (schematic representation); **B.** Isolated colonies grown by following streak culture; **C.** Lawn culture of a bacterial isolate to perform the antimicrobial susceptibility testing

Source: Department of Microbiology, JIPMER, Puducherry (with permission).

Lawn culture is useful for:

- Carrying out antimicrobial susceptibility testing by disk diffusion method (Fig. 4.7C)
- Bacteriophage typing
- Producing large amount of bacterial growth required for preparation of bacterial antigens and vaccines.

Stroke Culture

This is carried out on agar slopes or slants by streaking the straight wire in a zigzag fashion (Fig. 4.8A).

- Stroke culture provides a pure growth of bacterium for carrying out diagnostic tests
- Stroke culture is used in urease test.

Stab Culture

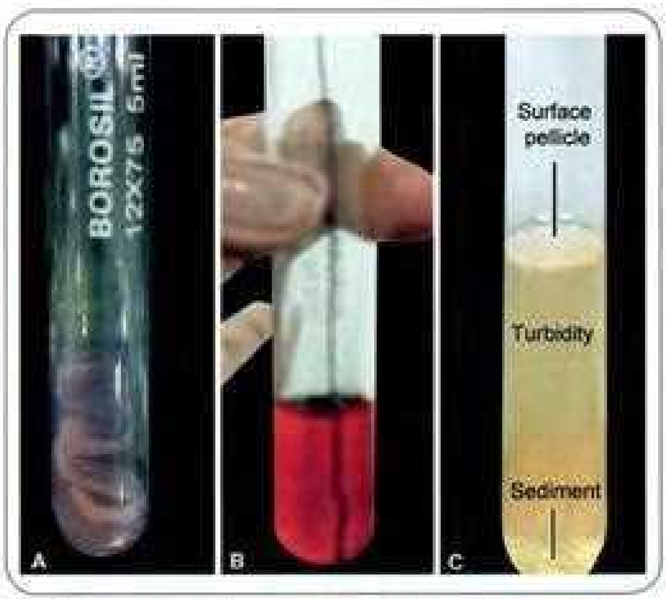
Stab culture is performed by stabbing the semisolid agar butt by a straight wire (Fig. 4.8B).

- Stab culture is used for—(1) maintaining stock cultures, (2) for demonstration of oxygen requirement of the bacteria by oxidative-fermentative (OF) test, (3) motility testing using semisolid agar
- Examples where stab culture is used are mannitol motility medium, nutrient agar semisolid butts, triple sugar iron agar test or TSI (here both stroke and stab cultures are made).

Liquid Culture

Liquid cultures in test tubes, screw-capped bottles (e.g. McCartney bottle) or flasks may be inoculated by touching with a loop or by adding the inoculum with pipettes or syringes.

- **Bacterial growth** is detected by observing the turbidity in the medium—(1) some bacteria produce uniform turbidity, (2) some produce granular turbidity with sediment at the bottom of the tube, (3) some aerobic bacteria form surface pellicles (Fig. 4.8C)
- **Uses:** Liquid cultures are useful for (1) blood culture, (2) for sterility testing, (3) water analysis
- **Advantages:** Liquid cultures are preferable for culture of—(1) specimen containing small quantity of bacteria, (2) specimens (e.g. blood) containing antibiotics and other antibacterial substances, as these inhibitory agents are neutralized by dilution in the medium. (3) It is also preferred when large yields of bacteria are required



Figs 4.8A to C: A. Stroke culture; **B.** Stabbing with inoculation wire (stab culture); **C.** Liquid culture in test tube (turbidity indicates growth)

Source: Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (with permission).

- **Disadvantages:** (1) Liquid cultures do not provide a pure culture from a mixed inoculum, (2) Identification of bacteria is not possible.

Pour-plate Culture

This is a quantitative culture method, used to estimate viable bacterial count.

Pour-plate Method

This is one of the best method to determine the number of bacteria present per mL of liquid broth/specimen.

- **Serial 10-fold dilutions** of the original bacterial suspension are made. This is achieved by:
  - 9 mL of nutrient broth is poured into a set of test tubes
  - 1 mL of the bacterial suspension added to the first test tube, mixed and then 1 mL is serially transferred to the subsequent tube and so on.
- **Pour plating:** 1 mL from each tube is added to a measured quantity of molten nutrient agar (which has been cooled to 45°C), mixed properly, and then is poured into a Petri dish
- After being cooled and solidified, the Petri dishes are incubated overnight at 37°C
- **Colony counting:** Next day, the total number of colonies formed are counted from one among the plate that contains colonies between 50–500 colonies/plate. The lower dilutions will produce much crowded colonies, hence not suitable for counting. Each colony represents one bacterium in the specimen (Fig. 4.9)
- **Viable count/mL** of the specimen is calculated by multiplying the number of colonies/plate with the dilution factor.

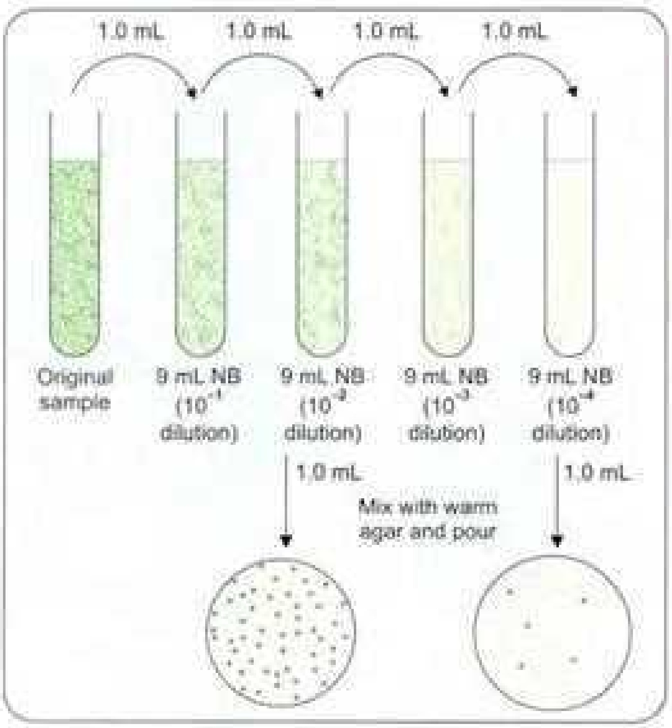


Fig. 4.9: Pour-plate method

Abbreviation: NB, Nutrient broth.

Fig. 4.10: Bacteriological incubator

Source: Department of Microbiology, JIPMER, Puducherry (with permission).

Spread-plate Method

This is another method for estimating the viable bacterial count. After serial dilution of the sample, known volume of individual dilutions are spread evenly on the surface of a suitable agar plate to obtain a lawn culture. After incubation, colonies are counted and multiplied by dilution factor to estimate the colony count.

Incubation of Culture Media

Most of the pathogenic organisms grow best at 37°C, i.e. body temperature of human beings. For aerobic bacteria, inoculated culture plates are incubated at 37°C for overnight in an incubator.

Bacteriological Incubator

It is a device used to grow and maintain bacteriological cultures or cell cultures (Fig. 4.10). The incubator maintains optimal temperature. Some incubators are specially designed to maintain other conditions, such as humidity and the carbon dioxide (CO_2).

Incubatory Conditions

There are various incubatory conditions required, depending up on the bacteria to be isolated.

- **Candle jar:** Inoculated media are placed in a jar with a lighted candle and then jar is sealed. The burning candle reduces oxygen to a point where the flame goes off (Fig. 4.11). This provides an atmosphere of approximately 3–5% CO_2. This is useful for capnophilic bacteria, such as *Brucella abortus*, *Streptococcus*, pneumococcus and gonococcus

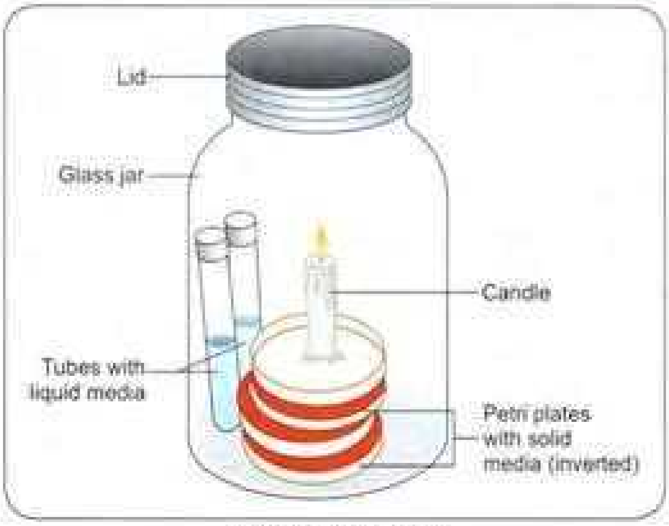


Fig. 4.11: Candle jar

- Microaerophilic bacteria, such as *Campylobacter* and *Helicobacter* require 5% oxygen for optimum growth
- Anaerobic culture methods (see below).

ANAEROBIC CULTURE METHODS

Obligate anaerobic bacteria can grow only in the absence of oxygen, hence for the growth of such bacteria, anaerobic environment is needed. The following are the methods used to create anaerobiosis:

1. Production of Vacuum

This is achieved by incubating cultures in a vacuum desiccator. It is not an effective method, hence not used.

2. By Displacement and Combustion of Oxygen

This involves evacuation of the air from jar and replacement with inert gas like hydrogen followed by removal of the residual oxygen by use of a catalyst. It is carried out by:

- McIntosh and Filde's anaerobic jar (Fig. 4.12A)
- Anoxomat instrument (Fig. 4.12B).

McIntosh and Filde's Anaerobic Jar

It is one of the most effective and popular method for creating anaerobiosis. It consists of a metal or glass jar with a metal lid, attached with a screw (to close airtight), pressure gauge and two openings (inlet and outlet).

- **Evacuation of air and replacement with hydrogen gas:** This is done manually, by using a Kipp's apparatus. Because this is a cumbersome procedure, hence this method is less commonly used now
- **Removal of residual oxygen:** This is done by using a catalyst (sachet containing aluminium pellets coated with palladium) which is suspended from the inner side of the lid. It helps in removing the residual oxygen by combining with hydrogen to form water.

$$[O_2 + H_2 \xrightarrow{\text{Catalyst}} H_2O]$$

Anoxomat

This is an automated equipment (Fig. 4.12B) which evacuates the air from jar and replaces by hydrogen gas from a cylinder. The same catalyst is used here to remove the traces of oxygen. It is easier to operate than McIntosh jar method and claims to be highly effective for creating anaerobiosis.

3. Absorption of Oxygen by Chemical Methods

Principle

Instead of displacing the oxygen from the jar, the oxygen is removed by chemical reactions (e.g. alkaline pyrogallol) in contrast to evacuation and replacement technique used

Figs 4.12A to C: A. McIntosh and Filde's anaerobic jar; **B.** Anoxomat anaerobic system; **C.** Anaerobic work station (Whitley pvt. Ltd.)

Source: A. Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry, B. Department of Microbiology, JIPMER, Puducherry, and C. Dr Padmaja A Shenoy, Associate Professor, Department of Microbiology, Kasturba Medical College, Manipal, Karnataka (with permission).

in McIntosh Filde's jar. This principle is used in GasPak system (BD diagnostics).

GasPak System

At present, it is the most commonly used method for anaerobiosis. It is very simple to perform and is perfect for a laboratory having less sample load.

- It uses a sachet containing sodium bicarbonate and sodium borohydride which react chemically in presence of water, to produce hydrogen and CO_2 gas (Fig. 4.13)
- The traces of oxygen is removed by using the same catalyst used for McIntosh Fildes method (aluminum pellets coated with palladium) placed below the jar (Fig. 4.13)
- **Indicator of anaerobiosis:** The effectiveness of anaerobiosis can be checked by the following methods:
  - Chemical indicator: Reduced methylene blue remains colorless in anaerobic conditions, but turns blue on exposure to oxygen
  - Biological indicator: Plate inoculated with *Pseudomonas* is incubated along with other inoculated plates for anaerobic culture. Absence of growth of *Pseudomonas* (which is an obligate aerobe) indicates that perfect anaerobiosis has been achieved.

4. Anaerobic Glove Box (or Anaerobic Chamber)

It is a self-contained anaerobic system that allows microbiologists to process the specimen and perform most bacteriological techniques for isolation and identification of anaerobic bacteria without exposure to oxygen.

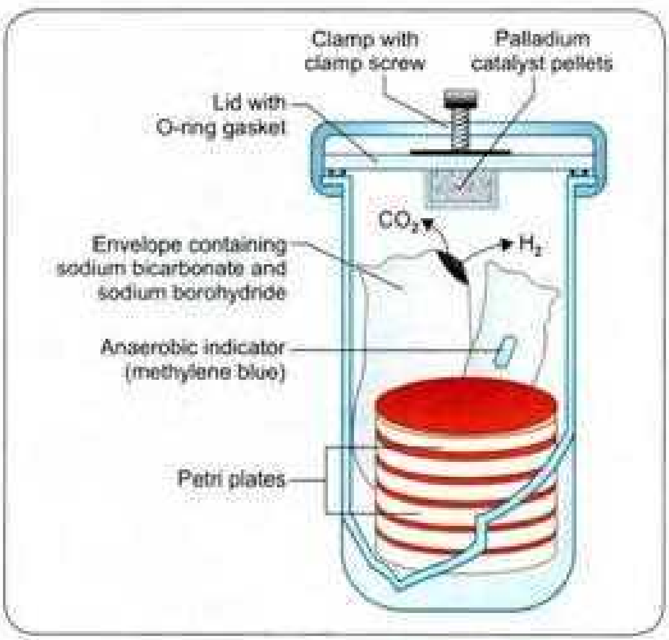


Fig. 4.13: Gas-pak anaerobic system

5. Anaerobic Work Station

This is a work station which provides facility for easy processing, incubation and examination of the specimen without exposure to atmospheric oxygen (Fig. 4.12C).

6. By Using Various Reducing Agents

Oxygen in culture media can be reduced by various reducing agents, such as glucose, thioglycollate, cooked meat pieces, cysteine and ascorbic acid. Robertson cooked meat broth is the most widely employed anaerobic culture medium which uses chopped meat particles (beef heart) as reducing agent.

7. Prereduced, Anaerobically Sterilized (PRAS)

PRAS media are prepared entirely under oxygen-free conditions from initial sterilization to packaging in sealed foil packets.

PRESERVATION OF MICROORGANISMS

Preservation of microorganisms has been a long tradition in microbiology. Preservation of organisms is necessary for epidemiological investigation, future research and educational purposes. Both short-term (weeks to months) and long-term (up to years) preservation methods are available.

Various methods of preservation of microorganisms are as follows:

Short-term Preservation Methods

Short-term preservation methods are cheaper, easy to perform. However, the phenotypic and genotypic properties of bacteria may get altered as organism is more liable to undergo mutations.

Subculturing

Regular subculturing on to various media (e.g. semisolid butts or nutrient agar slopes) is the most common and routinely followed method for bacterial preservation. By this method cultures can be preserved for not more than a few weeks. Cooked-meat medium is used for the preservation of anaerobes.

Other Short-term Methods

- Preservation by immersing the culture in mineral oil, glycerol, or sterile distilled water
- Freezing at -20°C
- Drying: This may be useful for moulds and spore bearing bacteria.

Long-term Preservation Methods

These methods are used for preserving the microorganisms for several years. Though the equipment cost is high, these methods have several advantages—(1) cultures occupy less space, (2) phenotypic and genotypic characters are well maintained, (3) reduce chance of organism undergoing mutations, (4) viability is well maintained. Long-term preservation methods are of two types; described below.

Ultra Temperature Freezing

This involves mixing the cultures with cryopreservative agents, such as glycerol, skimmed milk, sucrose, etc. and incubating at -70°C.

Lyophilization (Freeze-drying)

It is the ideal method for successful storage of most bacteria.

- This process involves freezing of the liquid culture followed by dehydration to remove water from frozen bacterial suspensions
- Lyophilization of bacterial cultures results in a stable, readily rehydrated product. Lyophilized cultures are best maintained at 4°C.

METHODS OF ISOLATING BACTERIA IN PURE CULTURES

Several methods are followed to obtain pure culture of individual bacterium from specimen containing mixture of bacteria:

- **Surface plating:** Streaking with intermittent heating is a routinely used method in the laboratory
- **Selective media and enrichment broth:** It is employed for isolating pathogens from specimens containing normal flora, e.g. feces
- **Pre-treatment of specimens:** Suitable bactericidal substances are used for pre-treatment of specimens to isolate a particular bacterium, e.g. concentration and decontamination of sputum sample with 4% NaOH before culturing it for *Mycobacterium tuberculosis*
- **Anaerobiosis:** Obligate aerobes and anaerobes may be separated by incubating the plates under aerobic and anaerobic conditions
- **Heating:** Mixture of bacteria in liquid medium with different optimum growth temperatures can be separated by heating the medium at different temperatures. For example, heating at 60°C would allow only the thermophilic bacteria to grow
- **Filters:** Filters of different pore diameters are widely used for separating bacteria of different sizes and also for separating viruses from bacteria
- **Based on motility:** Motile bacteria can be separated from non-motile bacteria by sub culturing them on to Craigie tube
- **Animal inoculation:** Pathogenic bacteria can be separated from non-pathogenic bacteria by inoculating the mixture into susceptible animals followed by their isolation from the lesions which would be produced only by the pathogenic bacteria. For example, *Bacillus anthracis* can be separated from other aerobic spore bearing bacilli by inoculation into guinea pigs.

EXPECTED QUESTIONS

I. Write short notes on:
1. Enriched media.
2. Selective media.
3. Transport media.
4. Anaerobic culture methods.
5. Methods of isolating bacteria in pure culture.

II. Multiple Choice Questions (MCQs):

1. Recommended transport medium for stool specimen suspected to contain *Vibrio cholerae* is:
a. Buffered glycerol saline medium
b. Venkatraman-Ramakrishnan medium
c. Nutrient broth d. Blood agar

2. Which is an enriched medium?
a. Selenite F broth b. Peptone water
c. MacConkey agar d. Chocolate agar

3. Agar concentration required to prepare nutrient agar is:
a. 2% b. 6%
c. 0.25% d. 0.5%

4. Robertson cooked meat broth is an example of:
a. Enriched media b. Enrichment media
c. Anaerobic media d. Nutrient media

5. Blood culture bottle contains:
a. Peptone water broth b. BHI broth
c. Selenite F broth d. Tryptic soy broth

6. Automated blood culture bottles contain:
a. Peptone water broth b. BHI broth
c. Selenite F broth d. Tryptic soy broth

7. Lowenstein-Jensen (LJ) medium is used for isolation of:
a. *M. tuberculosis* b. *Vibrio* species
c. *Salmonella* d. *Shigella*

8. Thiosulfate citrate bile salt sucrose (TCBS) agar is used for:
a. *Vibrio species* b. *Salmonella*
c. *Shigella* d. *Escherichia coli*

9. Which is NOT a selective medium?
a. DCA (deoxycholate citrate) agar
b. XLD (xylose lysine deoxycholate) agar
c. Selenite F medium
d. Wilson Blair bismuth sulfite medium

10. Which culture method is used for carrying out antimicrobial susceptibility testing:
a. Streak culture b. Lawn culture
c. Stroke culture d. Stab culture

Answers

1. b 2. d 3. a 4. c 5. b 6. d 7. a 8. a 9. c 10. b

Identification of Bacteria

5
CHAPTER

Chapter Preview

- Conventional method
- Automated culture techniques
- Molecular methods
  - Polymerase chain reaction (PCR)
  - Real-time PCR (rt-PCR)
- Microbial typing

Identification of bacteria can be done by various methods, such as (1) conventional methods of culture and identification, (2) automated culture techniques; and (3) molecular methods (Fig. 5.1).

CONVENTIONAL METHOD

Conventional method consists of specimens subjected to direct microscopy (Gram stain or any other special stain), followed by conventional culture on blood agar and MacConkey agar or any other special media. Colonies grown on culture media are subjected to culture smear and motility testing. Based on the culture smear report, the appropriate biochemical reactions are put for bacterial identification.

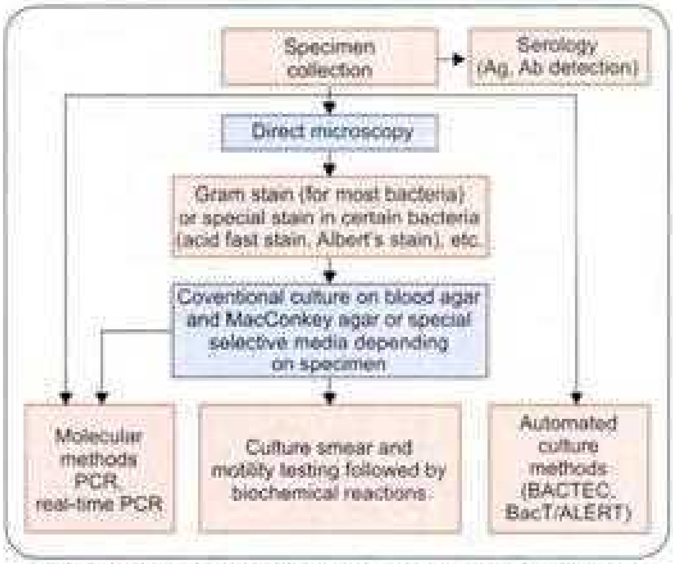


Fig. 5.1: Schematic representation of microbial identification
Abbreviations: Ag, antigen; Ab, antibody; PCR, polymerase chain reaction.

Specimen Collection

Specimen collection depends upon the type of underlying infections (Table 5.1). The proper collection of specimen is the key for correct and accurate identification of the bacteria. The following general principles should be followed while collecting the specimen:

- Standard precautions should be followed for collecting and handling all specimens (Chapter 53 for detail)
- Whenever possible, culture specimens should be collected prior to administration of any antimicrobial agents
- Contamination with indigenous flora should be avoided
- Swabs are convenient but inferior to tissue, aspirate and body fluids
- All specimens must be appropriately labeled

Table 5.1: Type of infections and various specimens collected

Type of infections	Specimens collected
Wound and soft tissue infection	Pus or exudate, swabs, aspirates for abscess
Bloodstream infection	Blood
Urinary tract infection	Midstream urine Suprapubic aspirated urine
Diarrheal diseases	Stool (mucus flakes), rectal swab
Respiratory tract infection	Sputum, throat swab, bronchoalveolar lavage or endotracheal aspirate
Eye and ear infection	Conjunctival swabs, Corneal scrapings Swabs from outer ear/eye
Infections of the sterile area	Sterile body fluids; e.g. CSF, pleural fluid, synovial fluid, peritoneal fluid, etc.

Abbreviation: CSF, cerebrospinal fluid.

- The specimens should be delivered to the laboratory as soon as possible after collection. If required appropriate transport media should be used
- Specimens should be collected in tightly sealed, leak proof containers
- Specimens grossly contaminated or compromised may be rejected
- If anaerobic culture is requested, proper anaerobic collection containers with media should be used.

Direct Microscopy

Direct smear examination—specimens are subjected to the following staining techniques.

- **Gram staining:** It is the first and foremost step done for bacterial identification. It divides bacteria into gram-positive and gram-negative
- **Albert's staining:** It is employed to identify *Corynebacterium diphtheriae*, the causative agent of diphtheria
- **Ziehl-Neelsen (ZN) acid fast staining:** It differentiates acid-fast bacilli (AFB), (e.g. *Mycobacterium tuberculosis*) from non-acid-fast bacilli.

Culture

Depending on the type of specimen, various culture media are used (Chapter 4). Combination of blood agar and MacConkey agar is most commonly employed for most specimens, such as pus, wound swab and other exudate specimens, sterile body fluids, urine, sputum and other respiratory specimens.

- Chocolate agar should be added for respiratory and sterile body fluid specimens
- Stool specimen should be inoculated on to selective media, such as:
  - Mildly selective media—MacConkey agar and
  - Highly selective media—Deoxycholate citrate agar (DCA), xylose lysine deoxycholate (XLD) and thiosulfate citrate bile salt sucrose (TCBS) agar
- Blood specimen should be directly inoculated into blood culture bottles without performing direct microscopy methods
- Cystine lactose electrolyte deficient (CLED) agar can be used for urine specimen as a substitute to blood agar and MacConkey agar combination.

Morphology of Bacterial Colony

The appearance of bacterial colony on culture medium is usually characteristic which helps in preliminary identification. The following features of the colony are studied.

- Size—In millimeters, e.g. pin head size is characteristic of staphylococcal colony and pin point size is characteristic of streptococcal colony
- Shape—Circular or irregular
- Surface—Glistening or dull
- Edge—Entire, crenated, lobate, undulated or filamentous
- Elevation—Flat, raised, convex, umbonate, or pulvinate
- Consistency—Mucoid, friable, firm, or butyrous
- Density—Opaque, translucent or transparent
- Hemolysis on blood agar (see below)
- Color of the colony—Colonies may be colored due to properties of the media used or due to pigment production
  - In some differential and selective media, a few bacteria produce colored colonies due to change of pH or enzymatic activity (e.g. pink colony of *E. coli* on MacConkey agar, black colony of *C. diphtheriae* on potassium tellurite agar)
  - Pigment produced by certain bacteria may also color the colony. Pigments are of two types
    1. **Diffusible pigments:** They diffuse throughout the media, e.g. blue green pigments produced by *Pseudomonas aeruginosa*.
    2. **Non-diffusible pigments:** They do not diffuse into surrounding media, hence only colonies are colored, not the surrounding media, e.g. golden yellow colonies of *Staphylococcus aureus*.

Hemolysis on Blood Agar

Certain bacteria produce hemolysin enzymes that lyse the red blood cells surrounding the colonies on blood agar, forming a zone of hemolysis (Fig. 5.2). Hemolysis may be:

- **Partial or α hemolysis:** Partial clearing of blood around the colonies occurs with green discoloration of the surrounding medium, outline of the RBCs is intact (e.g. pneumococci)
- **Complete or β hemolysis:** Zone of complete clearing of blood around the colonies due to complete lysis of the RBCs (e.g. *Staphylococcus aureus*)
- **No hemolysis (γ hemolysis, a misnomer):** There is no color change surrounding the colony (e.g. *Enterococcus*)

Contd...

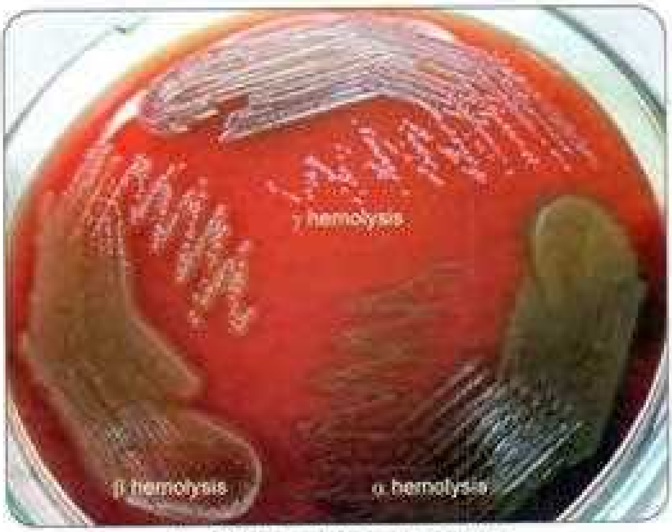


Fig. 5.2: Hemolysis on blood agar

Source: Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (with permission).

Contd...

- **a prime hemolysis:** Halo of incomplete lysis immediately surrounding the colonies with a second zone of complete hemolysis at the periphery (e.g. few streptococci)
- **Target hemolysis:** It is seen in *Clostridium perfringens*; characterized by a narrow zone of complete hemolysis, surrounded by incomplete zone of hemolysis due to alpha toxin.

Culture Smear and Motility Testing

The colonies grown on the culture media are subjected to Gram staining and motility testing by hanging drop method.

Biochemical Reactions

Based on the type of organisms observed in culture smear, the appropriate biochemical tests are employed.

1. Initially, catalase and oxidase tests are done on all types of colonies grown on the media.
2. For gram-negative bacilli: Common biochemical tests done routinely are abbreviated as 'ICUT':
   - Indole test
   - Citrate utilization test
   - Urea hydrolysis test
   - Triple sugar iron test (TSI).

   If there is any doubt in correct identification of bacteria, then further biochemical tests are performed, such as:
   - Sugar fermentation test
   - MR (methyl red) test
   - VP (Voges-Proskauer) test
   - OF test (oxidation-fermentation test)
   - Nitrate reduction test
   - Decarboxylase test
   - PPA test (phenyl pyruvic acid test).
3. For gram-positive cocci: the useful biochemical tests are:
   - Coagulase test (for *Staphylococcus aureus*)
   - DNase test (for *Staphylococcus aureus*)
   - CAMP (Christie-Atkins-Munch-Petersen) test for group B *Streptococcus*.
   - Bile esculin hydrolysis test (for *Enterococcus*)
   - Heat tolerance test (for *Enterococcus*)
   - Sugar fermentation test is useful for:
     - Pneumococcus (inulin fermentation) and
     - Species identification of coagulase negative *Staphylococcus* and *Enterococcus*).
   - PYR test (for *Streptococcus pyogenes* and *Enterococcus*)
   - Bile solubility test (for pneumococcus)
   - Antimicrobial susceptibility tests done for bacterial identification are as follows:
     - Novobiocin susceptibility test—done for *Staphylococcus saprophyticus* (resistant)
     - Optochin susceptibility test—done for pneumococcus (sensitive)
     - Bacitracin susceptibility test-done to differentiate group A (sensitive) and group B (resistant) *Streptococcus*.

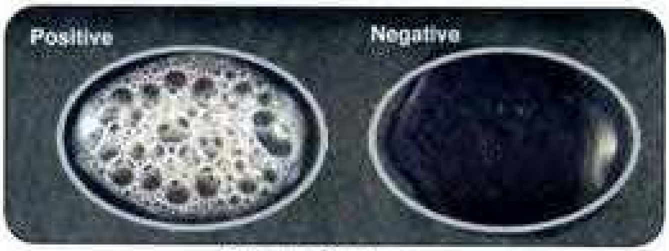


Fig. 5.3: Catalase test

Some of the important biochemical tests are described below. Coagulase test and other biochemical reactions for gram-positive cocci are described in the respective chapters.

Catalase Test

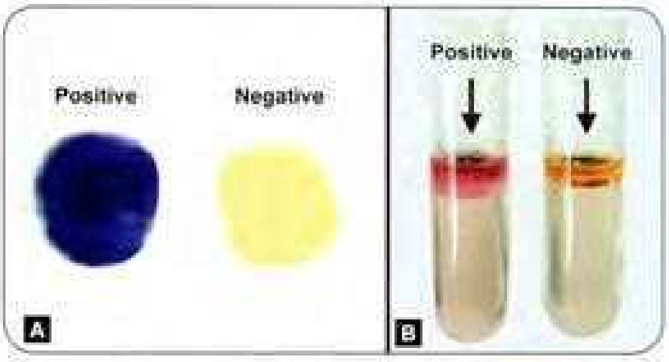
When a drop of hydrogen peroxide (3% H_2O_2) is added to a colony (or when the colony is mixed to a drop of H_2O_2 placed on a slide) of any catalase producing bacteria, effervescence or bubbles appear due to breakdown of H_2O_2 by catalase to produce oxygen (Fig. 5.3).

- Catalase test is primarily used to differentiate between *Staphylococcus* (catalase positive) from *Streptococcus* (catalase negative)
- It is also positive for members of the families Enterobacteriaceae, Vibrionaceae, Pseudomonadaceae, etc.
- False-positive: Since blood contains catalase, colonies from blood agar may result in false-positive reaction. Use of iron wire/loop for picking up colonies may also produce false-positive test
- Nutrient agar is the ideal medium to perform the catalase test and the colonies should be picked by glass/wooden sticks (e.g. tooth picks).

Oxidase Tests

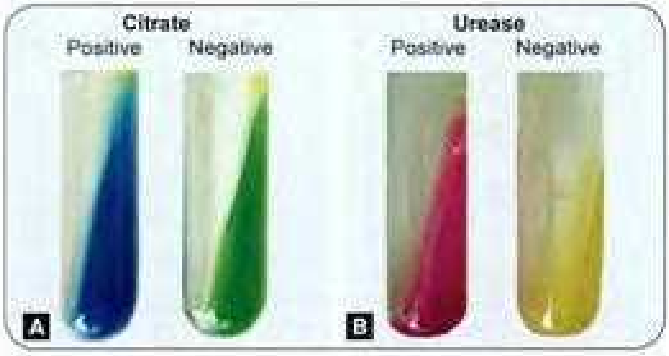
It detects the presence of cytochrome oxidase enzyme in bacteria, which catalyzes the oxidation of reduced cytochrome by atmospheric oxygen.

- When a filter paper strip or disk, soaked in oxidase reagent (1% tetramethyl para-phenylenediamine dihydrochloride), is smeared with a bacterial colony producing cytochrome oxidase enzyme, the smeared area turns deep purple within 10 seconds due to oxidation of the dye to form a purple colored compound indophenol blue
- Interpretation (Fig. 5.4A) and examples:
  - **Oxidase positive (deep purple):** Examples include *Pseudomonas, Vibrio, Neisseria, Bacillus, Haemophilus*, etc.



Figs 5.4A and B: A. Oxidase test; **B.** Indole test

Source: Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (*with permission*).



Figs 5.5A and B: A. Citrate utilization test; **B.** Urea hydrolysis test

Source: Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (*with permission*).

- **Oxidase negative (no color change):** Examples include; members of family Enterobacteriaceae, Acinetobacter, etc.

Indole Test

It detects the ability of certain bacteria to produce enzyme tryptophanase that breaks down amino acid tryptophan present in the medium into indole.

- When Kovac's reagent (para-dimethylamino-benzaldehyde) is added to an overnight incubated broth of a bacterial colony, it complexes with indole to produce a cherry red color ring near the surface of the medium
- **Indole positive** (Fig. 5.4B): A red colored ring is formed near the surface of the broth. Examples include *Escherichia coli, Proteus vulgaris, Vibrio cholerae*, etc.
- **Indole negative** (Fig. 5.4B): Yellow colored ring is formed near the surface of the broth, e.g. *Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas, Salmonella*, etc.

Citrate Utilization Test

It detects the ability of a few bacteria to utilize citrate as the sole source of carbon for their growth, with production of alkaline metabolic products. Citrate test is performed on a citrate containing medium, such as Simmon's (solid) or Koser's (liquid) medium.

- **Simmon's citrate medium:** Citrate utilizing bacteria produce growth and a color change, i.e. original green color changes to blue. Here bromothymol blue is used as an indicator (Fig. 5.5A)
- **Koser's (liquid) medium:** It becomes turbid, by the growth of citrate utilizing bacteria
- Citrate test is positive for *Klebsiella pneumoniae, Citrobacter, Enterobacter*, etc.
- The test is negative for *Escherichia coli, Shigella*, etc.

Urea Hydrolysis Test

Urease producing bacteria can split urea present in the medium to produce ammonia that makes the medium alkaline.

- Test is done on Christensen's urea medium, which contains phenol red indicator that changes to pink color in alkaline medium (Fig. 5.5B)
- **Urease test is positive for:** *Klebsiella pneumoniae, Proteus* species, *Helicobacter pylori, Brucella*, etc.
- **Urease test is negative for:** *Escherichia coli, Shigella, Salmonella*, etc.

Triple Sugar Iron (TSI) Agar Test

TSI is a very important medium employed widely for identification of gram-negative bacteria.

Composition

It is a composite solid agar medium in tube having a butt and a slant. Its constituents include:

- Three sugars—glucose, sucrose and lactose in the ratio of 1:10:10 parts
- Phenol red as an indicator of acid production
- Ferric salts as an indicator of hydrogen sulfide (H_2S) production.

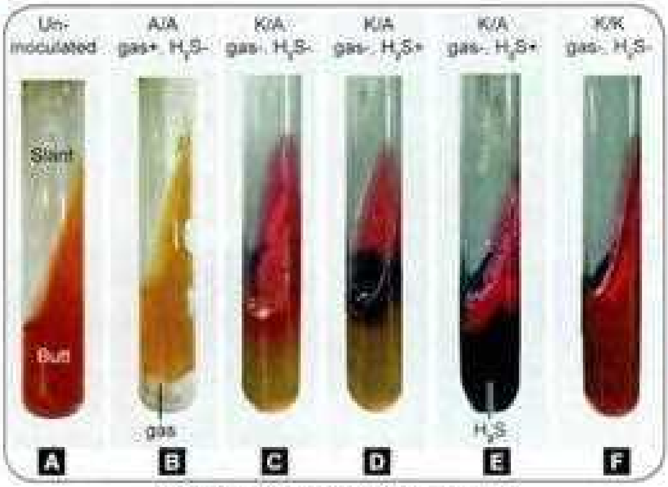
Procedure

Medium is inoculated with a pure bacterial culture by a straight wire pierced deep in the butt (stab culture) and then doing a stroke culture on the slant area. The tube is incubated at 37°C for 18–24 hours. Under incubation or over incubation may lead to false interpretation of result.

Interpretation

TSI detects three properties of bacteria, such as fermentation of sugars to produce acid and/or gas and production of H_2S.

- **Ability to ferment sugars to produce acid:** Uninoculated TSI medium is red in color (Fig. 5.6A) and on acid production the color changes to yellow. Based on fermentation of sugar present in TSI, the organisms are categorized into three groups (Table 5.2 and Fig. 5.6)
  1. **Nonfermenters:** They do not ferment any sugars, hence an alkaline slant and alkaline butt (no change)



Figs 5.6A to F: Triple sugar iron test

Source: Department of Microbiology, JIPMER, Puducherry (*with permission*).

Table 5.2: Various reactions in TSI with examples

Reactions in TSI	Examples
Acidic slant/acidic butt	**≥2 sugars fermented** (1) glucose, (2) lactose or/and sucrose
A/A, gas produced, no H_2S (Fig. 5.6B)	*Escherichia coli* *Klebsiella pneumoniae*
Alkaline slant/acidic butt	**Only glucose-fermenter group**
K/A, no gas, no H_2S (Fig. 5.6C)	*Shigella*
K/A, no gas, H_2S produced (small amount) (Fig. 5.6D)	*Salmonella* Typhi
K/A, no gas, H_2S produced (abundant) (Fig. 5.6E)	*Proteus vulgaris*
K/A, gas produced, H_2S produced (abundant)	*Salmonella* Paratyphi B
K/A, gas produced, no H_2S	*Salmonella* Paratyphi A
Alkaline slant/alkaline butt	**Non-fermenters group**
K/K, no gas, no H_2S (Fig. 5.6F)	*Pseudomonas, Acinetobacter*

reaction is observed, (K/K reaction or alkaline (red) slant/alkaline (red) butt (Fig. 5.6F).

2. **Glucose only fermenters:** They ferment only glucose and produce little acid. Initially at 8 hours, the whole medium turns acidic (yellow). Later on, the organism begins oxidative degradation of the peptones present in the slant, resulting in alkaline by-products in slant, which change the indicator back to red color (in slant). At 18–24 hours, the medium appears alkaline (red) slant/acidic (yellow) butt or K/A reaction (Fig. 5.6C).
3. **All sugars fermenters:** They ferment glucose and also ferment lactose and/or sucrose to produce large amount of acid so that the medium turns acidic at 8 hours and also at 18–24 hours (maintains the acidic pH in both in slant and butt) producing an acidic (yellow) slant/acidic (yellow) butt or A/A reaction (Fig. 5.6B).

- **Ability to produce gas:** Some bacteria produce gas by sugar fermentation; which is denoted by breaks/ cracks in the medium or the medium is lifted up (Fig. 5.6B)
- **Ability to produce H_2S:** Certain bacteria produce hydrogen sulfide (H_2S), which is a colorless gas. H_2S combines with ferric ions (from ferric salts present in the medium) to form ferrous sulfide, that produces blackening of the medium (Figs 5.6D and E).

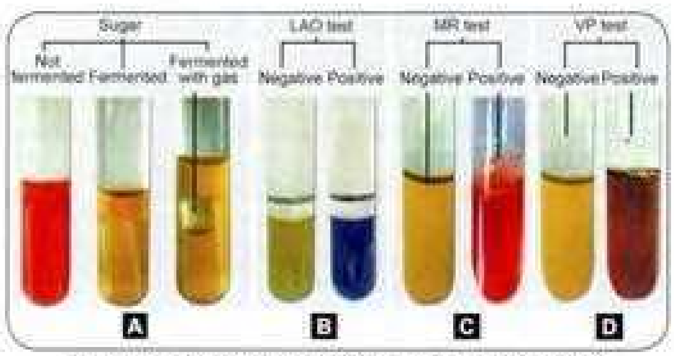
Carbohydrate (Sugar) Fermentation Test

It detects the ability of an organism to ferment a specific carbohydrate (sugar) incorporated in a medium producing acid with/without gas. Glucose, lactose, sucrose and mannitol are widely used for sugar fermentation.

- Ordinary sugar media contain 0.5% sugar and an indicator
- Enriched sugar media called serum (Hiss's) sugar media containing 1% sugar are used for detecting fermentation by fastidious organisms, such as pneumococci, *Neisseria*, *Corynebacterium*, etc.
- Acid production is detected by using indicators, such as:
  - Andrade's indicator (a solution of acid fuchsin and sodium hydroxide): It is colorless, turns pink in acidic medium
  - Phenol red indicator: It is red in color, turns yellow in acidic medium (Fig. 5.7A).
- Gas production is detected by using an inverted Durham's tube (Fig. 5.7A).

Decarboxylase (LAO) Test

It detects the presence of substrate specific decarboxylase enzyme in the bacteria that break down amino acids, such as lysine, arginine and ornithine to produce alkaline by-products which change the color of the indicator to purple (Fig. 5.7B). Cresol red and bromocresol purple are used as indicators.



Figs 5.7A to D: A. Carbohydrate fermentation test; **B.** Decarboxylase test; **C.** Methyl red test; **D.** Voges-Proskauer test

Source: Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (*with permission*).

Methyl Red (MR) Test

In glucose phosphate broth, certain bacteria ferment glucose to produce stronger acids (lactic, acetic or formic) that maintain the pH below 4.4, which turns methyl red indicator from yellow to red color (Fig. 5.7C).

- MR positive (red color): *Escherichia coli*
- MR negative (no change in color): *Klebsiella pneumoniae*.

Voges-Proskauer (VP) Test

This test is also done in glucose phosphate broth. Certain bacteria produce acetoin (acetyl methyl carbinol) as chief end product of glucose fermentation. In the presence of alkali (40% potassium hydroxide VP reagent-II) and atmospheric oxygen, acetoin is oxidized to diacetyl which reacts with α-naphthol (VP reagent-I) to give red color (Fig. 5.7D).

- **VP positive:** *Klebsiella pneumoniae, Enterobacter*, El Tor *Vibrio, Staphylococcus*, etc.
- **VP negative:** *Escherichia coli, Shigella, Salmonella*, etc.

Oxidation–Fermentation Test (OF test)

Hugh and Leifson OF test differentiates between fermenters and non-fermenters (that utilize sugars oxidatively). OF medium differs from ordinary sugar fermentation medium by containing:

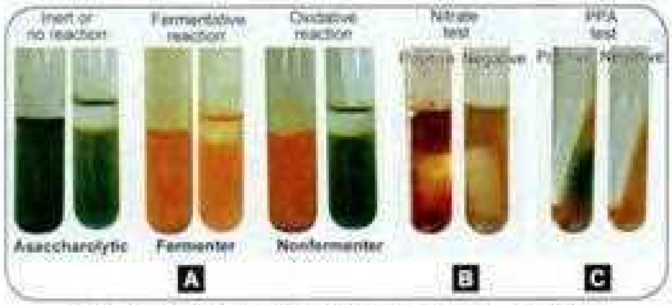
- Agar (0.3%)—making the medium semisolid that permits the diffusion of acids from the surface to throughout the medium changing the color of the bromothymol blue indicator to yellow (Fig. 5.8A)
- Increased sugar concentration from 0.5% to 1%
- Decreased peptone concentration from 1% to 0.2%.

The test organism is inoculated in OF medium in duplicate and one of the tubes is covered with 1 cm layer of liquid paraffin to create anaerobic environment. Acid production changes the color of the medium from green to yellow. If the sugar is fermentatively utilized, acid is produced in both the tubes, whereas the non-fermenters utilize the sugars oxidatively only in the tube without liquid paraffin overlay. Asaccharolytic bacteria cannot utilize the sugars in both the tubes (Fig. 5.8A).

Nitrate Reduction Test

This test detects the presence of an enzyme nitrate reductase in the organism, which reduces nitrate present in the medium (nitrate broth) to nitrite or free nitrogen gas.

- Nitrite production is detected by adding the nitrate reagent (sulfanilic acid and alpha-naphthylamine): Changes the medium to red from yellow (Fig. 5.8B)
- Free nitrogen gas is detected by using a Durham's tube



Figs 5.8A to C: A. Oxidation–fermentation (OF) test; **B.** Nitrate reduction test; **C.** PPA test

Source: Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (with permission).

- Nitrate test positive: For example, all the members of family Enterobacteriaceae.

Phenyl Pyruvic Acid Test (PPA test)

This is a specific test done for members of tribe Proteeae; which includes *Proteus, Morganella* and *Providencia*. They possess a specific enzyme that deaminates phenylalanine present in the medium to phenyl pyruvic acid (PPA). PPA reacts with few drops of 10% ferric chloride solution to produce green color (Fig. 5.8C).

AUTOMATED CULTURE TECHNIQUES

Automated or instrument-based techniques are available for various purposes, such as culture, identification, antimicrobial susceptibility testing, etc.

Automated Blood Culture Techniques

Conventional blood culture methods often yield poor results because of low bacterial load and increased chance of contamination. Therefore, various automated blood culture techniques have been in use since last two decades.

Advantages

The major advantages of automated blood culture techniques are:

- **Continuous automated monitoring:** Following inoculation, the culture bottles are kept within the automated culture system, where they are incubated and periodically monitored for the microbial growth once in every 10 minutes by the instrument. Once positive for microbial growth, the instrument gives a signal (producing beep or color change on the screen)
- **More sensitive:** It gives a higher yield of positive cultures from clinical specimens
- **Rapid:** Takes much less time than conventional methods
- **Less labor intensive:** Saves manpower.

Table 5.3: Common automated blood culture systems

BacT/ALERT 3D (Fig. 5.9A)

Principle of BacT/ALERT is based on colorimetric detection of growth in contrast to fluorometric detection by BACTEC.

- BacT/ALERT bottles (Fig. 5.9B) contain Tryptic soy broth and brain heart infusion broth added with adsorbent polymeric beads which neutralize the antimicrobials present in blood specimen
- A sensor (liquid emulsion sensor) is bonded to the bottom of each bottle and separated from the broth medium by a differentially permeable membrane
- CO_2 produced by growing microorganisms diffuses across the membrane into the sensor where it reacts with water generating hydrogen ions
- As the concentration of hydrogen ions increases and the pH decreases, the blue-green sensor becomes yellow, a change that is detected by colorimetric method
- The algorithm for detection of growth is based on an analysis of the rate of change of CO_2 concentration occurring in each individual bottle

BacT/ALERT VIRTUO (bioMérieux) (Fig. 5.10)

It is an advanced form of BacT/ALERT which offers several advantages such as: (i) automatic loading and unloading of bottles, (ii) faster detection of growth, (iii) can determine the volume of blood present in bottle

BACTEC (BD Diagnostics)

Earlier versions of this system were based on the use of radioisotopes to detect growth. They are not in use currently. The current versions are based on fluorescent detection

- The BACTEC bottle contains soybean-casein digest broth added with polymeric resin beads
- It uses an oxygen sensitive fluorescent compound, dissolved in the broth
- In uninoculated broth, the large amount of dissolved oxygen quenches the fluorescence dye
- Later, actively dividing microorganisms consume the oxygen removing the quenching effect and allowing the fluorescence to be detected

VersaTREK (Thermofisher)

It works on the principle that the CO_2 liberated from bacteria, causes a pressure change which is then detected by manometry

Disadvantages

Automated culture methods do have several disadvantages like (1) high cost of the instrument and culture bottles, (2) inability to observe the colony morphology as liquid medium is used.

There are several companies that manufacture automated blood culture systems which detect the bacterial growth based on different principles (Table 5.3).

Automated Systems for Bacterial Identification

Automated culture systems described above, give information only about a positive microbial growth; however, it does not help in identification of the organism. Traditionally, the bacterial identification is carried out by performing a series of biochemical tests. In modern era, various automated systems are available which help in bacterial identification, such as:

Figs 5.9A and B: A. BacT/ALERT automated blood culture system; **B.** BacT/ALERT blood culture bottle

Source: Department of Microbiology, JIPMER, Puducherry (*with permission*).

Fig. 5.10: BacT/ALERT VIRTUO automated blood culture system

Source: Department of Microbiology, JIPMER, Puducherry (*with permission*).

- MALDI-TOF (Matrix-assisted laser desorption/ionization time-of-flight), e.g. VITEK MS (bioMérieux). Refer the highlight box and Fig. 5.11 for detail
- VITEK 2 (bioMérieux) for automated identification and antimicrobial susceptibility test. Refer the highlight box and Figure 5.12 for detail
- Phoenix (BD Diagnostics) for automated identification and antimicrobial susceptibility test
- MicroScanWalkAway system (Beckman Coulter) for automated identification and antimicrobial susceptibility test.

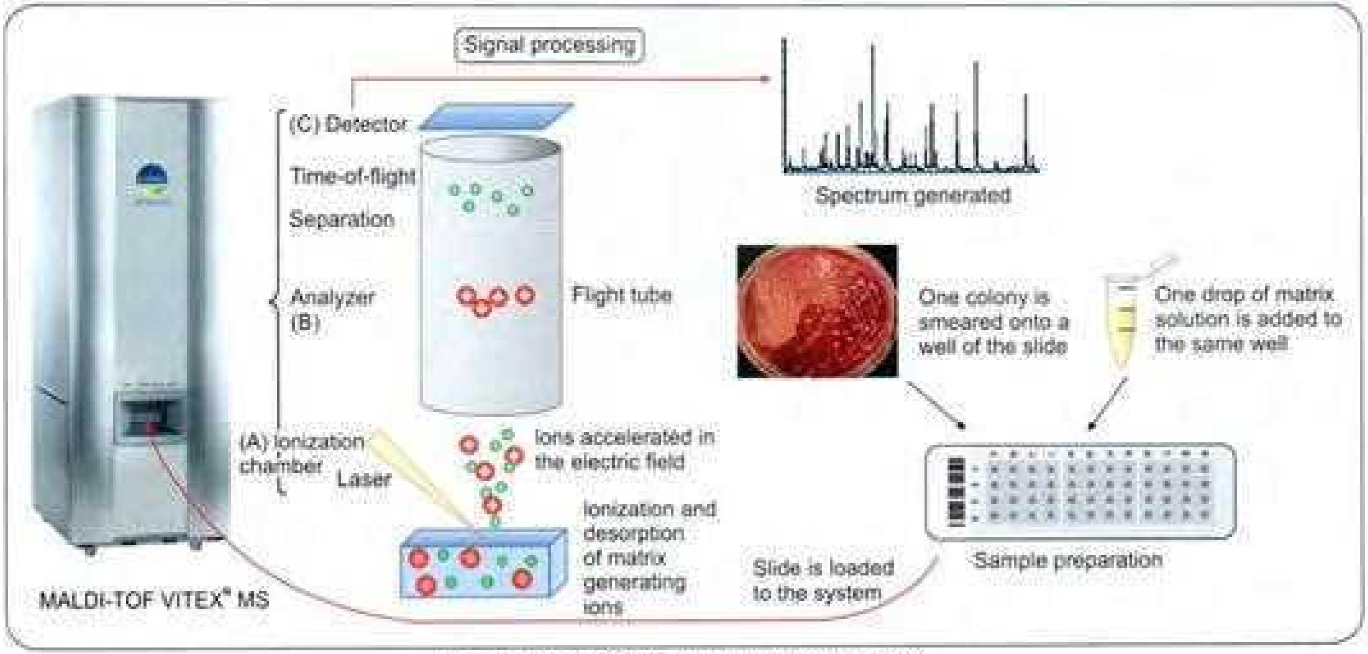


Fig. 5.11: MALDI-TOF and its working principle

Source: Department of Microbiology, JIPMER, Puducherry (*with permission*).

Automated techniques for antimicrobial susceptibility testing have been described in Chapter 7. Automated techniques for mycobacterial culture and susceptibility testing have been described in Chapter 27.

MALDI-TOF

MALDI-TOF technology (Matrix Assisted Laser Desorption Ionization Time-of-Flight) has revolutionized the identification of organisms in clinical microbiology laboratories.

- It can identify bacteria, fungi, and mycobacteria with a turnaround time of few minutes and with absolute accuracy
- Two systems are commercially available: VITEK MS (bioMérieux) and Biotyper system (Bruker).

Principle (Fig. 5.11)

MALDI-TOF examines the patterns of ribosomal proteins present in the organism.

Sample preparation: The colony of an organism is mixed a matrix solution (composed of cyano- hydroxy-cinnamic acid). The mixture is applied to the wells of the target slide and then the slide is loaded in the system.

Steps after loading: Overall, mass spectrometry can be divided into three steps occurring in three chambers of the system.

1. **Ionization chamber:** Here, the wells are irradiated with the laser beam. The matrix absorbs the laser light causing desorption and ionization generating singly protonated ions.
2. **Analyzer:** These ions are then accelerated into an electric field which directs them to the analyser chamber. The analyzer (mass spectrometer) separates them according to their time-of-flight (TOF) in the flight tube. The smaller molecules travels faster, followed by the bigger, according to the mass to charge (m/z) ratio.
3. **Detector:** It converts the received ion into electrical current which is amplified and digitized (D) to generate a characteristic spectrum which is unique to a species due to its conserved ribosomal proteins. Identification is done by comparing the spectrum of the test organism with the database.

VITEK 2 automated system

The VITEK 2 (Fig. 5.12) is an automated system used for identification and antimicrobial susceptibility testing (AST) of bacteria and yeast.

- It uses colorimetric reagent card containing 64 wells; each well contains an individual test substrate. Separate cards are available for gram-negative, gram-positive bacteria, fastidious bacteria and yeasts
- **Identification:** Substrates in the well measure various metabolic activities such as acidification, alkalinization, enzyme hydrolysis, etc. which helps in identification of the organism. The reaction pattern obtained from the test organism is compared with the database and the identification is reported with confidence level of matching (excellent matching to unidentified organism)
- **Antimicrobial susceptibility test (AST):** It works on the principle of microbroth dilution. The wells in the card contain doubling dilution of antimicrobial agents. The organism suspension (of the turbidity as recommended by manufacture) is added to the wells. The minimum inhibitory concentration (MIC) is determined as the highest dilution of the antimicrobial agent which inhibits the growth of organism

Fig 5.12: VITEK 2 system with its panels (reagent cards) for identification and antimicrobial susceptibility test

Source: Department of Microbiology, JIPMER, Puducherry (*with permission*).

- **Incubation:** The cards are incubated in the system at 35.5 ± 1°C. The reading is taken once every 15 minutes by the optical system of the equipment which measures presence of any colored products of substrate metabolism (for identification) or turbidity for AST
- The result of identification is usually available within 4–6 hours and AST within 16–18 hours.

VITEK 2 is the most widely used automated system in India; can perform AST of bacteria and yeasts (other automated AST systems can perform AST of bacteria only).

MOLECULAR METHODS

Molecular methods are broadly grouped into amplification based and non-amplification based methods. Nucleic acid amplification techniques (NAATs) have been increasingly used in diagnostic microbiology. Various NAATs used are:

- Polymerase chain reaction (PCR)
- Real-time polymerase chain reaction (rt-PCR)
- Ligase chain reaction (LCR)
- Transcription-mediated amplification (TMA)
- Nucleic acid sequence-based amplification (NASBA)
- Strand displacement amplification (SDA).
- Loop mediated isothermal amplification (LAMP)
- Automated PCR such as Biofire FilmArray
- Cartridge based nucleic acid amplification test (CB-NAAT)- described in chapter 27.

Non-amplification molecular methods include DNA hybridization method such as line probe assay (described in chapter 27).

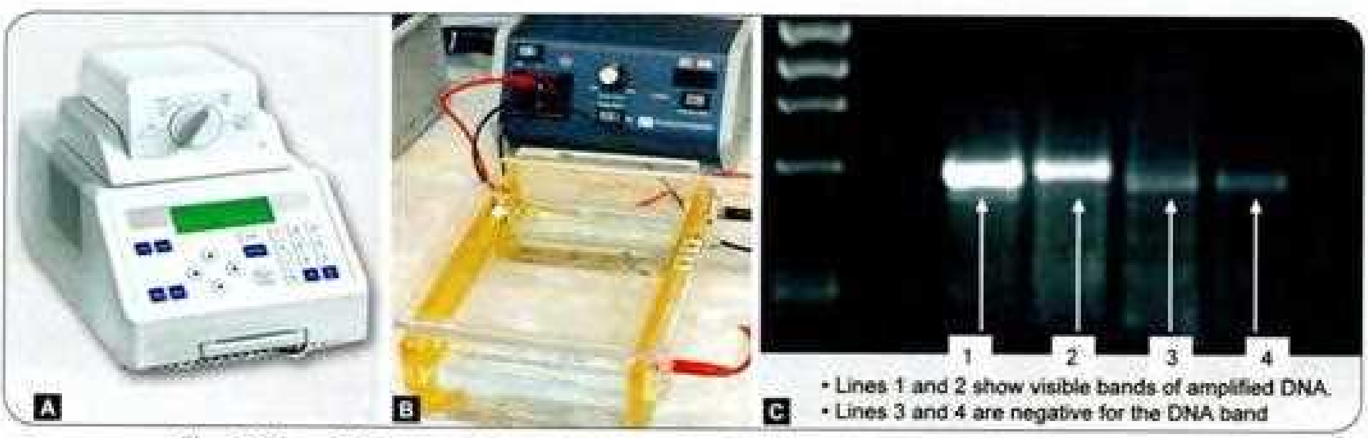
Polymerase Chain Reaction (PCR)

PCR is a technology in molecular biology used to amplify a single or few copies of a piece of DNA to generate millions of copies of DNA. It was developed by Kary B Mullis (1983) for which he and Michael Smith were awarded the Nobel prize in Chemistry in 1993.

Principle

PCR involves three basic steps.

1. **DNA extraction from the organism:** This involves lysis of the organisms and release of the DNA which may be done by various methods, such as boiling, adding enzymes, such as lysozyme, proteinase K, etc. phenol chloroform isoamyl alcohol method. DNA extraction kits are also available commercially.
2. **Amplification of extracted DNA:** This is carried out in a special PCR machine called thermocycler (Fig. 5.13A). The extracted DNA is subjected to repeated cycles (30–35 numbers) of amplification which takes about 3–4 hours. Each amplification cycle has three steps (Fig. 5.14).
   - **Denaturation at 95°C:** This involves separation of the dsDNA into two separate single strands
   - **Primer annealing (55°C):** Primer is a short oligonucleotide complementary to a small sequence of the target DNA. It anneals to the complementary site on the target ssDNA



Figs 5.13A to C: A. Thermocycler machine (Eppendorf); **B.** Gel electrophoresis of amplified product; **C.** Visualization of amplified DNA under UV light

Source: Department of Microbiology, JIPMER, Puducherry (*with permission*).

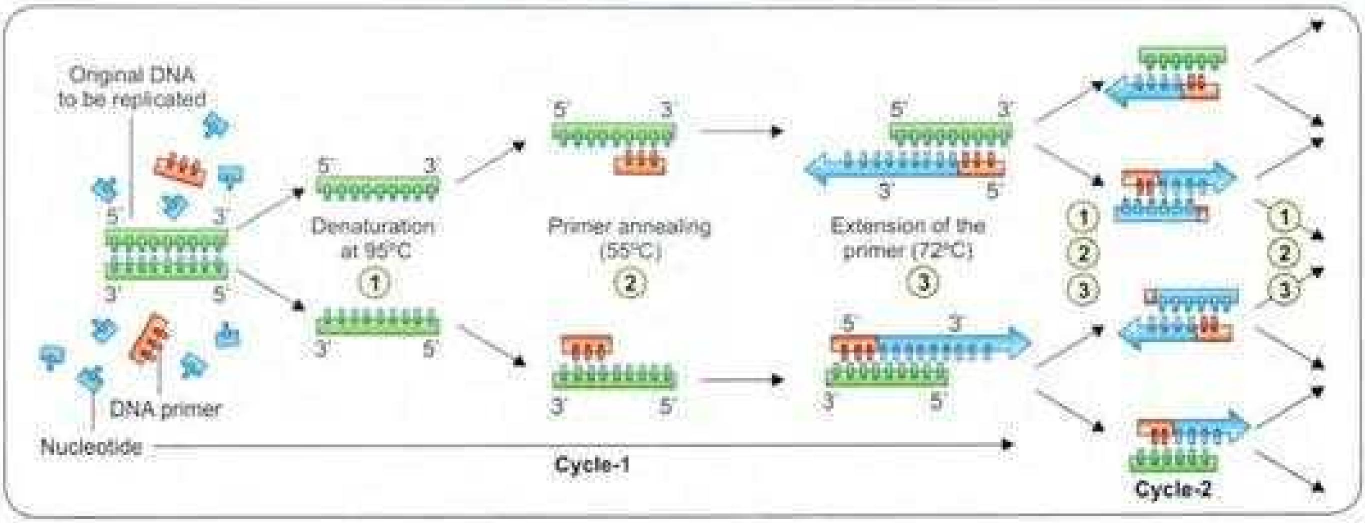


Fig. 5.14: Polymerase chain reaction cycle—3 basic steps of amplification

- **Extension of the primer (72°C):** This step is catalyzed by Taq Polymerase enzyme which keeps on adding the free nucleotides to the growing end of the primer. Taq Polymerase is a special type of DNA polymerase (isolated from the plant bacterium *Thermus aquaticus*), capable of withstanding the high temperature of PCR reaction.

3. **Gel electrophoresis of amplified product:** The amplified DNA is electrophoretically migrated according to their molecular size by performing agarose gel electrophoresis (Fig. 5.13B). The amplified DNA forms clear bands which can be visualized under ultraviolet (UV) light (Fig. 5.13C).

Applications of PCR

Polymerase chain reaction is now a common and often indispensable technique used in medical diagnostics and research laboratories for a variety of applications. It has the following advantages compared to the conventional culture methods:

- **More sensitive:** It can amplify very few copies of a specific DNA, so it is more sensitive
- **More specific:** Use of primers targeting specific DNA sequence of the organism makes the PCR assays highly specific
- PCR can be done to amplify the DNA of the organism: (1) either directly from the sample, or (2) to confirm the organism grown in culture
- PCR can also detect the organisms that are highly fastidious or noncultivable by conventional culture methods
- PCR can be used to detect the genes in the organism responsible for drug resistance (e.g. *mec* A gene detection in *Staphylococcus aureus*)
- Detects genetic diseases, such as sickle cell anemia, phenylketonuria, and muscular dystrophy.

Disadvantages of PCR

Conventional PCR detects only the DNA, but not the RNA (latter can be detected by reverse transcriptase PCR).

- **Qualitative, not quantitative:** Conventional PCR can only detect the presence or absence of DNA. It cannot quantitate the amount of DNA of the organism present in the sample. This is possible by real time PCR
- **Viability:** PCR cannot differentiate between viable or nonviable organisms. It only detects the presence of DNA in the sample which may be extracted from viable or nonviable organism
- **False-positive amplification:** It may occur due to contamination with environmental DNA. Hence, strict asepsis should be maintained in the PCR laboratory
- **False-negative:** The PCR inhibitors present in some specimens, such as blood, feces, etc. may inhibit the amplification of target DNA.

Modifications of PCR

1. **Reverse transcriptase PCR (RT-PCR):** Conventional PCR amplifies only the DNA. For amplifying RNA, RT-PCR is done.
   - After RNA extraction, the first step is addition of reverse transcriptase enzyme that coverts RNA into DNA. Then, the amplification of DNA and gel documentation steps are similar to that described for conventional PCR
   - It is extremely useful for detection of RNA viruses or 16S rRNA genes of the organisms.
2. **Nested PCR:** It is modification of PCR, where two rounds of PCR amplification are carried out by using two primers that are targeted against two different DNA sequences of same organism.

- The amplified products of the first round PCR is subjected to another round of amplification using a second primer which targets the same organism but a different DNA sequence
- More sensitive: Double round of amplification yields high quantity of DNA
- More specific: Use of two primers targeting two regions of DNA of the same organisms makes the test more specific
- Application: Nested PCR is used for detection of *Mycobacterium tuberculosis* (targeting IS6110 gene) in samples
- Disadvantage: There is more chance of contamination of the PCR tubes, which may lead to false-positive results.

3. **Multiplex PCR:** It uses more than one primer which can detect many DNA sequences of several organisms in one reaction.
   - Syndromic approach: Multiplex PCR is useful for the diagnosis of the infectious diseases that are caused by more than one organism
   - For example, for the etiological diagnosis of pyogenic meningitis, different primers targeting the common agents of pyogenic meningitis, such as pneumococcus, meningococcus and *H. influenzae* can be added simultaneously in the same reaction tube
   - Contamination issues: There are chances of the reaction tubes being contaminated with environmental DNA.

Biofire FilmArray

Biofire FilmArray (bioMérieux) is a completely automated multiplex nested PCR system where all the steps from sample preparation to amplification, detection and analysis are performed automatically by the system; giving result in about one hour (Fig. 5.15).

- Four panels are available such as respiratory, gastrointestinal, meningitis-encephalitis and blood culture identification panels; each panel comprises of primers targeting 20–25 common pathogens infecting the respective systems
- Except the blood panel which detects pathogens from positively flagged blood culture bottles, the other panels detect pathogen(s) directly from the specimen
- It has excellent sensitivity and specificity with turnaround time of 1 hour. However the higher cost of the panels limits its wide use.

Real-time PCR (rt-PCR)

It is based on PCR technology, which is used to amplify and simultaneously detect or quantify a targeted DNA molecule on real-time basis. Reverse transcriptase real-time PCR formats can detect and quantify RNA molecules of the test organism in the sample on real-time basis.

It uses a different thermocycler than the conventional PCR. It is very expensive, 5-10 times more than the cost of conventional PCR (Fig. 5.16).

Advantages: Real-time PCR has many advantages over a conventional PCR, such as:

- Quantitative: rt-PCR can quantitate the DNA present in the specimen; hence can be used for monitoring the disease progression in response to treatment, e.g. viral load monitoring in HIV or hepatitis B viral infection
- Takes less time: In rt-PCR, the amplification can be visualized simultaneously during the process of amplification unlike the conventional PCR where there

Fig. 5.15: Biofire FilmArray system
Source: bioMérieux (with permission).

Fig. 5.16: Real time PCR
Source: Department of Microbiology, JIPMER, Puducherry (with permission).

is an extra-step of gel electrophoresis to detect the amplicons
- ❖ Contamination rate is extremely less
- ❖ Sensitivity and specificity of rt-PCR assays are much more than the conventional PCR.

Detection of amplification products of real-time PCR: The detection of amplified nucleic acid in a real-time PCR reaction is carried out by using a variety of fluorogenic molecules which may be either nonspecific or specific.
- ❖ **Nonspecific methods:** They use SYBR green dye that stains any nucleic acid nonspecifically
- ❖ **Specific methods:** They use fluorescent labeled oligonucleotide probe which binds (i.e. hybridizes) only to a particular region of amplified nucleic acid. Three types of hybridization probes are commonly used:
  - TaqMan or hydrolysis probe
  - Molecular beacon
  - Fluorescence resonance energy transfer (FRET) probe.

Post-amplification melting curve analysis is used for quantitation of the nucleic acid load. Analysis of result of Real time PCR (for influenza) has been explained in detail in Chapter 44 (Table 44.5 and Fig. 44.4).

Loop Mediated Isothermal Amplification (LAMP)

LAMP is an isothermal nucleic acid amplification technique. It provides several advantages over PCR.
- ❖ Amplification is carried out at a constant temperature of 60–65° (in contrast to alternating temperature cycles in PCR)
- ❖ **Polymerase with strand displacement ability:** The isothermal nature of LAMP assay is due to the use of specific DNA polymerase enzymes which have additional strand displacement capacity, e.g. polymerase derived from *Geobacillus stearothermophilus*
- ❖ **Cheaper and easy to perform:** It does not require thermocycler or gel electrophoresis
- ❖ More specific, as it uses 4 primers targeting different regions on the target gene
- ❖ **Detection:** Amplicons are detected directly by naked eyes either by turbidity or visual fluorescence detection (in contrast to gel documentation in PCR)
- ❖ **Uses:** LAMP assay has been approved for tuberculosis
- ❖ **Drawback:** It shows high false positive results due to cross contamination between the reaction tubes

MICROBIAL TYPING

Microbial typing refers to characterization of an organism beyond its species level.

Applications: Microbial typing is an important tool for hospital microbiologists and epidemiologists. It is used to determine the relatedness between different microbial strains of the same species and thereby it helps to:
- ❖ Investigate outbreaks: All isolates tracked in an outbreak should belong to similar type
- ❖ Determine the source and routes of infections
- ❖ Trace cross-infection, i.e. transmission of healthcare-associated pathogens
- ❖ Differentiate virulent strains from avirulent strains of same species
- ❖ Differentiate between recurrence and infection with new strain
- ❖ Evaluate the effectiveness of control measures.

Classification: Typing methods are broadly classified as phenotypic and genotypic methods (Table 5.4).

Characteristic of Typing Methods

A good typing method should have the following properties:
- ❖ **Typeability:** Ability of the method to type and generate a result for each isolate tested
- ❖ **Reproducibility:** Ability to produce similar results when tested repeatedly in different laboratories
- ❖ **Discriminative power:** Ability to generate distinct units of information making fine distinctions between the types at the subspecies level
- ❖ **Practicality:** Ease of use and interpretation, cost and affordability.

In general, genotypic methods are more reliable and have better reproducibility and discriminative power than phenotypic methods, however they are expensive.

Phenotypic Methods

Bacteriophage Typing

Strains of an organism can be further differentiated into subspecies level based on their susceptibility to bacteriophages. Phase typing is obsolete now; was used

Table 5.4: Typing methods

Phenotypic methods	Genotypic methods
Bacteriophage typing	**Non-amplification-based methods**
Bacteriocin typing	Plasmid profile analysis
Biotyping	Chromosomal DNA analysis
Antibiogram typing	Restricted fragment length polymorphism (RFLP)
Auxotyping	Ribotyping (RFLP of ribosomal DNA)
Morphotyping	Pulse field gel electrophoresis (PFGE)
Serotyping	**Amplification-based methods**
	PCR-RFLP
	Amplified fragment length polymorphism (AFLP)
	Sequencing-based methods
	Microarrays

in the past for the typing of: *Staphylococcus aureus*, *Salmonella* Typhi, *Vibrio* species, *Brucella* species and *Corynebacterium diphtheriae*.

Bacteriocin Typing

Bacteriocin is an antibiotic like proteinaceous substance produced by one bacterium that inhibits other strains of the same or other closely related bacteria. Bacteriocin typing is based on the ability of a strain to produce particular bacteriocin which inhibits the growth of a set of selected indicator strains. It is done for:

- *Shigella sonnei* (colicin typing)
- *Klebsiella* (klebocin typing)
- *Escherichia coli* (colicin typing)
- *Proteus* (proticin typing)
- *Pseudomonas* (pyocin typing).

Biotyping

It refers to intra species classification based on different biochemical properties of the organism. It is used for:

- *Corynebacterium diphtheriae*: It is classified into gravis, intermedius and mitis
- *Vibrio cholerae* O1 is classified into two biotypes—(1) Classical and (2) El Tor
- *Yersinia pestis*.

Antibiogram Typing

It classifies the organism into different groups based on their resistance pattern to different antimicrobials. Since antimicrobial susceptibility testing is routinely done in any hospital, this typing system provides the first clue to a microbiologist about outbreaks occurring in a hospital.

Auxotyping

This is a typing method based on nutritional requirement of the organism. This is done for *Neisseria gonorrhoeae*.

Morphotyping

This is based on different morphological appearances of the colonies in the culture media. This is done for *Pseudomonas*.

Serotyping

It refers to a typing method based on the antigenic property of an organism. This is the most widely used and the most reliable phenotypic typing method. Serotyping is done for many organisms; important ones are given below.

- *Streptococcus* (Lancefield grouping, based on carbohydrate antigen)
- Based on capsular antigen—For example, pneumococcus, meningococcus and *Haemophilus influenzae*
- Based on somatic antigen—For example, *Escherichia coli*, *Shigella*, *Salmonella* and *Vibrio cholerae*.

Genotypic Methods

Plasmid Profile Analysis

It is a method of determining a number and size of plasmids present in bacterial isolates. Plasmids produced by various strains in an outbreak are compared. First the plasmids are extracted from the bacterial cell and are then separated on agarose gel based on molecular weight followed by their detection by staining with ethidium bromide.

Restricted Fragment Length Polymorphism (RFLP)

1. **Digestion of DNA:** This is done by using 2 or more restriction enzymes which cleave the DNA from a bacterial strain at different sites so that multiple DNA fragments are generated.
2. **Southern blot to detect DNA fragments:** The DNA fragments are separated by electrophoresis and transferred to a nitrocellulose membrane and then are detected by using specific DNA probes (Chapter 6).

The pattern of fragments generated by different strains tracked in an outbreak can be compared to know the relatedness between the strains.

Ribotyping

Ribotyping is a type of RFLP analysis which is done on chromosomal DNA coding for ribosomal RNA.

Pulse Field Gel Electrophoresis (PFGE)

PFGE is considered as a **gold standard** typing method. It is used for epidemiological investigation of pathogenic organisms. It comprises of the following steps.

- **Lysis:** First, the bacterial suspension is loaded into an agarose suspension. This is done to protect the chromosomal DNA from mechanical damage by immobilizing it into agarose blocks. Then the bacterial cell is lysed to release the DNA. The agarose-DNA suspension is also known as plug mold
- **Digestion of DNA:** The bacterial DNA is treated with rare cutting restriction enzymes so that it yields generation of less number of larger size DNA fragments (in contrast to frequent cutting restriction enzymes used in RFLP which produces large number of smaller fragments)
- **Electrophoresis:** The larger pieces of DNA are subjected to pulse field gel electrophoresis by applying electric current and altering its direction at a regular interval (in contrast to the conventional agarose gel electrophoresis done to separate the smaller fragments where the current is applied in a single direction)
- **Analysis:** The fragments generated by PFGE of various strains obtained during an outbreak are compared manually or by computer software BioNumerics.

The drawbacks of PFGE are (1) it is labor intensive, (2) requires many days to perform the procedure, (3) requires skilled personnel to interpret the results and (4) requires computer-assisted analysis of banding patterns.

Amplified Fragment Length Polymorphism (AFLP)

AFLP uses the principle of performing RFLP of the bacterial DNA followed by PCR.

- The genomic DNA is digested by restriction enzymes, followed by use of adaptors to ligate to the sticky ends of the restriction fragments
- PCR amplification of the restriction fragments is carried out by using primers complementary to both adaptor and restriction site sequences
- The amplified fragments are separated and visualized on denaturing polyacrylamide gels.

Sequencing-based Methods

The nucleotide sequence of a microbial gene can be obtained by specially designed equipment called *sequencer*. The variability within the sequences of particular genes can be used to determine the relatedness of bacteria. Sequence analysis can be done by two ways.

- **Nucleotide sequencing:** It is performed by Sanger sequencer. Sequencing can be done at one or multiple genes
  - *Sequencing at single gene:* This is useful for: (i) identification of organism, (ii) to find out polymorphism within the gene (called as single nucleotide polymorphism or SNP analysis)
  - *Sequencing at multiple genes* (called as multi-locus sequence typing or MLST).
- **Whole genome sequencing:** This is done an advanced sequencer called as next generation sequencer
  - It involves determining the complete DNA sequence of an organism's genome at a single time
  - Currently, it is largely been used as a research tool, in future the whole genome sequence data may serve as an important tool to guide therapeutic intervention.

Microarrays

Microarray technology offers a wide range of analysis of simultaneous detection of multiple gene products, such as antibiotic resistance determinants and virulence factors whose identification can be useful for epidemiological investigations.

- The principle of the microarray is based on generating labeled cDNA or cRNA molecules that are subsequently hybridized to an arrayed series of thousands of microscopic spots with specific complementary oligonucleotides (probes)
- DNA microarrays have been used to measure changes in expression levels and to detect SNPs. It is also used for genotyping.

EXPECTED QUESTIONS

I. Write short notes on:

1. Automations in Microbiology.
2. Polymerase chain reaction.
3. Real time PCR.
4. Microbial typing methods.

II. Multiple Choice Questions (MCQs):

1. Triple sugar iron agar consists of all of the following sugars except:

a. Glucose b. Lactose
c. Maltose d. Sucrose

2. All of the following are phenotypic typing methods of bacteria, *except*:

a. Ribotyping
b. Bacteriocin typing
c. Antibiogram typing
d. Bacteriophage typing

3. All of the following are oxidase negative bacteria, *except*:

a. *Escherichia coli* b. *Klebsiella*
c. *Proteus* d. *Pseudomonas*

4. Which type of hemolysis on blood agar is a misnomer?

a. α b. β
c. γ d. α prime

5. Coagulase test is done for identification of:

a. *Staphylococcus aureus*
b. *Streptococcus*
c. *Escherichia coli*
d. *Pseudomonas*

6. Urease test is positive for all, *except*:

a. *Klebsiella pneumoniae*
b. *Proteus* species
c. *Helicobacter pylori*
d. *E. coli*

7. Indole test is positive for:

a. *Salmonella*
b. *Klebsiella pneumoniae*
c. *Pseudomonas*
d. *Escherichia coli*

8. Triple sugar ion test shows alkaline slant and acidic butt, no gas and a speck of H_2S. The identification is:

a. *Escherichia coli* b. *Salmonella* Typhi
c. *Proteus* d. *Helicobacter pylori*

9. Which of the following is not an equipment for automated blood culture?

a. BacT/ALERT b. VITEK2
c. BACTEC d. VersaTREK

10. In BacT/ALERT, the principle is based on:
a. Colorimetric detection
b. Fluorescent detection
c. Detection of change of pressure
d. All of the above

11. MALDI-TOF technology is used for:
a. Bacterial culture
b. Bacterial identification
c. Antimicrobial susceptibility
d. All of the above

12. LAMP assay has all the advantages over PCR, *except*:
a. Isothermal amplification
b. No need of thermo cycler
c. No need of gel documentation
d. Less false positive

13. CB-NAAT technology is used for:
a. *Salmonella* b. HIV
c. *M. tuberculosis* d. Hepatitis B

14. Which modification of PCR is used to detect RNA:
a. Nested PCR b. Multiplex PCR
c. Reverse-transcriptase PCR
d. Real-time PCR

15. Which modification of PCR involves two rounds of amplification:
a. Nested PCR b. Multiplex PCR
c. Reverse-transcriptase PCR
d. Real-time PCR

16. Which modification of PCR is used for syndrome approach:
a. Nested PCR b. Multiplex PCR
c. Reverse-transcriptase PCR
d. Real-time PCR

17. Real-time PCR differs from conventional PCR by all, *except*:
a. More sensitive b. Quantitative
c. Takes more time
d. Real time visualization of amplicons

18. The gold standard typing method is:
a. PCR-RFLP b. Microarray
c. Pulse Field Gel Electrophoresis
d. Phage typing

19. The typing method based on nutritional requirement of the organism____
a. Morphotyping b. Biotyping
c. Auxotyping d. Serotyping

20. Biofire FilmArray system has the following advantages, *except*:
a. Result in 1 hour
b. Completely automated
c. Syndromic panels
d. Cheaper

21. Which of the following panel of Biofire FilmArray system cannot be performed directly from specimen:
a. Meningitis b. Respiratory
c. Blood d. Gastrointestinal

22. Which of the following automated blood culture instrument can automatic determine the volume of blood present in bottle?
a. BACTEC b. Bact/ALERT
c. VIRTUO d. VersaTREK

23. Which of the following automated blood culture instrument works on the principle of fluorometric detection?
a. BACTEC b. Bact/ALERT
c. VIRTUO d. VersaTREK

24. All the following are catalase positive, *except*:
a. *Staphylococcus* b. *Streptococcus*
c. *Escherichia coli* d. *Vibrio*

25. Which of the following is oxidase positive?
a. *Escherichia coli* b. *Klebsiella*
c. *Proteus* d. *Pseudomonas*

26. For the identification of gram-negative bacilli, the common biochemical tests used are abbreviated as 'ICUT'; which stands for all the following, *except*:
a. Indole test b. Coagulase test
c. Urease test d. Triple sugar iron test

27. Optochin susceptibility test is done for:
a. *Streptococcus* b. *Staphylococcus*
c. *Enterococcus* d. Pneumococcus

28. The three basic steps of PCR cycle involves all, *except*:
a. Denaturation b. Amplification
c. Extension d. Gel documentation

29. The three components of PCR involves all, *except*:
a. DNA extraction b. Amplification
c. Gel documentation d. Melting curve analysis

30. DNA polymerase used for LAMP assay is derived from:
a. *Thermus acquaticus*
b. *Geobacillus stearothermophilus*
c. *Escherichia coli* d. *H. influenzae*

31. All the following are specific methods for detection of amplification products of real-time PCR, *except*:
a. SYBR green dye
b. TaqMan hydrolysis probe
c. Molecular beacon
d. Fluorescence resonance energy transfer (FRET)

32. Which of the following automated system can perform antibiotic susceptibility test for yeasts?
a. Phonenix b. Microscan Walkaway
c. VITEK2 d. All of the above

33. The restriction enzymes used in Pulse Field Gel Electrophoresis (PFGE) cut the bacterial DNA into:
a. Less number of larger size DNA fragments
b. More number of larger size DNA fragments
c. Less number of smaller size DNA fragments
d. More number of smaller size DNA fragments

Answers

1. c **2.** a **3.** d **4.** c **5.** a **6.** d **7.** d **8.** b **9.** b **10.** a **11.** b **12.** d **13.** c **14.** c **15.** a **16.** b
17. c **18.** c **19.** c **20.** d **21.** c **22.** c **23.** a **24.** b **25.** d **26.** b **27.** d **28.** d **29.** d **30.** b **31.** a **32.** c
33. a

Bacterial Genetics

6
CHAPTER

Chapter Preview

- Principles of bacterial genetics
- Bacterial variation
- Horizontal gene transfer in bacteria
- Transformation
- Transduction
- Lysogenic conversion
- Conjugation
- Transposition
- Gene transfer by artificial methods

PRINCIPLES OF BACTERIAL GENETICS

Bacterial genetics deals with the study of heredity and gene variations seen in bacteria. All hereditary characteristics of the bacteria are encoded in their DNA (deoxyribonucleic acid). Bacterial DNA is present in chromosome as well in extrachromosomal genetic material as plasmid.

BACTERIAL DNA

Bacteria possess a single haploid chromosome, comprising of super coiled circular double stranded DNA of 1 mm length. The bacterial DNA lacks basic proteins. However, some bacteria have a linear DNA chromosome and some have two chromosomes (e.g. *Vibrio cholerae*). Bacteria do not have a true nucleus; but the genetic material is located in an irregularly-shaped region called the nucleoid. There is no nuclear membrane or nucleolus.

Structure of DNA (Watson and Crick Model)

The bacterial DNA molecule is composed of two strands of complementary nucleotides that are coiled together in the form of a double helix (Fig. 6.1) as described first by Watson and Crick.

- **Each strand is composed of three elements:** It has a backbone of deoxyribose sugar and phosphate groups. The nitrogenous bases are attached to the sugar group. The terms nucleotide and nucleoside are often used to describe the components of the DNA strand—
  - Nucleoside = Sugar + nitrogenous base
  - Nucleotide = Sugar + nitrogenous base + phosphate.
- There are four nitrogenous bases:
  - Two purines—adenine (A) and guanine (G)
  - Two pyrimidines—thymine (T) and cytosine (C).
- **Pairing:** The two DNA strands are held together by hydrogen bonds occurring between the nitrogenous bases on the opposite strands. The pairing follows a specific rule—
  - Adenine of one strand binds with thymine (A-T) of other strand by double hydrogen bonds.
  - Guanine of one strand binds with cytosine (G-C) of other strand by triple hydrogen bonds.
- Hence, in a molecule of DNA, the number of adenine molecules is equal to that of thymine, and the number of guanines is equal to cytosines.

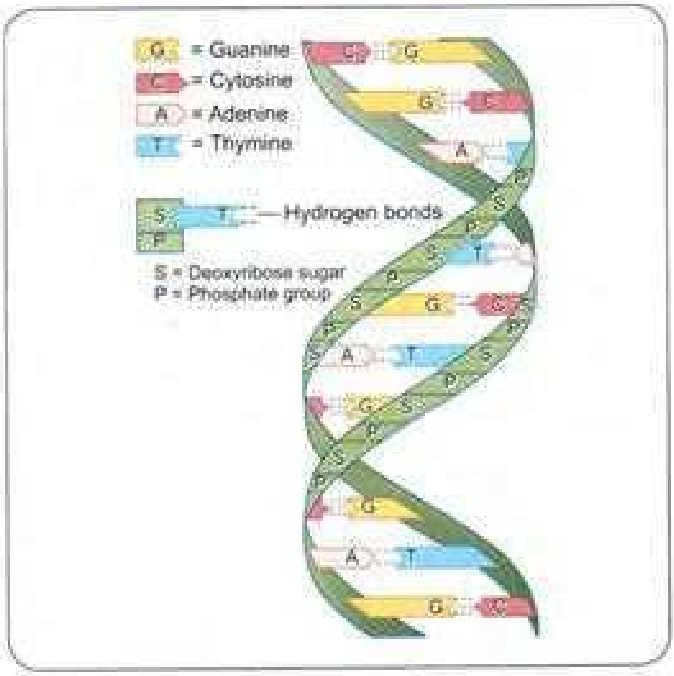


Fig. 6.1: Structure of DNA

Concept adapted and modified from Jawetz, Melnick, & Adelberg's Medical Microbiology. McGraw-Hill Education (with permission).

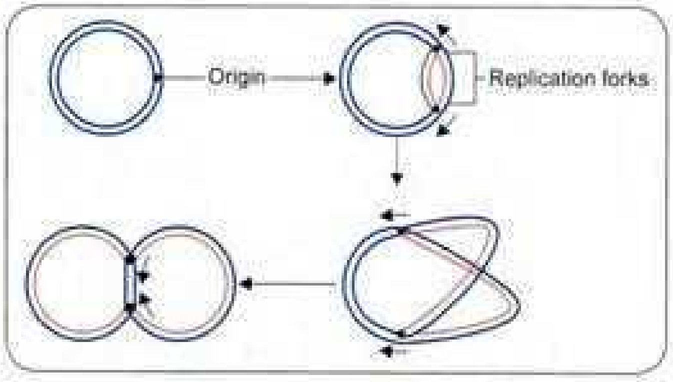


Fig. 6.2: Bidirectional replication

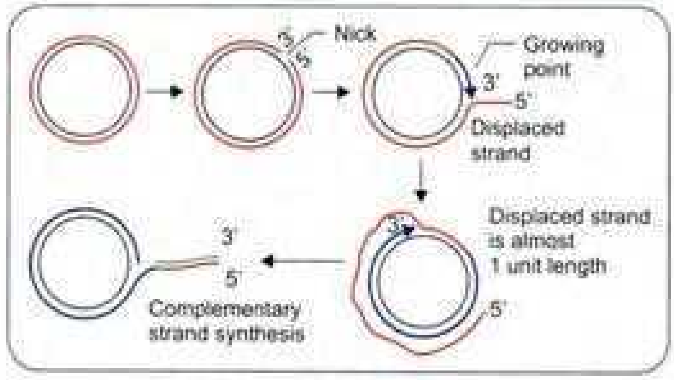


Fig. 6.3: Rolling-circle mechanism

- The ratio of A + T to G + C is constant for each species but varies widely from one bacterial species to another.

DNA Replication

In eukaryotes, during DNA replication, the two strands of the double helix unwind from one another and separate. Each strand acts as template for a new DNA strand which is synthesized through complementary base pairing—A with T, and G with C. In prokaryotic cells, DNA replication takes place in a similar way with some differences.

Bidirectional Replication

For example in *E. coli*, the replication begins at a single point, the origin. DNA helix is unwound at a region called replication fork. It is the site at which the DNA synthesis occurs and individual strands are replicated. Two replication forks move outwards from the origin until they have copied the whole replicon and a structure shaped like the Greek letter theta (θ) is formed (Fig. 6.2). Finally, since the forks meet on the other side and two chromosomes are separated.

Rolling-circle Mechanism

This is a different pattern of DNA replication which occurs during bacterial conjugation and during the reproduction of viruses and bacteriophages.

- The outer strand is nicked and the free 3' end is extended by replication enzymes in a manner that the growing point rolls around the circular inner strand (Fig. 6.3)
- At the same time, the 5' end of the outer strand is displaced and forms a single-stranded tail which later may be converted to the dsDNA by complementary strand synthesis.

The DNA replication in bacteria is catalyzed by several replication enzymes such as—

- **Helicase:** It is responsible for DNA unwinding
- **Topoisomerase** (e.g. DNA gyrase in *E. coli*): It relieves the tension generated by rapid unwinding by removing the super twists
- **DNA polymerase:** It forms complementary strand synthesis by adding nucleotides to the growing end of the strand. It catalyzes the synthesis of DNA in the 5' to 3' direction while reading the DNA template in the 3' to 5' direction. DNA polymerase III plays the major role in replication, although it is probably assisted by polymerase I. It is thought that polymerases I and II participate in the repair of damaged DNA
- **DNA ligase:** It helps in joining of the fragments.

BACTERIAL RNA

RNA (ribonucleic acid) is structurally similar to DNA, except for two differences.

- In sugar—ribose is present instead of deoxyribose and
- In nitrogenous base—uracil replaces thymine.

There are three different types of RNA in a cell, messenger RNA (mRNA), ribosomal RNA (rRNA) and transfer RNA (tRNA). The main function of RNA is protein synthesis.

POLYPEPTIDE SYNTHESIS

Gene is a segment of DNA that stores information for a particular polypeptide synthesis. The genetic information that is stored in DNA is transcribed into RNA and then translated to form the particular polypeptide.

Genetic Code

Codon: It is a sequence of three nucleotide bases present on mRNA that stores the information of an amino acid synthesis. It was discovered by Nirenberg and Khorana (1968).

- **Sense codons:** There are 64 codons, out of which 61 are sense codons, each directs the production of a single amino acid. As there are only 20 amino acids, so more than one codon exist for the same amino acid
- **Non-sense codons:** The remaining three codons (UGA, UAG, and UAA) do not code for any amino acids and are involved in the termination of translation; hence called as stop codons.

- **Start codon:** It is the first codon of an mRNA from which the translation begins. The most common start codon is AUG which codes for methionine in eukaryotes and modified methionine [N-Formyl methionine (fMet)] in prokaryotes.

Anticodon: It is a set of three nucleotide bases present on tRNA that is complementary to the nucleotide bases of codon on mRNA.

Transcription

Transcription is a process, in which a particular segment of DNA is copied into RNA by the enzyme RNA polymerase. Since DNA acts as a template for synthesis of mRNA, therefore, the bases in mRNA are complementary to that of DNA.

Translation

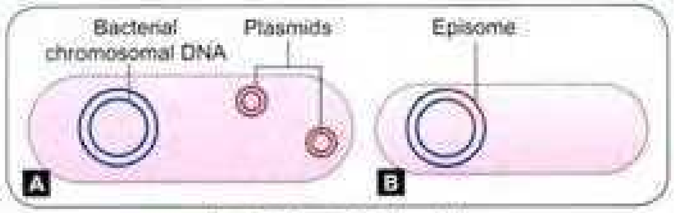
In translation, the mRNA transcribed from DNA is decoded by a ribosome to produce a specific amino acid chain, or polypeptide. It occurs in cytoplasm of the bacteria and proceeds in four phases:

1. **Initiation:** The ribosome assembles around the target mRNA. The first tRNA is attached at the start codon of mRNA.
2. **Elongation:** The tRNA transfers an amino acid to the adjacent tRNA, corresponding to the next codon.
3. **Translocation:** The ribosome then moves (translocates) to the next mRNA codon to continue the process, creating an amino acid chain.
4. **Termination:** When a stop codon is reached, the ribosome releases the polypeptide.

PLASMID

Plasmids are the extrachromosomal ds circular DNA molecules that exist in free state in the cytoplasm of bacteria (Fig. 6.4A) and also found in some yeasts.

- **Not essential:** Plasmids are not essential for life; bacteria may gain or lose plasmid during their lifetime
- **Numbers:** They may be present singly or in multiple numbers—up to 40 or even more per cell
- **Independent replication:** Plasmids are capable of replicating independently. They can behave as replicons, possessing an origin of replication and other genes that help in replication
- **Episome:** Sometimes, the plasmid may integrate with chromosomal DNA of bacteria and such plasmids are called as episomes. They replicate along with bacterial chromosome (Fig. 6.4B)
- **Curing:** The process of eliminating the plasmids from bacteria is known as curing. It may occur spontaneously or may be induced by treatment of the host cells with substances that inhibit plasmid replication without affecting the host cell, such as acridine, radiations, thymine starvation, and growth at higher temperatures.



Figs 6.4A and B: Plasmids

Classification of Plasmids

Plasmids can be classified in many ways:

1. **Based on ability to perform conjugation:**
   - Conjugative plasmids: Some plasmids have an ability to transfer themselves to other bacteria by means of conjugation. These are called self-transmissible or conjugative plasmids
   - Non-conjugative plasmids: There are also called as nontransmissible plasmids as they cannot transfer themselves.
2. **Based on compatibility between the plasmids**, they can be grouped into:
   - Compatible plasmids: Different plasmids can exist in a single bacterial cell only if they are compatible to each other
   - Incompatible plasmids: If two plasmids are not compatible, one or the other will be rapidly lost from the cell. They normally share the same replication or partition mechanisms, hence compete with each other.
3. **Based on function**, there are five main classes of plasmids:
   a. **Fertility or F-plasmids:** They contain *tra*-genes, which code for the expression of sex pili that help in bacterial conjugation by forming the conjugation tube.
   b. **Resistance (R) plasmids:** They contain genes that code for resistance to various antibiotics.
   c. **Col plasmids:** They contain genes that code for bacteriocins (antibiotic-like protein substances produced by bacteria that can kill other bacteria).
   d. **Virulence plasmids:** They code for certain virulence factors and toxins that help in bacterial pathogenesis. Examples include:
      - Heat labile and heat stable toxin of *E. coli*
      - Siderophore production
      - Adherence antigens (K88 plasmid in *E. coli*).
   e. **Metabolic plasmids:** They enable the host in various metabolic activities:

- Digestion of unusual substances, e.g. toluene and salicylate, camphor, etc.
- Urease synthesis
- Nitrogen fixation.

Plasmid as Vector

Plasmids by their ability to transfer DNA from one cell to another, they have become important vectors in genetic engineering. Plasmids contain certain sites where genes can be inserted artificially by recombinant DNA technology. Such plasmids can be used for various purposes such as protein production, gene therapy, etc. (described later in this chapter).

BACTERIAL VARIATION

There are two types of variations seen in bacteria:

1. **Phenotypic variation:** It refers to the variations in the expression of various characters by bacterial cells in a given environment, such as synthesis of flagella, expression of certain enzymes, etc.
2. **Genotypic variation:** It is the change in the genetic constitution of an organism; which occurs mostly as a result of mutation.

MUTATION

Definition: Mutation is a random, undirected heritable variation caused by change in nucleotide sequence of the genome of the cell.

Mutation can involve any of the numerous genes present in bacterial chromosome or rarely plasmid. The frequency of mutation ranges from 10^{-2} to 10^{-10} per bacterium per division.

Mutations occur in one of the two ways:

1. **Spontaneous mutations:** Mutations that occur naturally in any dividing cells that arise occasionally without adding any mutagen.
2. **Induced mutations:** These mutations on the other hand, are as a result of exposure of the organism to a **mutagen**, an agent capable of inducing mutagenesis. Examples of mutagens include—
   - Physical agent, e.g. ultraviolet (UV) radiations—cytosine and thymine are more vulnerable to UV rays
   - Chemical agents, e.g. alkylating agents, 5-bromouracil and acridine dyes.

Mutation is a natural event, taking place all the time, in all dividing cells. Most mutants go unrecognized as the mutation may be lethal or may involve some minor functions that may not be expressed. Mutation is best appreciated when it involves a function, which can be readily observed by experimental methods. For example, *E. coli* mutant that loses its ability to ferment lactose can be readily detected on MacConkey agar.

Mutation can affect any gene and hence may modify any characteristic of the bacterium, for example—

- Sensitivity to bacteriophages
- Loss of ability to produce capsule or flagella
- Loss of virulence
- Alteration in colony morphology
- Alteration in pigment production
- Drug susceptibility
- Biochemical reactions
- Antigenic structure.

The practical importance of bacterial mutation is mainly in the field of drug resistance and the development of live vaccines.

Classification of Mutation Types

Mutations may occur in two ways—

1. Small-scale mutations: They are more commonly seen in bacteria. Examples include (1) point mutations—occur at a single nucleotide, (2) addition or deletion of single nucleotide pair.
2. Large-scale mutations occur in chromosomal structure: These include deletion or addition of several nucleotide base pairs or gene duplications.

Various types of mutations observed in bacteria are described in Table 6.1.

Detection and Isolation of Mutants

Mutation can be recognized both by genetic method (gene sequencing) as well as by observing phenotypic changes such as fluctuation test and replica plating method. The carcinogenicity of a mutagen is tested by Ames test.

Fluctuation Test

Fluctuation test demonstrates the spontaneous mutation in bacteria. It was described by Luria and Delbruck (1943).

- It states that when bacterial suspension is subjected to selective pressure by subculturing on to agar plate containing a growth limiting substance (e.g. streptomycin or bacteriophage, etc.), they undergo spontaneous mutation
- However, the rate of mutation vary widely (some bacteria mutate early, some late) which leads to fluctuations
- Fluctuations in mutation are wide when small volume subcultures are made (which leads to more frequent mutations), as compared to large volume subcultures (where the mutations occur less frequently)
- This experiment was not widely appreciated, probably due to the complicated statistical evaluation.

Table 6.1: Types of mutations

Forward mutations	
Substitutions at single nucleotide base pair	
At DNA Level	
Transition	It is a point mutation that changes a purine nucleotide to another purine (A ↔ G) or a pyrimidine nucleotide to another pyrimidine (C ↔ T)
Transversion	It refers to the substitution of a purine for a pyrimidine or vice versa in DNA, (C/T ↔ A/G)
At codon level	
Silent mutation	The new codon codes for the same amino acid; e.g. AGG ↔ CGG, both code for arginine
Neutral mutation	The new codon forms different but functionally equivalent amino acid: AAA (lysine) AGA (arginine)
Missense mutation	The new codon codes for a different amino acid
Nonsense mutation	The new codon is a stop codon which causes termination, e.g. CAG (Glutamine) ↔ UAG (stop)
Addition or deletion at single or many nucleotide base pairs	
Frame-shift mutation	Any addition or deletion of base pairs that is not a multiple of three results in a shift in the normal reading frame of the coded message forming new set of triplet codon. They are usually very deleterious and may lead to synthesis of nonfunctional proteins
Reverse mutations	**It is a second mutation that nullifies the effect of the first mutation and results in gaining back the function of the wild phenotype**
True reversion	• A true reverse mutation converts the mutant nucleotide sequence back to the wild-type sequence. • AAA (Lysine) forward mutation GAA (Glutamine) reverse mutation AAA (Lysine) (wild type) ⟶ (mutant) ⟶ (wild type)
Equivalent reversion	• Second mutation produces a different codon which codes for the same amino acid of wild type sequence • UCC (Serine) forward mutation GAA (Cystine) reverse mutation AAA (Serine) (wild type) ⟶ (mutant) ⟶ (wild type)
Suppressor mutation	It is a second mutation in a different gene that reverts the phenotypic effects of an already existing mutation

Replica Plating Method

Replica plating method is used to detect auxotrophic mutants; described by Lederberg in 1952. It differentiates between the normal strains from auxotrophic mutants based on their ability to grow in the absence of a particular nutrient on which the mutant is dependent. For example, a lysine auxotroph will grow on lysine-supplemented media but not on a medium lacking lysine.

- Using a velvet template, mixture of colonies (some normal strains, some auxotroph mutants) of an organism are transferred from a master plate, onto two subculture plates—one of the plate is lacking a limiting nutritional substance, e.g. lysine
- After incubation, colonies similar to those on master plate are formed with relative position of all the colonies retained on the subculture plates, except for the lysine auxotroph which do not grow on the media lacking lysine (Fig. 6.5).

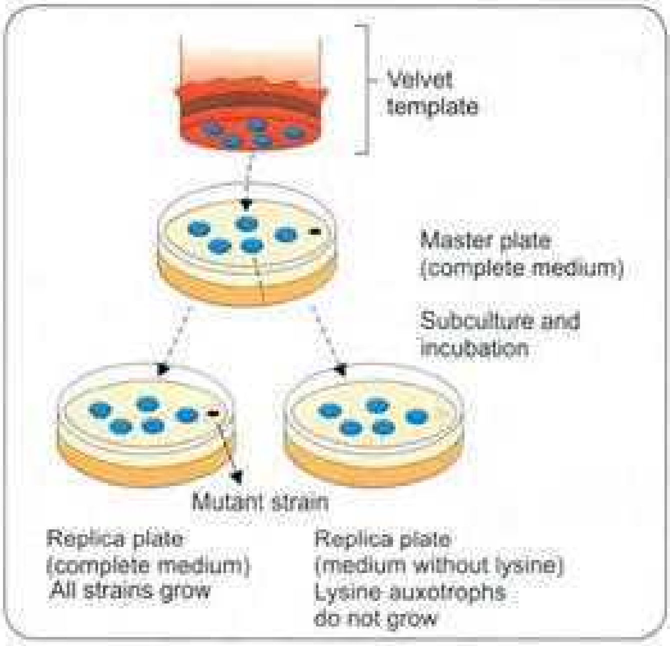


Fig. 6.5: Replica plating method

Ames Test (Carcinogenicity Testing)

Ames test is used to identify the environmental carcinogens. It was developed by Bruce Ames (1970).

- It is a mutational reversion assay that uses the mutant strains (histidine auxotroph) of *Salmonella* which are subcultured on two agar plates containing small amount of histidine; one of the plate is added with the test mutagen
- The plates are incubated for 2–3 days at 37°C

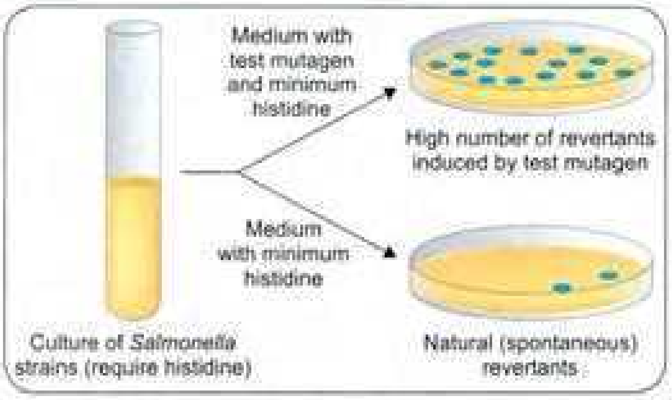


Fig. 6.6: Ames test (carcinogenicity testing)

Note: Revertant means conversion of *his-* strain to *his+*.

- All the histidine auxotrophs will grow for the first few hours until the histidine is depleted
- Once the histidine supply is exhausted, only revertants that have mutationally regained the ability to synthesize histidine will grow (Fig. 6.6)
- Reversed mutation may be induced due to carcinogen (can affect large number of strains) or occur spontaneously (affect only few strains)
- The relative mutagenicity of the carcinogen can be estimated by counting the colonies—the more colonies, the greater is the mutagenicity.

HORIZONTAL GENE TRANSFER IN BACTERIA

Gene transfer in bacteria can be broadly divided into—
- Vertical gene transfer (transmission of genes from parents to offspring during cell division)
- Horizontal gene transfer (transmission of genes from one bacterium to another neighbor bacterium).

Horizontal gene transfer occurs in bacteria by several methods, such as:
- Transformation (uptake of naked DNA)
- Transduction (through bacteriophage)
- Lysogenic conversion
- Conjugation (plasmid mediated via conjugation tube).

TRANSFORMATION

Definition

Transformation is a process of random uptake of free or naked DNA fragment from the surrounding medium by a bacterial cell and incorporation of this DNA fragment into its chromosome in a heritable form.

Natural transformation has been studied so far only in certain bacteria—*Streptococcus, Bacillus, Haemophilus, Neisseria, Acinetobacter* and *Pseudomonas*.

Mechanism of Transformation

When bacteria lyse, they release large amounts of dsDNA into the surrounding environment. Their uptake depends up on the competency of the bacteria present in the surroundings.

Competency for Transformation

Competent bacteria refers to the cells multiplying in log phase of cell division and expressing certain transformation promoting factors called competence factors.

- Bacteria expressing competence factors (e.g. *S. pneumoniae*) can uptake any DNA fragment irrespective of source.
- But competence factors are not expressed by all bacteria that mediate transformation e.g. *Haemophilus influenzae*. In such case, the uptake of DNA occurs only from the closely related species.

The transformation frequency of very competent cells is around 10^{-3} for most genera. Steps involved in transformation are as follows (Fig. 6.7):

1. A long dsDNA fragment comes in contact with a competent bacterium and binds to DNA-binding protein present on its surface and then it is nicked by a nuclease.
2. One strand is degraded by the recipient cell exonucleases.
3. The other strand associates with a competence specific protein and is internalized, which requires energy expenditure.
4. The single strand enters into the cell and is integrated into the host chromosome in place of the homologous region of the host DNA.

Griffith Experiment

The famous Griffith experiment (1928) on mice using pneumococci strains provided the direct evidence of existence of transformation.

- Griffith found that mice died when they were injected with a mixture of live noncapsulated pneumococci and

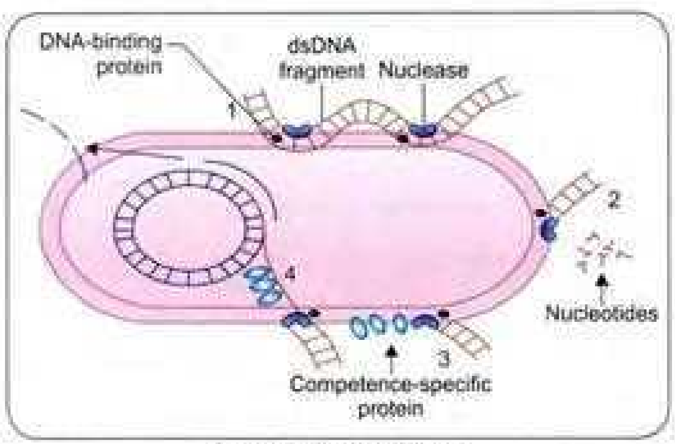


Fig. 6.7: Transformation

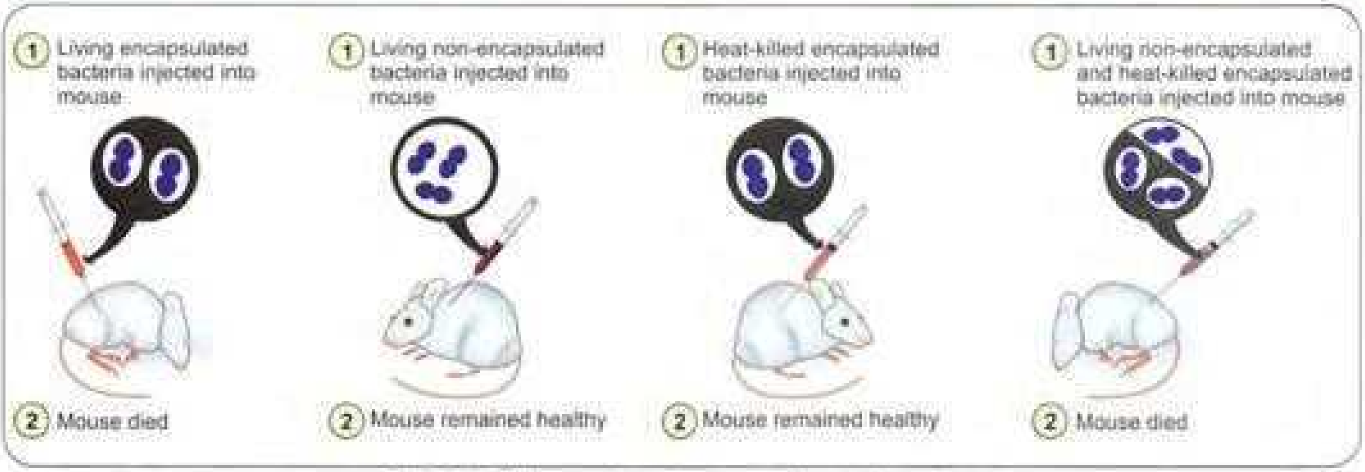


Fig. 6.8: Griffith experiment demonstrating transformation

heat killed capsulated pneumococci strains. However, neither of which separately proved fatal to mice (Fig. 6.8)

- He stated that the live noncapsulated strains were transformed into the capsulated strains due to transfer of the capsular genes released from the lysis of the killed capsulated strains, which was confirmed later by Avery, Macleod and McCarty in 1944.

TRANSDUCTION

Definition

Transduction is defined as transmission of a portion of DNA from one bacterium to another by a bacteriophage (bacteriophage is a virus that infects and multiplies inside the bacterium).

Mechanism of Transduction

During the transmission of bacteriophages from one bacterium to other, a part of the host DNA may accidentally get incorporated into the bacteriophage and then gets transferred to the recipient bacterium. This leads to acquisition of new characters by the recipient bacterium coded by the donor DNA.

Bacteriophages can perform two types of life cycle inside the host bacteria.

1. **Lytic or virulent cycle:** Bacteriophage multiplies in host cytoplasm, produces large number of progeny phages, which subsequently, are released causing death and lysis of the host cell.
2. **Lysogenic or temperate cycle:** In contrast to virulent cycle, here the host bacterium is unharmed. The phage DNA remains integrated with the bacterial chromosome as the prophage, which multiplies synchronously with bacterial DNA. However, when the phage DNA tries to come out, it is disintegrated from host chromosome, comes out into the cytoplasm, and behaves as a lytic phage. It replicates to produce daughter phages, which are subsequently released by host cell lysis.

Types of Transduction

Transduction is of two types, either generalized or restricted.

Generalized Transduction

It involves transfer of any part of the donor bacterial genome into the recipient bacteria. Generalized transduction usually occurs as a result of defective assembly during the lytic cycle of virulent and some temperate phages.

- Packaging errors may happen occasionally due to defective assembly of the daughter phages. Instead of their own DNA, a part of host DNA may accidentally be incorporated into the daughter bacteriophages
- The resulting bacteriophage (called transducing phage) often injects the donor DNA into another bacterial cell but does not initiate a lytic cycle as the original phage DNA is lost
- The donor DNA may have three fates inside the recipient bacterium (Fig. 6.9):
  - **Abortive transduction:** About 70-90% of the transferred DNA is not integrated with the recipient bacterial chromosome, but often is able to survive and express itself. Such bacteria containing this non-integrated, transduced DNA are called abortive transductants
  - **Stable gene transfer:** The donor DNA gets integrated with recipient bacterial chromosome
  - **Unstable gene transfer:** In some cases, the donor DNA gets disintegrated by the host cell enzymes.

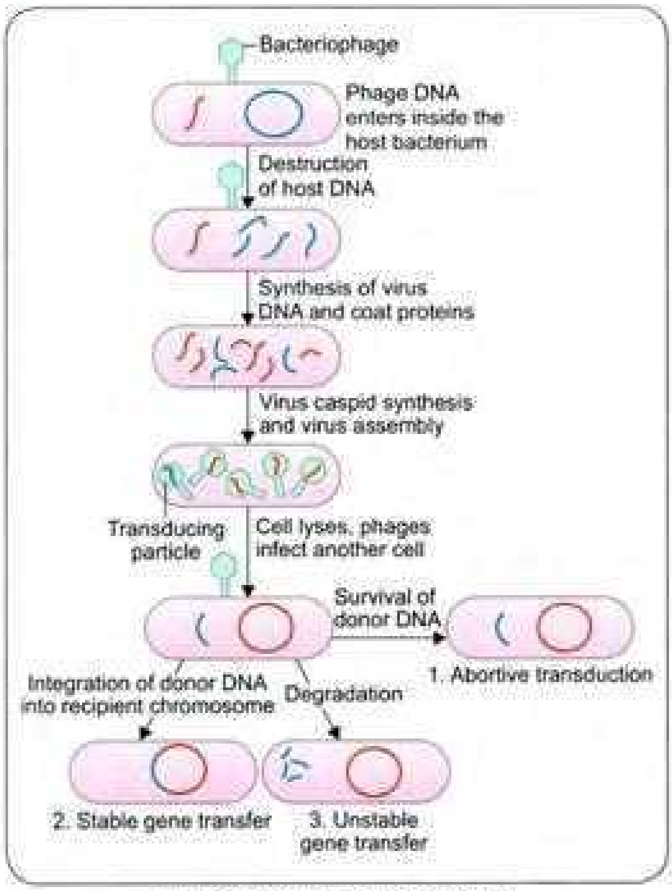


Fig. 6.9: Generalized transduction

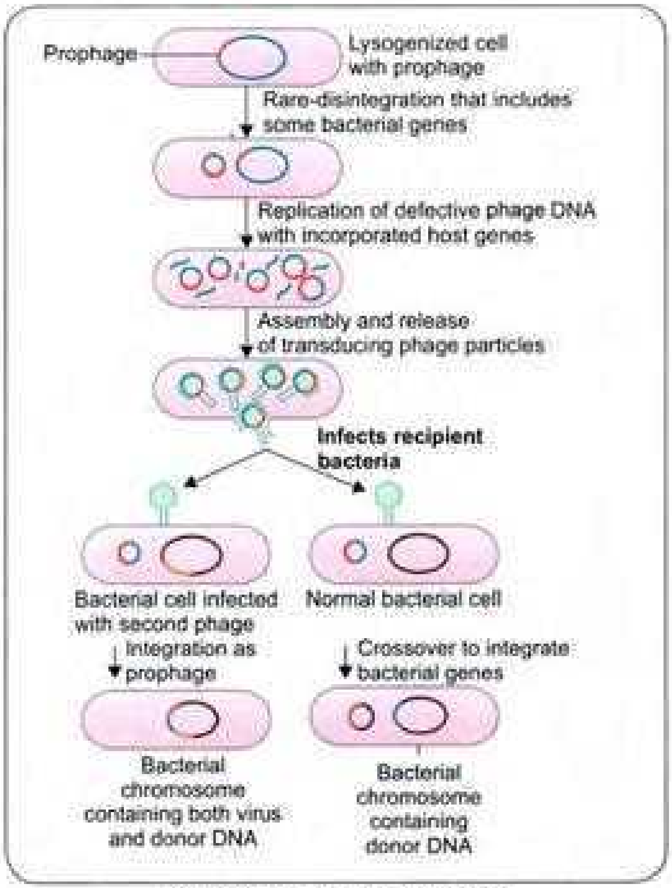


Fig. 6.10: Restricted transduction

Restricted or Specialized Transduction

In contrast to generalized transduction, the restricted transduction is capable of transducing only a particular genetic segment of the bacterial chromosome that is present adjacent to the phage DNA.

It occurs as a result of defect in the disintegration of the lysogenic phage DNA from the bacterial chromosome.

- Restricted transduction has been studied intensively in the 'lambda' phage of *E. coli*
- When a prophage (i.e. lysogenic bacteriophage is integrated with the bacterial chromosome) leaves the host chromosome, portions of the bacterial chromosome present adjacent to the phage DNA may get wrongly excised along with it
- Such transducing phages carrying a part of bacterial DNA in addition to their own DNA, when infect another bacterium, the transfer of the donor DNA takes place in two ways (Fig. 6.10)
  - The entire transducing genome (i.e. phage DNA + donor DNA) acts as a prophage and gets integrated to the recipient chromosome. This occurs if the recipient bacterium is already infected by another helper bacteriophage
  - Crossover between the donor DNA and a part of recipient DNA—leads to integration of the donor DNA into the recipient chromosome and a part of recipient DNA into the phage DNA.

Role of Transduction

In addition to chromosomal DNA, transduction is also a method of transfer of episomes and plasmids.

- **Drug resistance:** Transduction may be a mechanism for the transfer of bacterial genes coding for drug resistance; for example, plasmid coded penicillin resistance in staphylococci
- **Treatment:** Transduction has also been proposed as a method of genetic engineering in the treatment of some inborn metabolic defects.

LYSOGENIC CONVERSION

During the temperate or lysogenic life cycle, the phage DNA remains integrated with the bacterial chromosome as prophage, which multiplies synchronously with the bacterial DNA.

- The prophage acts as an additional chromosomal element which encodes for new characters and is transferred to the daughter cells. This process is known as lysogeny or lysogenic conversion
- **Imparts toxigenicity to the bacteria:** Phage DNA may be responsible for bacterial virulence by coding for their toxin production. For example, in *Corynebacterium diphtheriae*, the diphtheria toxin is coded by a lysogenic phage DNA which is integrated with the bacterial chromosome. Elimination of the phage from a toxigenic strain renders the bacterium nontoxigenic

Phage Coded Toxins

Bacterial toxins that are coded by lysogenic phages include:

- Diphtheria toxin
- Cholera toxin
- Verocytotoxin of *E. coli*
- *Streptococcus* pyrogenic exotoxin (SPE)— A and C
- Botulinum toxin C and D

- In lysogenic conversion, the phage DNA itself behaves as the new genetic element, in contrast to transduction where the phage acts only as a vehicle carrying bacterial genes.

CONJUGATION

Conjugation refers to the transfer of genetic material from one bacterium (donor or male) to another bacterium (recipient or female) by mating or contact with each other and forming the conjugation tube. It was discovered first by Lederberg and Tatum (1946).

F^+ X F^- Mating

The F^+ cell (also called as the donor or the male bacterium) contains a plasmid called as F factor or fertility factor. The bacteria lacking the F factor are called as recipient or female bacteria or F^- cell.

- F factor is a conjugative plasmid; carries genes that encode for the formation of sex pilus (that helps in conjugation) and self plasmid transfer
- The F pilus brings the donor and nearby recipient cells close to each other and form a conjugation tube that bridges between the donor and recipient cells (Fig. 6.11A)
- During conjugation, the plasmid DNA replicates by the rolling-circle mechanism, and a copy moves to the recipient bacterium through the conjugation tube. Then, in the recipient, the entering strand is copied to produce complete F factor with ds DNA
- As a result, recipient (F^-) becomes (F^+) cell and can in turn conjugate with other (F^-) cells. Therefore, it is said that this character of maleness (F^+) in bacteria is transmissible or infectious
- During F^+ X F^- conjugation, chromosomal genes from donor bacterium may rarely be transferred along with F factor. Here, though the donor chromosomal gene may undergo recombination with the recipient chromosome; but with a lower frequency.

HFR Conjugation

F factor being a plasmid, it may integrate with bacterial chromosome and behave as episome.

- Such donor cells are able to transfer chromosomal DNA to recipient cells with high frequency in comparison to F^+ cells, therefore, named as Hfr cells (high frequency of recombination)
- During conjugation of Hfr cell with an F^- cell, only few chromosomal genes along with a part of the F factor get transferred. Connection between the cells usually breaks before the whole genome is transferred
- As the entire F factor does not get transferred, hence following conjugation, F^- recipient cells do not become F^+ cells (Fig. 6.11B).

F' Conjugation

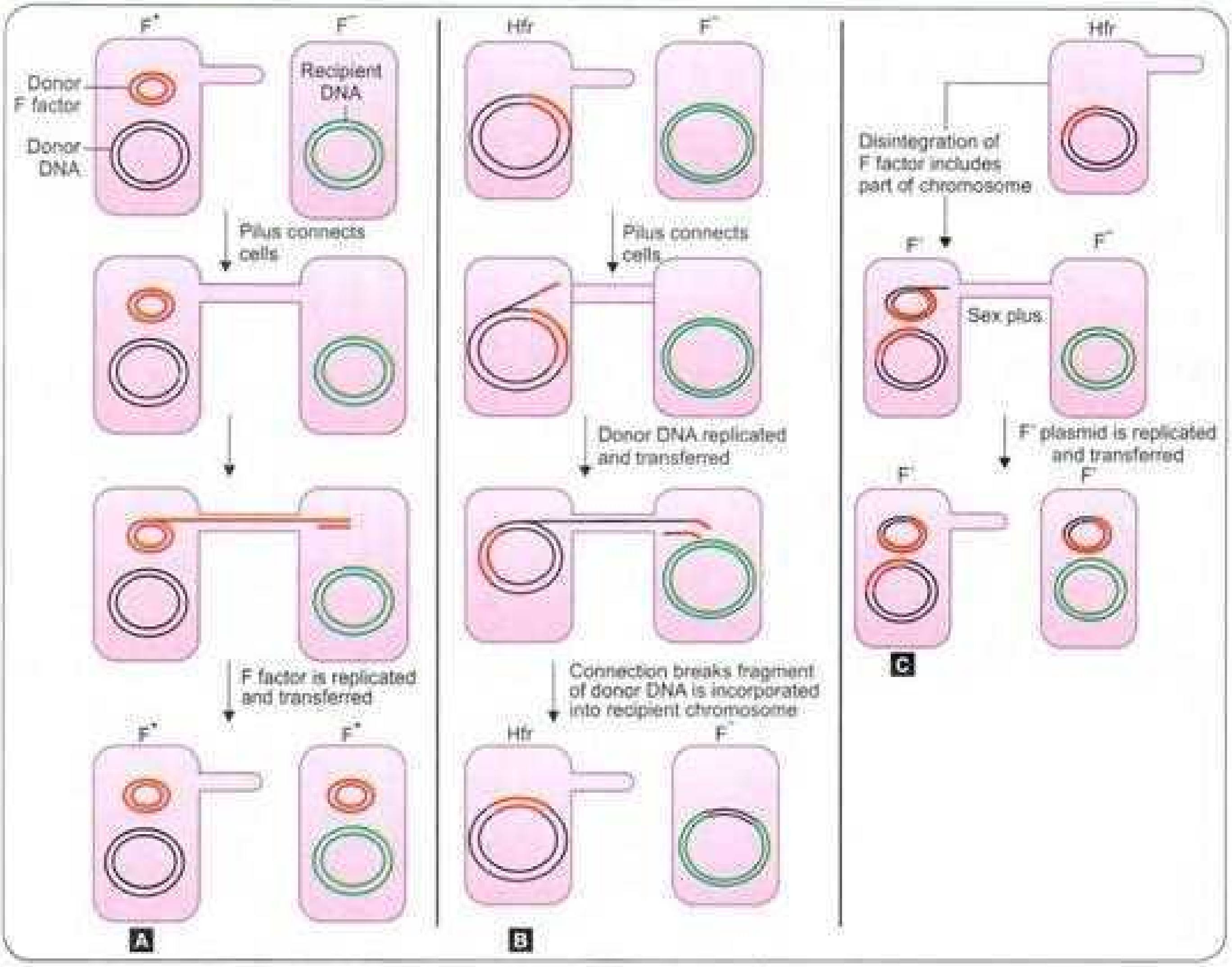
The conversion of a F^+ cell into a Hfr cell is reversible.

- When the F factor reverts from the integrated to free-state, it may sometimes carry with it some chromosomal DNA from the adjacent site of its attachment. Such an F factor carrying some chromosomal DNA is named as F' factor (F prime factor)
- When F' cell conjugates with a recipient (F^-), it transfers the host DNA incorporated with it along with the F factor. The recipient becomes F' cell. This process is called sexduction (Fig. 6.11C).

Conjugation plays an important role in the transfer of plasmids coding for antibacterial drug resistance [resistance transfer factor (RTF), see the box in the proceeding text] and bacteriocin production [Colicinogenic (Col) factor].

Colicinogenic (Col) Factor

The bacteriocin production in bacteria is plasmid coded which may be transferred by conjugation. Such plasmids are called as the col factors. Bacteriocins are the antibiotic-like substances produced by one bacterium that inhibit other bacteria. Bacteriocin produced by coliform bacteria are called as colicin. Bacteria other than coliforms also produce similar kind of substances e.g. pyocin by *Pseudomonas*, diphthericin by *Corynebacterium diphtheriae*.



Figs 6.11A to C: Bacterial conjugation. **A.** F+ X F- mating; **B.** Hfr X F- mating; **C.** F' X F- mating

Resistance Transfer Factor (RTF)

Conjugation is also an important method of transfer of plasmids coding for multiple drug resistance among bacteria.

- R factor (or the resistance factor) is a plasmid which has two components. (R factor = RTF+ r determinants)
  - Resistance transfer factor (RTF): It is the plasmid responsible for conjugational transfer (similar to F factor)
  - Resistance determinant (r): R factor can have several r determinants and each r determinant coding for resistance to one drug.
- Sometimes, the R factor dissociates and both RTF and the r determinants exist as separate plasmids. In such cases, the resistance is not transferable though the host cell remains drug resistant
- In addition to r determinants, the RTF can also attach to other genes; for example, genes coding for enterotoxin and hemolysin production in some enteropathogenic *E. coli*.

Mutational and transferrable drug resistance have been discussed in Chapter 7.

Fate of the Donor DNA Following Horizontal Transfer

Following horizontal gene transfer by any of the methods described above, the donor DNA enters inside the recipient cell, and remains in the cytoplasm temporarily. At this stage, the recipient cell is called **merozygote**. The donor DNA has one of the following fate inside the recipient cell (Fig. 6.12).

- **Recombination:** The donor DNA integrates with the recipient chromosome either as a replacement piece (usually occurs in transformation) or as an extra piece
- **Partially diploid cells:** The donor DNA persists outside the host chromosome and the host cell becomes partially diploid for a portion of the genome that is homologous to the donor DNA. Such cells may or may not replicate to produce a clone of partially diploid cells
- **Host restriction:** The host cell nucleases may degrade the donor DNA if it is not homologous to any part of bacterial chromosome.

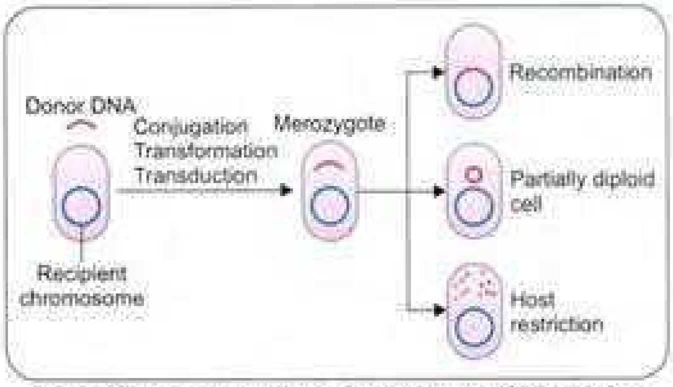


Fig. 6.12: Fate of the donor DNA following horizontal transfer

BACTERIAL RECOMBINATION

Recombination takes place between the donor DNA and recipient chromosome in two ways—general recombination and site specific recombination.

General or Homologous Recombination

It is the most common form, can occur at any place on the recipient's chromosome in general, and occurs between DNA of similar sequences (homologous).

- *Rec genes:* The *rec* genes present in recipient's chromosome and their products, such as the *recA* proteins are crucial to bring out recombination
- **Crossing-over:** The donor and recipient DNA strands breakage takes place followed by their reunion by crossing-over of strands
- The **Holliday model** has been put forward (named after Robin Holliday, 1964), to describe the process of reunion.

Reciprocal vs Nonreciprocal General Recombination

Most of the donor DNA fragments entering into the recipient cell by horizontal gene transfer are double stranded; except those transferred by transformation which are single stranded. General recombination can be of two types:

1. **Reciprocal exchange:** In most cases (except in transformation), the general recombination usually involves a reciprocal exchange between a pair of homologous DNA sequences between donor and recipient strands.
2. **Nonreciprocal exchange:** In bacterial transformation, a nonreciprocal form of general recombination takes place. The single strand of donor DNA is inserted into the host chromosome (by replacing a piece of host chromosome) to form a stretch of heteroduplex DNA.

Site Specific Recombination

In restricted transduction, the integration of bacteriophage DNA into bacterial chromosome is site-specific.

- The donor DNA is not homologous with the chromosome it joins, and
- The enzymes responsible for this event are specific for the particular bacteriophage and its host bacterium.

TRANSPOSITION

Transposons or transposable elements are the bacterial genes that are capable of intracellular transfer between chromosome to chromosome, plasmid to plasmid, and chromosome to plasmid or vice versa and the process of such intracellular transfer of transposons is called as transposition. As transposons move around the genome in a cut-and-paste manner, they are also called **jumping genes** or **mobile genetic elements**.

- Transposition does not require any DNA homology between transposon and the site of insertion. It is, therefore, different from recombination
- Unlike plasmids, transposons are not self-replicating and are dependent on chromosomal or plasmid DNA for replication
- Transposons were first discovered in the 1940s by Barbara McClintock during her studies on maize genetics for which she won the Nobel prize in 1983
- Transposons are also discovered in the virus and in eukaryotic genome.

Types of Transposons

Insertion Sequence Transposon

The simplest form of transposon is an insertion sequence. It is about 1-2 kilo basepairs (kbp) in length and consists of a **transposase gene** (that helps in transposition) which is flanked at both the ends by **inverted repeat sequences** of nucleotides, i.e. nucleotide sequences complementary to each other but in the reverse order (Figs 6.13A and B).

Because of this feature, each strand of the transposon can form a single-stranded loop carrying the transposase gene, and a double stranded stem formed by hydrogen bonding between the terminal inverted repeat sequences.

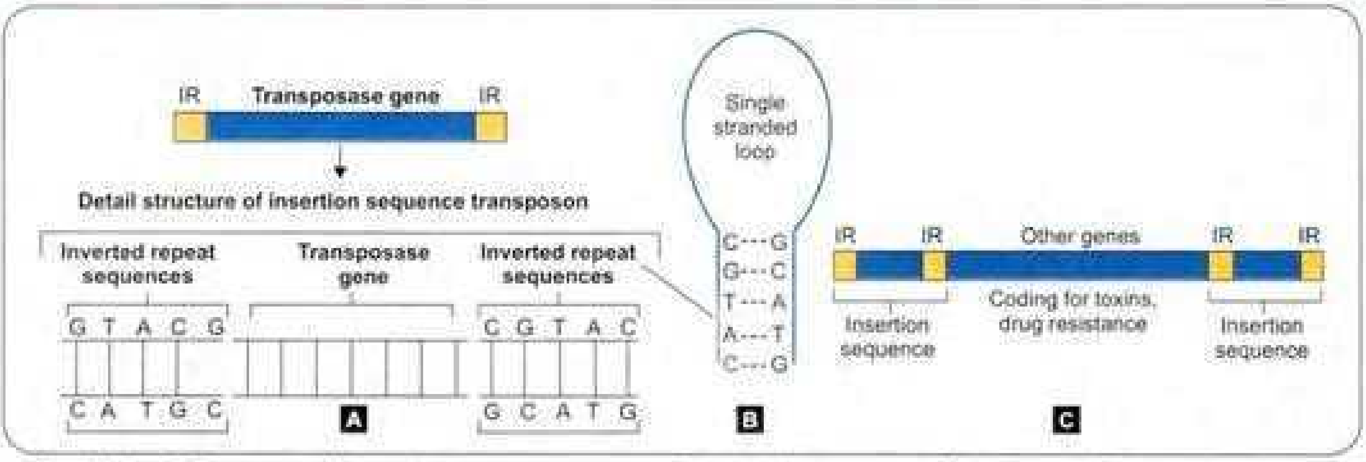
Composite Transposon

They are larger transposons carrying additional genes, such as genes coding for antibiotic resistance or toxin production in the center and both the ends are flanked by insertion sequences that are identical or very similar in sequence (Fig. 6.13C).

GENE TRANSFER BY ARTIFICIAL METHODS

GENETIC ENGINEERING

Genetic engineering refers to deliberate modification of an organism's genetic information by directly altering its



Figs 6.13A to C: Transposons. **A.** Insertion sequence transposon; **B.** Hairpin loop structure of transposon; **C.** Composite transposon

nucleic acid genome. Genetic engineering is accomplished by a precise mechanism known as recombinant DNA technology.

The gene coding for any desired protein is isolated from an organism, and then inserted into suitable vector, which is then cloned in such a way that it can be expressed in the formation of specific (desired) protein.

Recombinant DNA Technology

The procedure of recombinant DNA technology involves the following steps:

1. **Treatment with restriction enzyme:** The DNA from the microorganism is extracted and then is cleaved by enzymes called restriction endonucleases to produce mixture of DNA fragments.
2. **Southern blot:** The fragment containing the desired gene is isolated from the mixture of DNA fragments. This is done by:
   - Electrophoresis: DNA fragments are electrophoretically separated by subjecting to agar gel electrophoresis
   - Transfer to nitrocellulose membrane: The separated DNA fragments are transferred from the gel to a nitrocellulose membrane
   - Detection of desired gene: The DNA fragment containing the desired gene is detected adding a specific DNA probe, complementary to the gene of interest
   - Isolation: The band containing the desired gene is isolated by DNA extraction and then, is subjected to electrophoresis in a different gel.
3. **Recombination with a vector:** The isolated DNA fragment is annealed with a vector by DNA ligase enzyme.
4. **Introduction of the vector into bacteria:** The vector is introduced into bacteria usually by transformation (injecting by electroporation) and rarely by phage vector by transduction.
5. **Cloning:** Culture of the bacteria containing the desired gene followed by expression of the gene products, yields a large quantity of desired protein.

Applications of Genetic Engineering

- **Production of vaccines:** Preparation of certain vaccines is done by DNA recombination technology by producing the desired antigen that can be used as immunogen in vaccine, against which the protective antibody will be produced, e.g. vaccines for hepatitis B and human papilloma virus
- **Production of antigens used in diagnostic kits:** The antigens used in diagnostic techniques for antibody detection (e.g. ELISA) are prepared by DNA recombinant technology
- **Production of proteins used in therapy:** Genetic engineering has also been used for the production of proteins of therapeutic interest. These include human growth hormone, insulin, interferons, interleukin-2, tumor necrosis factor, and factor VIII
- **Transgenic animals:** Recombinant DNA technology can be used to artificially introduce a foreign DNA into the genome of animals. The process is called transfection and the recombinant animals produced in this way are named transgenic or genetically modified animals. Transgenic mice are available for a variety of biotechnological applications
- **Gene therapy:** Genetic diseases can be cured by replacing the defective gene by introducing the normal gene into the patient.

Vector

A vector is a small piece of DNA, into which a foreign DNA fragment can be inserted and that can be stably maintained in an organism and used for cloning purposes. There are four major types of vectors, such as: 1. Plasmids,

2. bacteriophages, 3. cosmids, 4. artificial chromosomes, such as bacterial/yeast artificial chromosomes.

NUCLEIC ACID PROBE

Nucleic acid probes are radiolabeled or fluorescent labeled pieces of single stranded DNA or RNA, which can be used for the detection of homologous nucleic acid by hybridization.

- Hybridization is the technique in which two single-strands of nucleic acid come together to form a stable double stranded molecule
- There are two types of nucleic acid probes—DNA probes (hybridizes with DNA) and RNA probes (hybridizes with RNA).

Nucleic acid probes are used to detect the specific nucleic acid either—

- Directly in the clinical sample or
- Following amplification of small quantity nucleic acid present in the clinical sample (e.g. in real time PCR) or
- Following enzymatic digestion of the extracted nucleic acid—so that it detects only the specific DNA fragment from the mixture (e.g. in Southern blot).

BLOTTING TECHNIQUES

A blot, in molecular biology refers to a method of transferring DNA, RNA, or proteins, from gel onto a carrier (e.g. nitrocellulose membrane), followed by their detection by using specific nucleic acid probes (for DNA or RNA detection) or enzyme immunoassay (for protein detection). There are various blotting techniques:

- Southern blot is used to detect DNA
- Northern blot is used to detect RNA
- Western blot is used to detect proteins
- Eastern blot: It is a modification of Western blot, used to analyze proteins for post-translational modifications using probes that may detect lipids, carbohydrate, phosphorylation or any other protein modification.

Southern blotting technique is described above, under genetic engineering. The methodology of Northern blot is similar to Southern blot, but uses a RNA probe to detect the specific RNA fragment. Western blot is described in Chapter 12.

EXPECTED QUESTIONS

I. Essay:

1. Name various methods of horizontal gene transfer. Discuss in detail about mechanism of conjugation.

II. Write short notes on:

1. Transformation.
2. Transposition.
3. Transduction.

III. Multiple Choice Questions (MCQs):

1. Mechanism of direct transfer of free DNA:
a. Transformation b. Conjugation
c. Transduction d. Transposition

2. Phage mediated transfer of DNA from one bacterium to another bacterium is known as:
a. Transformation b. Transduction
c. Transmission d. Conjugation

3. Northern blotting is used for separation of:
a. DNA b. RNA
c. Proteins d. None

4. Horizontal transmission of 'R' factor is by:
a. Transduction b. Transformation
c. Conjugation d. Fusion

5. F factor carrying chromosomal DNA is called as:
a. F factor b. Hfr
c. RTF d. F' factor

6. Regarding the blotting techniques, which of the following is false?
a. Southern blot is used to detect DNA
b. Northern blot is used to detect RNA
c. Western blot is used to detect antigens
d. Eastern blot is used to analyze proteins for post-translational modifications

7. Fate of the donor DNA following horizontal transfer:
a. Recombination b. Partially diploid cell
c. Host restriction leads to digestion
d. All of the above

8. Competence factor mediated transformation occurs in:
a. *Haemophilus* b. *Neisseria*
c. *S. pneumoniae* d. *Pseudomonas*

9. The Griffith experiment on mice using pneumococci strains provides the direct evidence of:
a. Transformation b. Transduction
c. Conjugation d. Transposition

10. Plasmids have the following functions, *except*:
a. Conjugation b. Transposition
c. Resistance d. Virulence

11. Phage DNA may code for various toxins, *except*:
a. Cholera toxin b. Anthrax toxin
c. Diphtheria toxin d. Verocytotoxin

12. Following $F^+ \times F^-$ mating, the recipient cell becomes:
a. F^+ cell b. F^- cell
c. Hfr cell d. F' cell

13. Following Hfr $\times F^-$ mating, the recipient cell becomes:
a. F^+ cell b. F^- cell
c. Hfr cell d. F' cell

14. Following $F' \times F^-$ mating, the recipient cell becomes:
a. F^+ cell b. F^- cell
c. Hfr cell d. F' cell

Answers

1. a **2.** b **3.** b **4.** c **5.** d **6.** c **7.** d **8.** c **9.** a **10.** b **11.** b **12.** a **13.** b **14.** d

Antimicrobial Agents, Antimicrobial Resistance and Antimicrobial Susceptibility Testing

7
CHAPTER

Chapter Preview

- Antimicrobial agents
- Antimicrobial resistance
    - Intrinsic and acquired
    - Mutational and transferable
    - Mechanism of resistance
- Antimicrobial susceptibility testing
    - Disk diffusion method
    - Dilution tests
    - Other methods

ANTIMICROBIAL AGENTS

Antimicrobials are the agents that kill or inhibit the growth of microorganisms.

Classification

Antimicrobial agents are classified in various ways:

1. According to microorganisms against which they are used—antibacterial, antifungal, antiparasitic, antiviral agents, etc.
2. According to their ability to kill (ends with suffix cidal) or inhibit (ends with suffix static) the microorganism, e.g. bactericidal and bacteriostatic.
3. According to the source:
    - Antibiotics: These are natural substances, produced by certain groups of microorganisms
    - Chemotherapeutic agents: These agents are chemically synthesized.
4. According to their site of action and usage:
    - Disinfectants destroy a wide range of microbes on non-living surfaces to prevent their spread
    - Antiseptics (which are applied to the living tissues and help to reduce infection), and
    - Antibiotics (which destroy microorganisms within the body).
5. According to the chemical structure and mechanism of action—the antimicrobial agents can be further divided into many classes, as described in Table 7.1 and Fig. 7.1.

Though incorrect, the word 'antibiotics' is loosely used to describe antimicrobial agents.

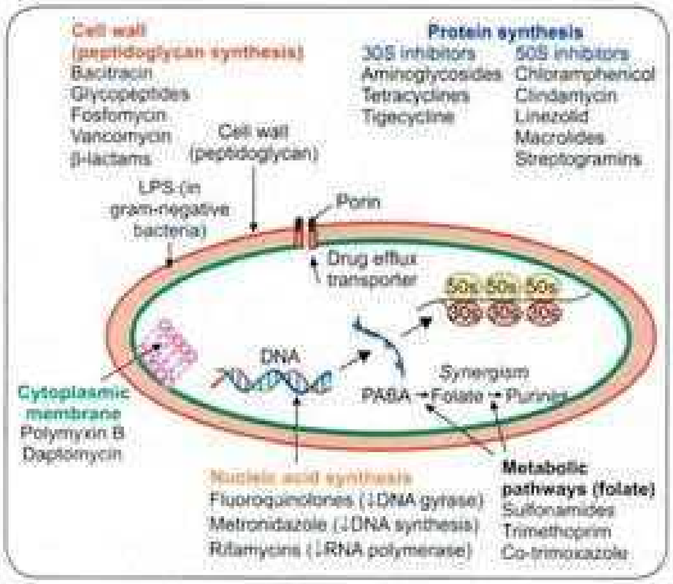


Fig. 7.1: Mechanism of action of antimicrobial agents

ANTIMICROBIAL RESISTANCE

Antimicrobial resistance refers to development of resistance to an antimicrobial agent by a microorganism. It can be of two types— acquired and intrinsic.

Acquired Resistance

This refers to the emergence of resistance in bacteria that are ordinarily susceptible to antimicrobial agents, by acquiring the genes coding for resistance. Most of the antimicrobial resistance shown by bacteria belong to this category.

Table 7.1: Antimicrobial agents—classification, indication, and mechanism of resistance

Class/mechanism	Drugs	Spectrum of activity	Mechanism of resistance
		A. Inhibit Cell Wall Synthesis	
β-Lactam Antibiotics (Bactericidal: block cross linking via competitive inhibition of the transpeptidase enzyme i.e. penicillin-binding protein)			
Penicillins			
Penicillin	Penicillin G Aqueous penicillin G Procaine penicillin G Benzathine penicillin G Penicillin V	Mostly gram-positive bacteria: • *Streptococcus pyogenes* • Pneumococcus • *Corynebacterium diphtheriae* (diphtheria) • *Clostridium tetani* (tetanus) • *Clostridium perfringens* (gas gangrene) • Meningococcal infection • Gonococcus (resistance has been reported) • *Treponema pallidum* (syphilis)	**1. Drug inactivation** (by producing β-lactamase enzyme): Seen in both gram-positive and gram-negative bacteria **2. Alteration of target site-PBP** (penicillin-binding protein) is altered to PBP2a, seen in gram-positive bacteria **3. Decreased permeability** as in gram-negative bacteria- due to altered outer-membrane porins
Penicillinase-resistant-penicillins	Methicillin, cloxacillin, oxacillin, dicloxacillin	**Same as penicillin *plus*** Penicillinase producing *Staphylococcus aureus*	
Aminopenicillins (extended spectrum)	Ampicillin Amoxicillin	**Same as penicillin *plus*** • *Enterococcus faecalis* • *Escherichia coli, Klebsiella* • *Helicobacter pylori* • *Salmonella* (resistance reported) • *Shigella* (bacillary dysentery)	
Antipseudomonal penicillins	Carbenicillin Ticarcillin, piperacillin	**Same as aminopenicillins *plus*** *Pseudomonas aeruginosa*	
β lactam + β lactamase inhibitors	Aminopenicillins or antipseudomonal penicillins plus • Clavulanic acid • Sulbactam, tazobactam	Same as aminopenicillins or anti-pseudomonal penicillins spectrum plus β lactamase producing bacteria	
Cephalosporins			
1st generation	Cefazolin Cephalexin	• *Staphylococcus aureus* • *Staphylococcus epidermidis* • Some gram-negative bacteria like *Escherichia coli* and *Klebsiella*	
2nd generation	Cefoxitin Cefaclor, Cefuroxime	• Same as 1st generation *plus* • ↑ Gram-negative activity	
3rd generation	Ceftriaxone Cefotaxime Ceftazidime	• Decreased activity against gram-positives compared to 1st, 2nd generations • ↑ Gram-negative activity • Some are active against *Pseudomonas* (Ceftazidime)	ESBL (extended spectrum β-lactamases)
4th generation	Cefepime Cefpirome	• Good activity against gram-positive and negative bacteria including *Pseudomonas*	
5th generation	Ceftobiprole Ceftaroline	• Same as 4th generation *plus Pseudomonas* and MRSA (only β lactam to be effective against Methicillin resistant *Staphylococcus aureus*, i.e. MRSA)	
Carbapenems	Imipenem Meropenem Doripenem Ertapenem	• Broadest range of activity against most bacteria, which include gram-positive cocci, Enterobacteriaceae, *Pseudomonas, Listeria*, anaerobes like *Bacteroides fragilis* and *Clostridium difficile*; except-MRSA, *Mycoplasma*, etc.	1. Produce carbapenemases 2. Efflux pump

Contd...

Contd...

Class/mechanism	Drugs	Spectrum of activity	Mechanism of resistance
Aztreonam	Aztreonam	• Gram-negative rods	ESBL (extended spectrum β-lactamases)
Other cell wall inhibitors			
Glycopeptides (bactericidal: disrupt peptidoglycan cross-linkage)	Vancomycin Teicoplanin	Active against most gram-positive bacteria MRSA (drug of choice), and indicated if the patient is allergic to β lactam drugs	Alteration of target (substitution of D-alanine—D-alanine side chain of peptidoglycan)
Fosfomycin	Fosfomycin	Inactivates the enzyme UDP-N-acetylglucosamine-3-enolpyruvyltransferase, also known as MurA; required for cell wall synthesis. Active against urinary tract pathogens; against both gram-positive and gram-negative bacteria such as *E. faecalis*, *E. coli* etc.	1. Alteration of target 2. Producing enzymes that inactivates fosfomycin
Bacitracin	Bacitracin	Topical gram-positive cocci infections	Not defined
		B. Protein Synthesis Inhibition	
Anti-30S ribosomal subunit			
Aminoglycosides (bactericidal: irreversible binding to 30S)	Gentamicin Neomycin Amikacin Tobramycin Streptomycin	Aerobic gram-negative bacteria, such as—Enterobacteriaceae and some are active against *Pseudomonas* (gentamicin and amikacin) Often used for empirical therapy in adjunct with third generation cephalosporins in respiratory infections, meningitis and subacute bacterial endocarditis	1. Drug inactivation by aminoglycoside-modifying enzyme 2. Decreased permeability through gram-negative outer membrane 3. Decreased influx of drug
Tetracyclines (bacteriostatic: bind to 30S subunit of ribosome and block tRNA attachment)	Tetracycline Doxycycline Minocycline Demeclocycline	Rickettsiae, Chlamydiae, *Mycoplasma*, Spirochetes *Yersinia pestis*, *Brucella*, *Haemophilus ducreyi*, *Campylobacter*, *Vibrio cholerae*	1. Decreased intracellular drug accumulation (active efflux) 2. Ribosomal target site alteration
Anti-50S ribosomal subunit			
Chloramphenicol (bacteriostatic: binds to 50S ribosomal subunit and interfere with peptide bond formation)	Chloramphenicol	*Haemophilus influenzae* Pyogenic meningitis Brain abscess Anaerobic infection Enteric fever (*Salmonella*)—not used now due to development of resistance	1. Drug inactivation by producing chloramphenicol acetyltransferase enzyme 2. Altered membrane transport (active efflux)
Macrolides (bacteriostatic: binds 50S ribosomal subunit and prevent translocation of elongated peptide	Erythromycin Azithromycin Clarithromycin	*Streptococcus* *Haemophilus influenzae* *Mycoplasma pneumoniae*	1. Alteration of ribosomal target 2. Active efflux of antibiotic
Linezolid (Inhibitit protein synthesis by binding to 50S)	Linezolid	Resistant gram-positives like MRSA	Alteration of target site
Streptogramins (Inhibit protein synthesis by binding to 50S	Quinupristin Dalfopristin	*Streptococcus pyogenes* and *Staphylococcus aureus* skin infections MRSA infections VRE (Vancomycin resistant enterococci) infections	1. Alteration of target (dalfopristin) 2. Active efflux (quinupristin) 3. Drug inactivation (quinupristin and dalfopristin)
Mupirocin (Inhibits isoleucyl-tRNA synthetase)	Mupirocin	Topical ointment is given for- Skin infections Nasal carriers of MRSA	Mutation of gene for target site protein

Contd...

Contd...

Class/mechanism	Drugs	Spectrum of activity	Mechanism of resistance
C. Nucleic Acid Synthesis Inhibitors			
DNA synthesis inhibitors			
Fluoroquinolones	Inhibit DNA gyrase (A subunit) and topoisomerase IV, thus inhibiting DNA synthesis		
Nalidixic acid		Coliform gram-negative bacilli	1. Alteration of target (mutation of DNA gyrase genes) 2. Poor transport across cell membrane
Fluoroquinolones: 1st generation	Norfloxacin, ciprofloxacin, ofloxacin	**Enterobacteriaceae:** such as *E.coli, Klebsiella, Enterobacter, Salmonella, Shigella, Proteus, Yersinia*	
Fluoroquinolones: 2nd generation	Levofloxacin, lomefloxacin, moxifloxacin, sparfloxacin	**Others:** *Neisseria, Haemophilus, Campylobacter, Vibrio cholerae, Pseudomonas, Staphylococcus aureus*	
Metronidazole	Intracellularly generates metabolic by-products that damage DNA	Anaerobic organisms	Not defined
RNA synthesis inhibitors			
Rifampin	Inhibits RNA polymerase	*Staphylococcus* *Mycobacterium tuberculosis*	Alteration of target (mutation of *rpoB* gene)
D. Mycolic Acid Synthesis Inhibitors			
Isoniazid	Inhibits mycolic acid synthesis	Tuberculosis Latent TB	Mutations in enzyme processing isoniazid into active metabolites (KatG enzyme)
E. Folic acid Synthesis Inhibitors			
Bacteriostatic: Competitively inhibit enzymes involved in two steps of folic acid biosynthesis			
PABA (para-amino-benzoic acid) —Folate synthase / Sulfonamide blocks→ **Dihydrofolic acid** —Dihydrofolate reductase / Trimethoprim blocks→ **Tetrahydrofolic acid**			
Sulfonamides and trimethoprim	Sulfadiazine Sulfacetamide Cotrimoxazole (Trimethoprim + Sulfamethoxazole)	**Sulfadiazine:** Used topically in burn wound surface **Cotrimoxazole** is indicated in: • Urinary tract and respiratory tract infections-Active against *Serratia, Klebsiella, Enterobacter* • *Shigella* dysentery, *Vibrio cholerae* • *Toxoplasma gondii, Haemophilus ducreyi* • *Pneumocystis jirovecii*	Production of insensitive targets [dihydropteroate synthetase (sulfonamides) and dihydrofolate reductase (trimethoprim)] that bypass metabolic block
F. Antimicrobial agents that act on cell membrane			
Gramicidin	Forms pores	Topical use against cocci (gram-positive and negative)	Not defined
Daptomycin	Forms channels that disrupt membrane potential	Bactericidal against gram-positive bacteria including VRE and MRSA	Not defined
Polymyxins	Polymyxin B (topical) Colistin or Polymyxin E (topical and systemic use)	Gram-negative infections (disrupt both the outer and inner membranes in gram-negative cell wall)	1. Alteration of LPS 2. Efflux pump mediated

The emergence of resistance is a major problem worldwide in antimicrobial therapy. Infections caused by resistant microorganisms often fail to respond to the standard treatment, resulting in prolonged illness, higher healthcare expenditures, and a greater risk of death.

- Overuse and misuse of antimicrobial agents is the single most important cause of development of acquired resistance
- The evolution of resistant strains is a natural phenomenon, which can occur among bacteria especially when an antibiotic is over used
- Use of a particular antibiotic poses selective pressure in a population of bacteria which in turn promotes resistant bacteria to thrive and the susceptible bacteria to die off (Fig. 7.2)
- Thus the resistant bacterial populations flourish in areas of high antimicrobial use, where they enjoy a selective advantage over susceptible populations
- The resistant strains then spread in the environment and transfer the genes coding for resistance to other unrelated bacteria.

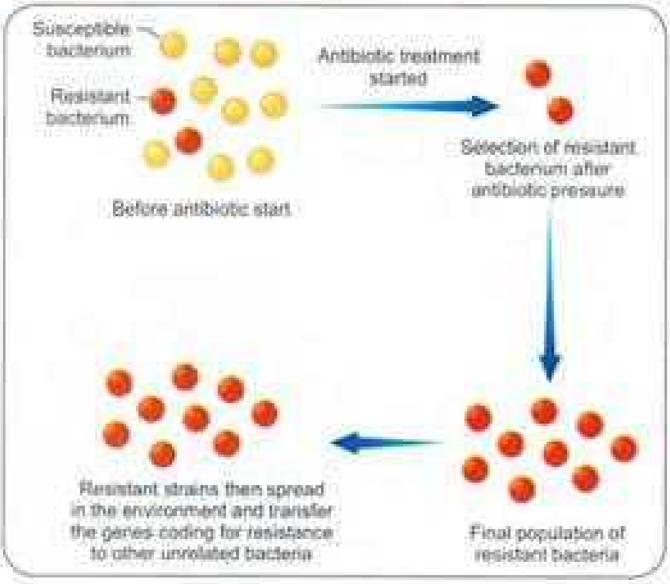


Fig. 7.2: Mechanism of development of acquired resistance

Other factors favouring the spread of antimicrobial resistance include—

- Poor infection control practices in hospitals—e.g. poor hand hygiene practices can facilitate transmission of resistant strains
- Inadequate sanitary conditions
- Inappropriate food-handling
- Irrational use of antibiotics by doctors, not following antimicrobial susceptibility report
- Uncontrolled sale of antibiotics over the counters without prescription.

Intrinsic Resistance

It refers to the innate ability of a bacterium to resist a class of antimicrobial agents due to its inherent structural or functional characteristics, (e.g. gram-negative bacteria are resistant to vancomycin). This imposes only little threat to the world as very few organisms show intrinsic resistance (Table 7.2).

Table 7.2: Intrinsic antimicrobial resistance

Organism	Intrinsic resistance against
Anaerobic bacteria	Aminoglycosides
Aerobic bacteria	Metronidazole
Gram-negative bacteria	Vancomycin
Klebsiella species	Ampicillin
Pseudomonas	Sulfonamides, trimethoprim, tetracycline or chloramphenicol
Enterococci	Aminoglycosides, all cephalosporins
Serratia, Proteus and *Burkholderia*	Polymyxin B and colistin
Stenotrophomonas maltophilia	Carbapenems

Mutational and Transferable Drug Resistance

In presence of selective antibiotic pressure, bacteria acquire new genes mainly by two broad methods:

Mutational Resistance

Resistance can develop due to mutation of the resident genes.

- It is typically seen in *Mycobacterium tuberculosis*, developing resistance to anti-tubercular drugs
- Mutational drug resistance differs from transferable drug resistance in many ways (Table 7.3)

Table 7.3: Mutational vs transferable drug resistance

Mutational drug resistance	Transferable drug resistance
Resistance to one drug at a time	Multiple drug resistance at the same time
Low-degree resistance	High-degree resistance
Resistance can be overcome by combination of drugs	Cannot be overcome by drug combinations
Virulence of resistance mutants may be lowered	Virulence not decreased
Resistance is not transferable to other organisms but spread to off- springs by vertical spread only	• Resistance is transferable to other organisms. • Spread by: Horizontal spread (conjugation, or rarely by transduction/transformation)

- Usually, it is a low level resistance, developed to one drug at a time; which can be overcome by using combination of different classes of drugs
- That is why multidrug therapy is used in tuberculosis using 4-5 different classes of drugs, such as isoniazid, rifampicin, pyrazinamide, ethambutol and streptomycin.

Transferrable Drug Resistance

In contrast, transferrable drug resistance is plasmid coded and usually transferred by conjugation or rarely by transduction, or transformation (refer Chapter 6).

- The resistance coded plasmid (called R plasmid) can carry multiple genes, each coding for resistance to one class of antibiotic
- Thus, it results in a high degree of resistance to multiple drugs, which cannot be overcome by using combination of drugs.

Mechanism of Antimicrobial Resistance

Bacteria develop antimicrobial resistance by several mechanisms.

1. Decreased Permeability across the Cell Wall

Certain bacteria modify their cell membrane porin channels; either in their frequency, size, or selectivity; thereby preventing the antimicrobials from entering into the cell. This strategy has been observed in many gram-negative bacteria, such as *Pseudomonas*, *Enterobacter* and *Klebsiella* species against drugs, such as imipenem, aminoglycosides and quinolones.

2. Efflux Pumps

Certain bacteria possess efflux pumps which mediate expulsion of the drug(s) from the cell, soon after their entry; thereby preventing the intracellular accumulation of drugs. This strategy has been observed in:
- *Escherichia coli* and other Enterobacteriaceae against tetracyclines, chloramphenicol
- Staphylococci against macrolides and streptogramins
- *Staphylococcus aureus* and *Streptococcus pneumoniae* against fluoroquinolones.

3. By Enzymatic Inactivation

Certain bacteria can inactivate the antimicrobial agents by producing various enzymes, such as:
- **β lactamase** enzyme production (observed in both gram-positive and gram-negative bacteria): It breaks down the β lactam rings, there by inactivating the β lactam antibiotics (see the highlight box)
- **Aminoglycoside modifying enzymes** like (acetyltransferases, adenyltransferases, and phosphotransferases, produced by both gram-negative and gram-positive bacteria)—they destroy the structure of aminoglycosides
- **Chloramphenicol acetyl transferase:** It is produced by members of Enterobacteriaceae; it destroys the structure of chloramphenicol.

4. By Modifying the Target Sites

Modification of the target sites of antimicrobial agent (which are within the bacteria) is a very important mechanism. It is observed in:
- MRSA (Methicillin-resistant *Staphylococcus aureus*): In these strains, the target site of penicillin i.e. penicillin binding protein (PBP) gets altered to PBP-2a. The altered PBP coded by a chromosomally coded gene *mec A*, do not sufficiently bind to β-lactam antibiotics and therefore prevent them from inhibiting the cell wall synthesis. (Described in detail in Chapter 21)
- Streptomycin resistance in *Mycobacterium tuberculosis*: It is due to modification of ribosomal proteins or 16S rRNA
- Rifampicin resistance in *Mycobacterium tuberculosis*—due to mutations in RNA polymerase
- Quinolone resistance seen in many gram-positive bacteria, particularly *S. aureus* and *S. pneumoniae*—due to mutations in DNA gyrase enzyme.

Vancomycin resistance in enterococci (VRE): These strains have a change in the target site of vancomycin (i.e. D-alanine D-alanine side chain of peptidoglycan) (Chapter 22).

Beta Lactamase Enzymes

β lactamase enzymes are capable of hydrolyzing the β-lactam rings (the active site) of β lactam antibiotics; thereby deactivating their antibacterial properties.
- They can be produced by both gram-positive and gram-negative organisms
- They are plasmid coded, and transferred from one bacterium to other mostly by conjugation, (except in *Staphylococcus aureus* where they are transferred by transduction).

Beta-lactamases can be classified in two ways:
- Ambler's classification (structural or molecular classification): According to this, β lactamases are classified into four classes (Table 7.4)
- Bush Jacoby Medeiros classification or functional (phenotypic) classification: This is the most advanced and complex classification.

Table 7.4: Ambler's classification of beta lactamases

Class A: Extended spectrum β lactamases (ESBL)
Organisms producing ESBL enzymes are resistant to all penicillins and 1st, 2nd and 3rd generation cephalosporins and monobactam, however remain sensitive to carbapenems and cephamycins. • Resistance can be overcome by use of β lactam along with β lactamase inhibitor (e.g. sulbactum or clavulanic acid) • Detected by- (i) Combination disk tests (e.g. Ceftazidime + Ceftazidime-clavulanate and Cefotaxime + Cefotaxime-clavulanate) (ii) molecular methods
Class B: Metallobetalactamase (MBL)
These organisms are resistant to all those antibiotics to which AmpC beta-lactamase producers are resistant. In addition, they are also resistant to carbapenems. • Resistance cannot be overcome by β lactam + β lactamase inhibitor combination • Detected by- (i) Carba NP test, (ii) mCIM (Modified Carbapenem Inactivation Method), (iii) EDTA mCIM and (iv) Molecular methods
Class C: AmpC beta-lactamase
These organisms are resistant to all those antibiotics to which ESBL producers are resistant plus they are resistant to cephamycins (e.g. cefoxitin and cefotetan). But they are sensitive to carbapenems. Resistance cannot be overcome by β lactam + β lactamase inhibitor combination
Class D: Oxacillinase
Resistance can be overcome by β lactam + β lactamase inhibitor combination

ANTIMICROBIAL SUSCEPTIBILITY TESTING

Bacteria exhibit great strain variations in susceptibility to antimicrobial agents. It is, therefore, essential to determine

Table 7.5: Classification of antimicrobial susceptibility testing methods

- Disk diffusion methods:
  - Kirby-Bauer disk diffusion method
  - Stokes disk diffusion method
- Dilution tests:
  - Broth dilution method (macro and micro broth dilution)
  - Agar dilution method
- Epsilometer test (E-test)
- Automated methods (Vitek, Phoenix and Microscan systems)
- Molecular methods (PCR detecting drug resistant genes)

the susceptibility of pathogenic bacteria isolated from the clinical specimens to antibiotics that are likely to be used in the treatment.

Antimicrobial susceptibility test (AST) is performed only for pathogenic bacteria isolated from the specimen, and not for the commensal bacteria (Table 7.5). For example, *E. coli* isolated from urine specimen should be subjected to AST, whereas *E. coli* isolated from stool is a commensal; hence, AST is not performed.

Disk Diffusion Method

Disk diffusion tests are the most widely used method. They are suitable for rapidly growing pathogenic bacteria; however, they are not suitable for slow growing bacteria. The disk diffusion method is so named because:

- It uses filter paper disks impregnated with appropriate concentration of the antibiotic solution
- The test bacterium is inoculated (as lawn culture) on the solid medium and then the antibiotic disks are applied
- The antibiotic in the disks diffuses through the solid medium, so that the concentration is highest near the site of application of the antibiotic disk and decreases gradually away from it
- Sensitivity to the drug is determined by the zone of inhibition of bacterial growth around the disk.

Medium (Mueller-Hinton Agar)

Mueller-Hinton agar (MHA) is considered as the best medium to use for routine susceptibility testing of non-fastidious bacteria for the following reasons:

- It shows acceptable batch-to-batch reproducibility for susceptibility testing
- It supports satisfactory growth of most nonfastidious pathogens
- It has minimal inhibitory effect on sulfonamide and trimethoprim. Hence, these antibiotics are better tested in MHA than any other media.

Modifications of MHA

- Lysed horse blood is added to MHA to support the growth of fastidious organisms, such as *S. pneumoniae* and *S. pyogenes*
- Sodium chloride (2-4% NaCl) should be added to the medium for testing MRSA isolates.

Inoculum

Isolated pure colonies of the test organism are inoculated in a suitable liquid medium (peptone water broth) and incubated at 35–37°C for 4–6 hours.

- The density of the organisms in broth is adjusted to approximately 1.5×10^8 cfu/mL by comparing its turbidity with that of 0.5 McFarland opacity standard tube
- **Lawn culture:** The broth is then inoculated on the medium by spreading with sterile swabs
- The ideal inoculum after overnight incubation gives an even semiconfluent growth. Too heavy inoculum reduces the size of inhibition zones
- **Control strains:** Similar to the test isolate, the inoculum of control strain should also be made and tested for AST. The following ATCC (American Type Culture Collection) strains are used as standard control strains
  - *Escherichia coli* ATCC 25922
  - *Pseudomonas aeruginosa* ATCC 27853
  - *Staphylococcus aureus* ATCC 25923
  - *Enterococcus feacalis* ATCC 29212.

Antibiotic Disk

Antibiotic disks are available commercially or prepared in in-house. Sterile filter paper (Whatman number 1) disks of 6 mm diameter are impregnated with standard quantity of antibiotic solution.

Choice of Antibiotic Disk

It is neither possible nor desirable to test the susceptibility against all the drugs. The panel of the drugs to be tested against an isolate depends upon various factors:

- Antibiotics should likely to be used for therapy
- Organism against which the drug has to be tested
- Local prescribing habits of the antimicrobial agents
- Resistant pattern of the locally prevalent pathogens
- Cost, toxicity, pharmacokinetics, and spectrum of activity of an antimicrobial agent for the management of illness in a particular patient.

First-line and Second-line Drug Testing

- **First-line drugs:** Those antibiotics that are commonly used in a locality with respect to the organism isolated should be tested first. First-line should also include the antibiotic currently being administered to the patient, their spectrum should be relevant to the organism isolated
- **Second-line drugs** include the panel of those antibiotics for which the prescription is restricted only to special circumstances. They are reserved for testing later if the organism is found to be resistant to all the first-line antibiotics tested before.

Kirby-Bauer Disk Diffusion Method

A sterile cotton swab is dipped into inoculum and squeezed to drain out the excess fluid. Then the swab is inoculated on to the Mueller-Hinton agar plate by streaking the swab three times over the entire agar surface.

- After drying the surface of agar plate for 3–5 minutes the antibiotic disks are applied using either sterile forceps or multidisk dispenser
- Disks should not be placed closer than 20 mm (center to center) on the MHA plate
- Ordinarily, maximum up to 6 disks can be applied on a 100 mm plate (five in the periphery and one in the center) (Fig. 7.3)
- The plates are then incubated at 37°C for 16–18 hours. When tested for MRSA, result should be read only after 24 hours of incubation
- The zones of complete growth of inhibition around each of the disks are measured using a ruler or Vernier caliper. The diameter of the disk (6 mm) is also included in this measurement (Fig. 7.4)
- The interpretation of zone size into sensitive, intermediate or resistant is based on the standard zone size interpretation chart, Clinical and Laboratory Standards Institute(CLSI) guideline (Table 7.6)
- Control strains should be tested each time when a new batch of disks or Mueller-Hinton agar (MHA) is used.

Stokes Disk Diffusion Method

The MHA plate is divided into two halves. The test strain and control strain are inoculated separately in each half.

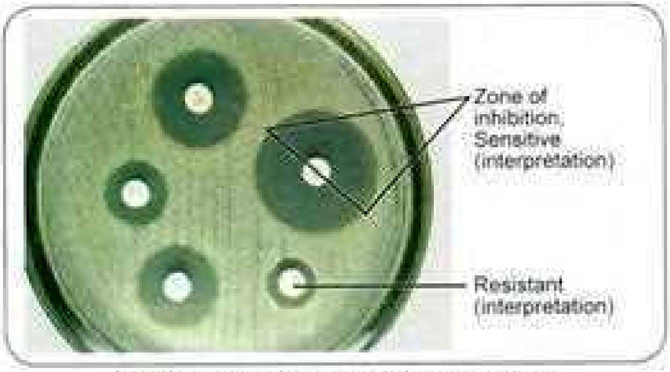


Fig. 7.3: Kirby-Bauer disk diffusion method

Source: Department of Microbiology, Pondicherry Institute of Medical sciences, Puducherry (*with permission*).

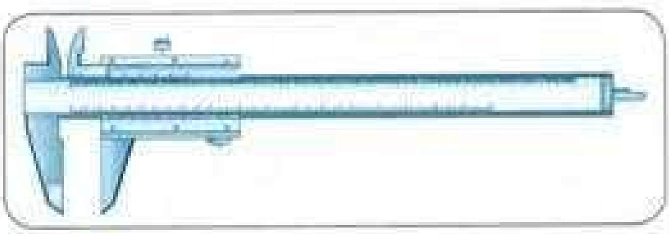


Fig. 7.4: Vernier caliper

Table 7.6: Commonly used disk concentrations and interpretation of disk diffusion test (as per CLSI 2018 guideline)

Antimicrobial agents	Disk (μg) strength	Diameter of zone of inhibition (in mm)		
		Resistant	**Intermediate**	**Sensitive**
Benzyl penicillin (*S. aureus*)	10 units	≤28	–	≥29
Cefoxitin (*S. aureus*)	30	≤21	–	≥22
Gentamicin (G)	10	≤12	13–14	≥15
Amikacin (Ak)	30	≤14	15–16	≥17
Erythromycin (E)	15	≤13	14–22	≥23
Tetracycline (T)	30	≤14	15–18	≥19
Nitrofurantoin (Nf)	300	≤14	15–16	≥17
Ciprofloxacin (Cf)	5	≤15	16–20	≥21
Ceftriaxone (Ci)	30	≤19	20–22	≥23
Imipenem (I)	10	≤19	20–22	≥23
Vancomycin (for *Enterococcus*)	30	≤14	15–16	≥17

Abbreviation: CLSI, Clinical and Laboratory Standards Institute.

- An uninoculated gap of 2–3 mm wide should separate the test and the control area on which the antibiotic disks are applied
- The plates are then incubated at 37°C for 16–18 hours
- The sensitivity report is prepared by comparing the zones of inhibition of control and test strain (Fig. 7.5).

Primary or Direct Disk Diffusion Test

The primary or direct disk diffusion test may be performed when results are required urgently and single pathogenic bacterium is suspected in the specimen (for sterile fluid, urine or positively flagged blood culture bottle). Here, the specimen is directly inoculated uniformly on the surface of an agar plate and the antibiotic disks are applied. The results of the primary test should be verified by testing the isolates subsequently. This test is of no use when mixed

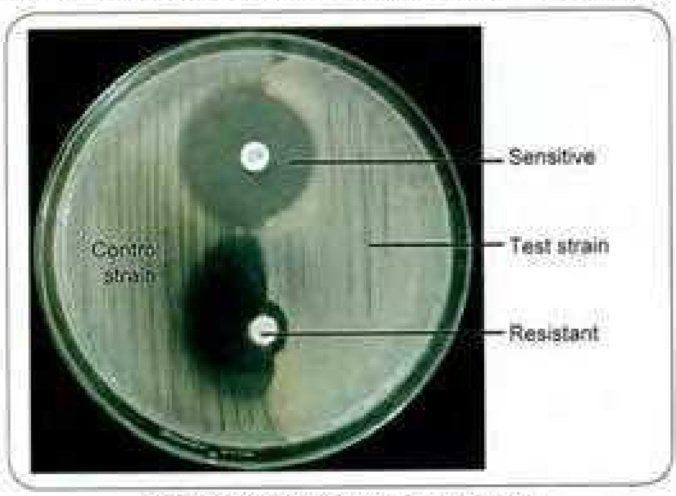


Fig. 7.5: Stokes disk diffusion method

Source: Dept. of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (*with permission*).

growth of different bacteria is suspected to be there in the specimen, e.g. pus, stool, sputum, etc.

Dilution Tests

Here, the antimicrobial agent is serially diluted, each dilution is tested with the test organism for antimicrobial susceptibility test and the MIC is calculated.

- MIC (minimum inhibitory concentration) is the lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism after overnight incubation
- Depending upon whether the dilutions of the antimicrobial agent are made in agar or broth, there are two types of dilution tests.

Broth Dilution Method

It is of two types: macro broth dilution (performed in tubes) and micro broth dilution (performed in microtiter plate). The procedure of macro broth dilution is explained below.

- Serial dilutions of the antimicrobial agent in Mueller-Hinton broth are taken in tubes and each tube is inoculated with a fixed amount of suspension of the test organism. A control organism of known sensitivity should also be tested. Tubes are incubated at 37°C for 18 hours
- The MIC is determined by noting the lowest concentration of the drug at which there is no visible growth, i.e. broth appears clear (Fig. 7.6)
- The minimum bactericidal concentration (MBC) can be obtained by subculturing from each tube (showing no growth) onto a nutrient agar plate without any antimicrobial agent. The tube containing the lowest concentration of the drug that fails to show growth, on subculture, is the MBC of the drug for that test strain (Fig. 7.6).

The MIC determination is useful in the following situations:

- For confirming the antimicrobial susceptibility test results obtained by disk diffusion tests
- For testing antimicrobial sensitivities of—
  - Slow growing bacteria, such as tubercle bacilli
  - Bacteria for which disk diffusion test is not standardized.
- When a very small degree of resistance has to be demonstrated
- When the therapeutic dose of the drug has to be regulated accurately as in the treatment of bacterial endocarditis.

Agar Dilution Method

Here, the serial dilutions of the drug are prepared in molten agar and poured into Petri dishes. The test strain is spot inoculated. This method is more convenient than broth dilution and has the added advantage of:

- Several strains can to be tested at the same time by using the same plate
- It directly measures the MBC; there is no need of subculturing as it is done with broth dilution method.

Epsilometer or E-test

This is a quantitative method of detecting MIC by using the principles of both dilution and diffusion of antibiotic into the medium.

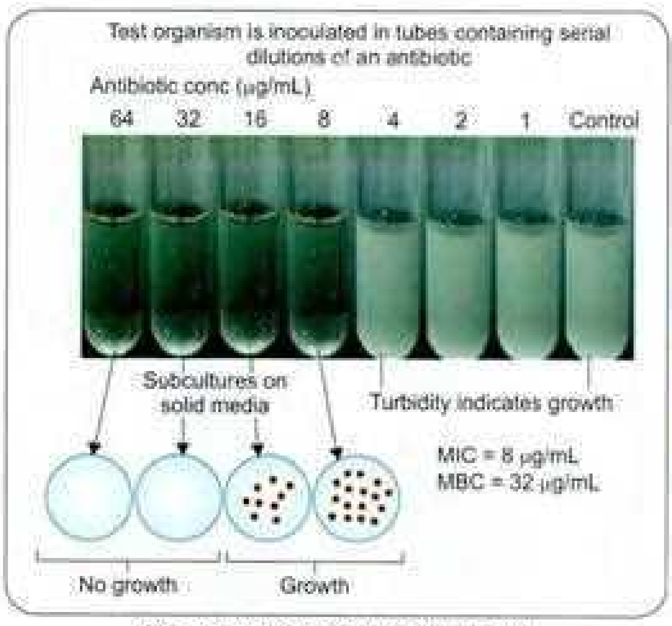


Fig. 7.6: Macro broth dilution method

Source: Department of Microbiology, Pondicherry Institute of Medical sciences, Puducherry (*with permission*).

Fig. 7.7: Epsilometer or E-test

Source: Department of Microbiology, Pondicherry Institute of Medical sciences, Puducherry (*with permission*).

- It uses an absorbent strip containing predefined gradient (serial dilution) of antibiotic concentration immobilized along its length
- It is applied to a lawn inoculum of a bacterium. Following incubation of the test organism, an elliptical zone of inhibition is produced surrounding the strip
- The antibiotic concentration at which the ellipse edge intersects the strip, is taken as MIC value (Fig. 7.7).

Automated Antimicrobial Susceptibility Tests

Several automated systems are available now, such as:

- VITEK 2 identification and antimicrobial sensitivity system (bioMerieux) (described in detail in chapter 5)
- Phoenix System (Becton Dickinson)
- Micro Scan Walk Away system.

Most systems are computer assisted and have sophisticated softwares to analyze the growth rates and determine the antibiotic susceptibility report. They work in principle of micro broth dilution. They use commercially available panels that contain antibiotic solution in serial dilutions. They provide more rapid results compared with traditional methods.

Molecular Methods

PCR based assays are available targeting specific drug resistant genes; for example *mecA* gene for MRSA detection.

EXPECTED QUESTIONS

I. Write short notes on:

1. Mechanism of antibiotic resistance.
2. Mutational and transferable drug resistance.
3. Antimicrobial susceptibility testing method.

II. Multiple Choice Questions (MCQs):

1. MRSA is mediated due to
a. Plasmid b. Chromosome
c. Transposons d. None

2. All of the following are diffusion methods for antimicrobial susceptibility except:
a. Kirby Bauer
b. Stokes
c. Broth dilution method
d. E test

3. Which of the following uses both diffusion and dilution methods for antimicrobial susceptibility:
a. Kirby Bauer
b. Stokes
c. Broth dilution method
d. E-test

4. All of the following antimicrobial agents act on cell membrane, *except*:
a. Gramicidin b. Daptomycin
c. Polymyxins d. Vancomycin

5. Fosfomycin- all are true, *except*:
a. Inactivates the enzyme MurA
b. Active against urinary tract pathogens
c. Active against both gram-positive and gram-negative bacteria
d. Resistance has not been reported yet

6. All of the following antimicrobial agents act on 50S ribosomal subunit, *except*:
a. Aminoglycosides b. Macrolides
c. Streptogramins d. Chloramphenicol

7. All of the following are examples of intrinsic antimicrobial resistance, *except*:
a. Anaerobic bacteria-aminoglycosides
b. *Pseudomonas*- carbapenems
c. Aerobic bacteria-metronidazole
d. Gram-negative bacteria-vancomycin

8. Extended spectrum β-lactamases (ESBL) producing organisms are resistant to all, *except*:
a. All penicillins
b. 3rd generation cephalosporins
c. Monobactam
d. Carbapenems

9. All of the following can be given for the treatment of Extended spectrum β-lactamases (ESBL) producing organisms, *except*:
a. Carbapenems
b. β-lactam/ lactamase inhibitor combination
c. 3rd generation cephalosporins
d. Aminoglycoside

10. Which of the following can be given for the treatment of carbapenamase producing organisms:
a. Carbapenems
b. β-lactam/lactam inhibitor combination
c. 3rd generation cephalosporins
d. Aminoglycoside

11. All the following automated systems can perform antimicrobial susceptibility testing, *except*:
a. VITEK 2 system b. MALDI-TOF system
c. Phoenix system
d. Micro Scan WalkAway system

12. What should be the minimum distance (center to center) between two adjacent antimicrobial disks on the Mueller-Hinton agar plate?
a. 5 mm b. 10 mm
c. 20 mm d. 30 mm

13. How many maximum antimicrobial disks should be placed on a 100 mm Mueller-Hinton agar plate?
a. up to 5 disks b. up to 6 disks
c. up to 7 disks d. up to 8 disks

14. Choice of antibiotic disk to perform antimicrobial susceptibility test depends up on:
a. Local prescribing habits of antimicrobial agents
b. Resistant pattern of locally prevalent pathogens
c. Cost, toxicity, pharmacokinetics, and spectrum of activity
d. All of the above

Answers

1. b 2. c 3. d 4. d 5. d 6. a 7. b 8. d 9. c 10. d 11. b 12. c 13. b 14. d

Microbial Pathogenicity

8

CHAPTER

Chapter Preview

- Infection
- Sources and reservoir of infection
- Mode of transmission
- Mechanism of microbial pathogenicity

Depending upon the relationship of microbes with respect to humans, microorganisms may be of following types:

- **Saprophytes:** They are the free-living microbes that live on dead or decaying organic matter. They are usually found in soil and water and are generally unable to invade the living host
- **Parasites:** They are the microbes that live on a living host, derive nutrition from the host and also cause harm to the host. They are of two types—microparasites (which include bacteria, viruses, fungi and protozoa) and macroparasites (which include helminths)
- **Commensals:** They are harmless microbes that harbor on a living host as normal flora of the host without causing any injury to the host
- **Pathogens:** They are the microbes capable of causing disease. However, they represent only a very small proportion of the microbial world
- **Opportunistic pathogens:** They are capable of causing disease only in immunocompromized people; when the usual defense mechanisms of humans are reduced or altered by accident, surgery, or by an underlying metabolic or an infectious disorder (e.g. AIDS).

INFECTION

Following the entry of the microorganism into the body, it may lead to either infection or colonization: both the terms need to be distinguished.

- **Infection:** It is a process in which a pathogenic organism enters, establishes itself, multiplies and invades the normal anatomical barrier of the host resulting in disease. When infection becomes apparent results in clinical manifestation and it is referred as 'infectious disease'
- **Colonization:** Here, the pathogenic organism enters, multiplies but does not invade, and neither causes disease or nor elicits specific immune response
  - Colonizers are different from normal flora. They have pathogenic potential and may invade and cause disease in another host or the same host later
  - However commensals are limited to a particular anatomical site, e.g. intestine, respiratory and genital tract. When they enter through other routes, they may behave as pathogens. For example, *Escherichia coli* is a normal flora in intestine, but it causes infection when enters through respiratory or urinary tract.
- **Infestation:** It is distinct from infection in that it applies specifically to parasites of macroscopic size, such as parasitic worms in intestine or arthropods (e.g. lice, itch mite, etc.) on the body surface.

Classification of Infections

Infections may be classified in various ways:

- **Primary infection:** It refers to the initial infection with a pathogenic microorganism in a host
- **Reinfection:** It results following subsequent infection by the same microbe
- **Secondary infection:** Here, a new pathogen sets up an infection in a host whose immunity is already lowered by a preexisting infection
- **Focal infection** (or focal sepsis): It indicates a condition where, due to infection or sepsis at localized sites (such as in the appendix or tonsils), generalized effects are produced
- **Superinfection:** It is defined as a second infection superimposed on an earlier one, by a different microbial agent of exogenous or endogenous origin, that is resistant to the treatment used against the first infection
- **Cross-infection:** When in a patient already suffering from a disease a new infection sets up from another host or another external source, it is termed as cross-infection

- **Nosocomial infection:** Cross-infections occurring in hospitals are called nosocomial infections. They are distinct from the patient's primary condition
- **Iatrogenic (or physician induced) infection:** This refers to the infection induced by the professional activity of the physician or other health care workers resulting from preventive, diagnostic, treatment or other procedures
- **Depending on the source of infection:**
  - Endogenous infections: The source of infection is within the body, either normal flora (if the anatomical barrier is breached), or endogenous reactivation of a latent infection
  - Exogenous infections: The source is outside the host's own body.
- **Based on the clinical manifestation produced:**
  - Asymptomatic or inapparent or subclinical infection: It is an infection that is active but does not produce noticeable symptoms
  - Symptomatic or apparent infections: It may be classified into:
    - Acute infection: The symptoms last for a short period, and
    - Chronic infection: The symptoms persist for a long period.
  - Latent infection: It refers to an infection that is inactive or dormant or in hidden form; but capable of reactivating later
  - Atypical infection: Here, the usual manifestations of the disease are not present. Instead, atypical symptoms may be present.

Epidemiological Pattern of Infection

Four epidemiological terms are often used to describe infection—endemic, epidemic, pandemic and sporadic.

- **Endemic:** The infections that occur at a persistent, usually low level in a certain geographical area are called endemic
- **Epidemic:** The infections that occur at a much higher rate than usual in a particular geographic area is known as epidemic
- **Pandemic:** Infection that spreads rapidly over large areas of the world is known as pandemic
- **Sporadic:** Infections occur at irregular intervals or only in a few places; scattered or isolated.

SOURCES AND RESERVOIR OF INFECTION

The starting point for the occurrence of an infectious disease is known as a source or/and reservoir of infection. Source and reservoir are not always synonymous.

- **Source:** The source of infection refers to the person, animal, or object from which a microorganism is transmitted to the host
- **Reservoir:** A reservoir is the natural habitat in which the organism lives, multiplies. Reservoir may be a person, animal, arthropod, plant, soil or substance (or combination of these) on which the organism is dependent for its survival; where it reproduces in, such a way that it can be transmitted to susceptible hosts.

The term source and reservoir may be same for many organisms but are not always synonymous. For example,

- In tetanus infection, the reservoir and source of the agent (*Clostridium tetani*) are same, i.e. the soil
- In hook worm infection, the reservoir is man, but the source of infection is the soil contaminated with the larva of hook worm
- In typhoid fever, the reservoir may be a case or carrier, but the source of infection is usually contaminated food and water.

Thus, the term 'source' refers to the immediate source of infection and may or may not be a part of reservoir.

The reservoir (and/or source) may be of three types.

Human Reservoir

By far the most important reservoir and/or source of infection for humans is man himself. Man is often described as his own enemy because most of the infectious diseases are contracted from human sources. The diseases that can be spread from one person to another are called **communicable diseases**. Human sources may be either cases or carriers.

- **Cases or patients:** They are the persons in a given population identified as having a particular disease. Cases may be of various types depending upon the appearance of symptoms—subclinical, clinical and latent cases
- **Carrier:** It refers to the persons/animals who harbor the infectious agent in the absence of any clinical symptoms and shed the organism from the body via contact, air or secretions
  - It results due to inadequate treatment or immune response occurring in some diseases, which leads to incomplete elimination of the organism from the body
  - Though, carriers are less infectious than cases, but are more dangerous as they often go undetected and continue to transmit the infection for a long period.

Types of Carriers

Carriers can be of various types:

- **Incubatory carriers** are those who shed the organism during the incubation period of disease. This usually occurs in the last few days of incubation period, e.g. measles, mumps, polio, diphtheria, pertussis, hepatitis B, influenza, etc.
- **Healthy carriers** refers to the subclinical cases who develop into carriers without suffering from overt disease, e.g. polio, cholera, salmonellosis, diphtheria, meningococcal meningitis, etc.

Contd...

Contd...

- **Convalescent carrier** is the one who has recovered from the disease and continues to harbor and shed the pathogen from his body.

Depending on the duration of carriage:
- Temporary carriers: They shed the organisms for less than six months. Incubatory, healthy and convalescent carriers are actually the types of temporary carriers
- Chronic carriers: They shed the organisms for indefinite period, e.g. in hepatitis B, typhoid fever, malaria, gonorrhea, etc.

Depending on the source:
- Contact carrier is a person who acquires the pathogen from a patient
- Paradoxical carrier refers to a carrier who acquires the pathogen from another carrier.

Animal Reservoir

The source of infection may sometime be animals and birds. The disease and the infections which are transmitted to man from vertebrates are called zoonoses. Common examples include:
- **From animals:** Rabies (from dog), leptospirosis (from rodents), influenza (from pigs), etc.
- **Birds** may be source of infection for various diseases like influenza, *Chlamydophila psittaci* infection (psittacosis), histoplasmosis, etc.

Amplifying host: It refers to the vertebrate reservoir in which the organism multiplies exponentially, e.g. pigs in Japanese B encephalitis.

Nonliving Things as Reservoir

Soil and inanimate matter can also act as reservoir/source of infection, for example soil may harbor the agents of tetanus, anthrax and some intestinal helminths.

MODE OF TRANSMISSION

Microorganisms may be transmitted from the reservoir or source to a susceptible host in different ways.

Contact

Infection may be transmitted by direct or indirect contact:
- **Direct contact** is via skin and mucosa of an infected person, e.g. through unclean hand, kissing, or sexual contact. Organisms transmitted by direct contact include agents of common cold, skin and eye infections and agents of sexually transmitted infection (STI), such as HIV, *Neisseria gonorrhoeae*, *Chlamydia trachomatis* and *Treponema pallidum*, etc.
- **Indirect contact** is through the agency of fomites, which are inanimate objects, such as clothing, toys, etc. These may be contaminated by a pathogen and act as a vehicle for its transmission, e.g. face towels shared by various persons may lead to spread of trachoma.

Inhalation

Agents causing respiratory infections are acquired by inhalational route. These organisms are shed into the environment by patients in secretions from the nose or throat during sneezing, coughing or speaking in the form of droplets.

Air-borne Transmission

Droplet nuclei (<10 µm size) can remain suspended in air for long periods, and can act as source of infection.
- **Droplet (dust) transmission:** Larger droplets (>10 µm size) travel for a short distance, settle down on clothing and other objects and become a part of the dust. They can infect only those persons who come in contact with the source. Organisms transmitted through this route include respiratory viruses (e.g. influenza, parainfluenza virus, adenovirus, respiratory syncytial virus) and *Bordetella pertussis*
- **Air-borne (droplet nuclei) transmission:** When the droplet is small (1-10 µm), it can travel a long distance; can infect any person it finds on its way. Primary contact with the source is not necessary. Organisms transmitted through this route include agents causing tuberculosis, measles and chickenpox.

Ingestion

Intestinal infections like cholera, dysentery, food poisoning and most of the parasitic infections are acquired by ingestion of food or water contaminated by pathogens. Food-borne infections occur mostly through carriers engaged in handling or preparation of food and contaminating the food stuffs. The water supply may get contaminated with the feces of the patients or carriers. All these may transmit infection.

Inoculation

Pathogens, in some instances, may be inoculated directly into the skin or tissues of the host:
- Animal bite—for example, rabies virus is inoculated directly by the bite of a rabid animal
- Inoculated directly into tissue—Spores of *Clostridium tetani* present in the soil, get deposited directly into the host tissues following severe wounds leading to tetanus.

Transmission of Blood-borne Infections

Blood-borne infections, such as hepatitis B, hepatitis C and HIV may be transmitted by:
- Needle prick and other sharp injuries
- Blood transfusion
- Intravenous drug abuse (contaminated needles).

Vector Borne (Mechanical or Biological)

Arthropod vectors, such as mosquitoes, flies, fleas, ticks, mite and lice are the vectors that transmit many diseases (Table 8.1). Vectors may be of two types:

Table 8.1: Arthropod vectors transmitting human infections

Vectors	Human infections transmitted
Mosquito	Malaria, filariasis, yellow fever, Japanese B encephalitis, dengue fever
Sandfly	Kala-azar, oriental sore, sandfly fever, Oroya fever
Hard tick	Tick typhus, Kyasanur forest disease (KFD), babesiosis, tularemia,
Soft tick	Q fever, endemic relapsing fever
Rat flea	Bubonic plague, endemic typhus, *Hymenolepis diminuta*
Mite	Scrub typhus, rickettsial pox
Tsetse fly	Sleeping sickness
House fly	Typhoid fever, paratyphoid fever, cholera, gastroenteritis, trachoma
Louse	Epidemic typhus, epidemic relapsing fever, trench fever
Reduvild bug	Chagas disease
Cyclops	Guinea worm disease, fish tape worm infection

- **Mechanical vectors:** These carry the microorganisms (do not multiply) and transmit them to the eatables
- **Biological vectors:** The pathogen multiplies in the body of the vector, often undergoing part of a development cycle in it, such vectors are named biological vectors (e.g. female *Anopheles* mosquito in malaria; *Culex* mosquito in filariasis).

Extrinsic incubation period: After the entry of pathogen into the vector, the time required for the vector to become infective is called extrinsic incubation period.

Vertical Transmission

It refers to transmission of infection from mother to the fetus. It may be categorized into:

- **Transplacental transmission:** Infection occurs via the placental barrier; leads to abortion, miscarriage or stillbirth. If babies are born, they suffer from congenital malformations., such infections are known as teratogenic infections. Examples include TORCH infections:
  - *Toxoplasma gondii*
  - Others (*Treponema pallidum* and varicella-zoster virus)
  - Rubella virus
  - Cytomegalovirus
  - Herpes simplex virus.
- **Transmission via the birth canal** without causing congenital malformation in the baby. Examples include: Group B *Streptococcus, N. gonorrhoeae, C. trachomatis, Listeria* and viruses (e.g. Hepatitis B, C and HIV).

MECHANISM OF MICROBIAL PATHOGENICITY

Ability of a microbe to produce disease or tissue injury is often referred to as two closely related but not synonymous term 'pathogenicity' and 'virulence'.

- 'Pathogenicity' is generally employed to refer to the ability of a microbial species to produce disease
- While the term 'virulence' is used more specifically to describe the relative degree of pathogenesis (tissue damage), which may vary between different strains of the same organism depending upon the expression of the virulence factors
- Virulence is a relative term: different strains of same species may exhibit varying degrees of virulence. Some strains are highly virulent; while some strains are low and some are avirulent (vaccine strains).

The virulence of a strain may undergo spontaneous or induced variation.

- **Exaltation:** Enhancement of virulence is known as exaltation, which can be induced experimentally by serial passage into susceptible hosts
- **Attenuation:** It refers to the reduction of virulence, which can be achieved by passage through unfavorable hosts, repeated cultures in artificial media, growth in high temperature or in the presence of weak antiseptics, desiccation or prolonged storage in culture.

Pathogenicity of viral, fungal and parasitic diseases are explained in the respective sections. Bacterial pathogenesis is described below.

Bacterial pathogenicity depends upon the sum total of several factors as described below.

Route of Transmission of Infection

Route of transmission of infection plays a crucial role in the pathogenicity of certain bacteria.

- Some bacteria, such as streptococci, can initiate infection whatever be the route of entry
- Others can survive and multiply only when introduced by the optimal routes. *Vibrio cholerae* are infective orally but are unable to cause infection when introduced subcutaneously
- This difference is probably related to the modes by which different bacteria are able to initiate tissue damage and establish themselves.

Infective Dose of the Organism

Infective dose of the organism is referred to as the minimum inoculum size that is capable of initiating an infection. Infective dose plays a major role in determining whether the disease is going to set in or not.

- **Low infective dose:** Certain organisms require a relatively small inoculum to initiate infection
  - *Shigella:* Very low (as low as 10 bacilli)
  - *Cryptosporidium parvum:* Very low (10–30 oocysts)
  - *Escherichia coli* O157: H7 (<10 bacilli)
  - *Entamoeba coli and Giardia:* few cysts
  - *Campylobacter jejuni* (500 bacilli).

- **Large infective dose:** In contrast, organisms with high infective dose can initiate the infection only when the inoculum size exceeds a particular critical size
  - *Escherichia coli* (10^6–10^8 bacilli)
  - *Salmonella* (10^2–10^3 bacilli)
  - *Vibrio cholerae* (10^6–10^8 bacilli).

Infective dose varies depending upon the factors, such as:
- Virulence of the organism: Higher the virulence, lower is the infective dose
- Host's age and overall immune status
- Ability of the organism resisting the gastric acidity: *Shigella* has an ability to survive in gastric acidity, even a low infective dose can initiate the infection. In contrast *Vibrio* is extremely acid labile, hence requires a heavy inoculum to bypass the gastric barrier.

Adhesion

Adhesion of the bacteria to body surfaces is the initial event in the pathogenesis of the disease. It is mediated by specialized molecules called adhesins which bind to specific host cell receptors. Adherence prevents the bacteria from being flushed away in secretions and also facilitates bacterial invasion into the host cells.
- **Fimbriae or pili:** They are the most important adhesins present in some bacteria. They directly bind to the sugar residues (glycolipids or glycoproteins) on host cells
- **Non-pilus adhesins:** Apart from pili, there are other adhesins found in certain bacteria, such as M protein (*Streptococcus pyogenes*), lipoteichoic acid (gram-positive cocci), cell surface lectin (*Chlamydia*), etc.
- **Biofilm formation:** It is another mechanism by which certain bacteria mediate strong adherence to certain structures, such as catheters, prosthetic implants, and heart valves. Biofilm is a group of bacterial cells which stick to each other on a surface and are embedded within layer (called slime layer) of a self-produced matrix of extracellular polymeric substance called glycocalyx.

Invasion

Invasion refers to entry of bacteria into host cells, leading to spread within the host tissues.
- Highly invasive pathogens produce spreading or generalized lesions (e.g. streptococcal infections), while less invasive pathogens cause localized lesions (e.g. staphylococcal abscess)
- Some pathogens though capable of causing fatal diseases, lack invasiveness but remain confined to the site of entry and produce disease by elaborating a potent toxin, e.g. *Clostridium tetani*.

Important virulence factors that help in invasion include:
- Virulence marker antigen or invasion plasmid antigens in *Shigella*
- Enzymes: Invasion of bacteria is enhanced by many enzymes such as: hyaluronidase, collagenase, streptokinase, IgA proteases.

Antiphagocytic Factors: Bacteria are rapidly killed once they are ingested by phagocytes, such as polymorphonuclear cells (neutrophils) or macrophages. Some pathogens develop strategies to evade phagocytosis by several antiphagocytic factors, the most important ones being—
- **Capsule:** It prevents phagocytosis of bacteria by preventing the phagocytes from adhering to the bacteria. Capsules are produced by—
  - *Neisseria meningitidis*
  - *Streptococcus pneumoniae*
  - *Haemophilus influenzae*
  - *Klebsiella pneumoniae*
  - *Cryptococcus neoformans* (fungus).
- **Cell wall proteins** may help in invasion, such as—
  - Protein A of *Staphylococcus aureus* binds to IgG and prevents the activation of complement
  - M protein of *Streptococcus pyogenes*.
- **Cytotoxins:** Certain bacteria produce cytotoxins that interfere with chemotaxis or killing of phagocytes. For example, *S. aureus* produces hemolysins and leukocidins that lyse and damage RBCs and WBCs.

Intracellular Survival

Some organisms survive in intracellular environment. They are grouped into obligate and facultative intracellular organisms (Table 8.2). Once engulfed, they develop strategies that inhibit various steps of phagocytosis (see highlight box above and Table 8.3).

Toxins

Endotoxins

Endotoxins are the lipid A portion of lipopolysaccharide (LPS). They are present as an integral part of the cell wall of gram-negative bacteria. They are released from the bacterial surface by natural lysis of the bacteria and are responsible for various biological effects in the host (Fig. 8.1).
- **Macrophage activation:** Endotoxin binds to specific receptors on macrophages and stimulates the release of acute-phase cytokines, such as interleukin (IL)-1, tumor necrosis factor-α, IL- 6, nitric oxide and prostaglandins which cause fever and inflammation and activation of immune system (T cells and B cells)
- **Complement activation:** High concentrations of endotoxin can activate the alternative pathway of complement → release C3a and C5a → promote inflammatory cells chemotaxis, high grade fever, hypotension, shock produced by vasodilatation and capillary leakage
- **Endothelial activation:** Leads to ↑ vascular permeability
- **Coagulation pathways activation:** It activates Hageman factor and other coagulation factors, leads to thrombosis, and disseminated intravascular coagulation (DIC)

Table 8.2: Intracellular organisms

Facultative intracellular organism	Obligate intracellular organism
Bacteria	
Salmonella Typhi, *Brucella*	*Mycobacterium leprae*
Legionella, Listeria, Nocardia	*Rickettsia*
Neisseria meningitidis, Yersinia	*Chlamydia*
Mycobacterium tuberculosis	*Coxiella burnetii*
Viruses	
	All viruses
Fungi	
Histoplasma capsulatum *Cryptococcus neoformans*	*Pneumocystis jirovecii*
Parasites	
	Toxoplasma, Cryptosporidium *Plasmodium, Leishmania* *Babesia, Trypanosoma*

Table 8.3: Mechanisms used by organisms for intracellular survival

Mechanism of intracellular survival	Organism
Inhibition of phagolysosome fusion	*Legionella* species *Mycobacterium tuberculosis* *Chlamydia* species
Resistance to lysosomal enzymes	*Salmonella* Typhimurium *Coxiella* species *Mycobacterium leprae* *Leishmania* species
Adaptation to cytoplasmic replication	*Listeria, Rickettsia* *Francisella tularensis*

- **Platelet activation:** Leads to release of mediators that cause ↑ vascular permeability, thrombosis, and DIC
- **Mast cell activation:** Leads to release of mediators (e.g. histamine) that causes muscle contraction and ↑ vascular permeability
- **In gram-negative septicemia:** Endotoxins are released in large quantity, causing high fever, petechiae (skin lesions resulting from the capillary leakage) and DIC which may result in shock and possibly death.

Exotoxins

They are heat labile proteins; secreted by certain species of both gram-positive and gram-negative bacteria and diffuse readily into the surrounding medium.

- **High potency:** Exotoxins are highly potent even in minute amounts. Botulinum toxin is the most potent, it has been estimated that 39.2 g of botulinum toxin would be sufficient to eradicate the entire humankind
- **Used for vaccine:** Exotoxins can be converted into toxoids by treatment with formaldehyde. Toxoids lack toxicity but retain antigenicity and thus induce protective immunity when used as vaccines
- **Specific action:** They are highly specific for a particular tissue, e.g. tetanus toxin for CNS. They have specific pharmacological activities (Table 8.4).

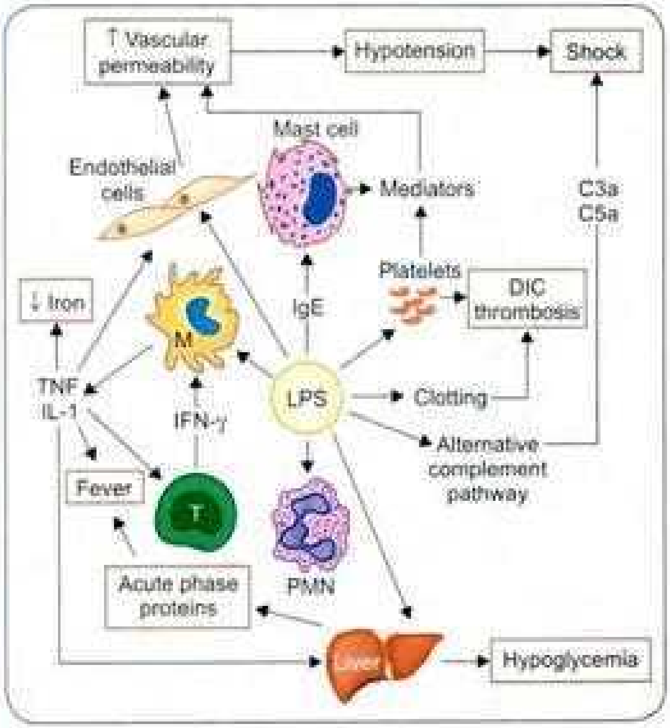


Fig. 8.1: Effects of bacterial endotoxins (cell wall lipopolysaccharide)

Abbreviations: DIC, disseminated intravascular coagulation; LPS, lipopolysaccharide; IL-1 interleukin-1; IFN-γ, interferon gamma; PMN, polymorphonuclear leukocyte; IgE, immunoglobulin E; T, T cells; M, macrophage.

Exotoxins differ from endotoxins in several ways (Table 8.5).

Pathogenicity Islands

Pathogenicity islands (PAIs) are large genomic islands located in the chromosomal regions of some bacteria containing sets of genes encoding numerous virulence factors, such as adhesins, secretion systems, toxins, invasins, iron uptake system, etc.

- Genes encoded in a pathogenicity island are expressed in a coordinated way to initiate the virulence process
- These genes may be turned on by a single stimulus (e.g. the temperature of the gut) and can be transferred as a unit by horizontal gene transfer mechanisms to different sites within a chromosome or to other bacteria
- Pathogenicity islands have been detected in some bacteria, such as *Shigella, Salmonella, Vibrio cholerae, Yersinia pestis, Staphylococcus aureus*, uropathogenic *E. coli*, and *Helicobacter*.

Bacterial Secretory System

Secretion in bacteria refers to the translocation of effector molecules, such as proteins, enzymes or toxins (such as cholera toxin) across the cell membrane from cytoplasm to its exterior. Secretion is a very important mechanism for bacterial survival and pathogenesis. There are at least six specialized secretion systems described especially in gram-negative bacteria (type I to type VI).

Table 8.4: Bacterial exotoxins and their mechanism of action

Organisms	Toxins (Exotoxins)	Mechanism
Staphylococcus aureus	Enterotoxin, Toxic shock syndrome toxin	Act as super antigen; stimulate T cell nonspecifically, to release large amounts of cytokines
Streptococcus pyogenes	Streptococcal pyrogenic exotoxin	
Corynebacterium diphtheriae	Diphtheria toxin (DT)	Inhibits protein synthesis (by inhibiting elongation factor-2)
Bacillus anthracis	Anthrax toxin	↑cAMP in target cell, edema
Clostridium perfringens	α toxin and other major and minor toxins	Lecithinase and phospholipase activity → causes myonecrosis
Clostridium tetani	Tetanus toxin (tetanospasmin)	Decrease in neurotransmitter (GABA and glycine) release from the inhibitory neurons → spastic paralysis
Clostridium botulinum	Botulinum toxin (BT)	Decrease in neurotransmitter (acetyl choline) release from neurons → flaccid paralysis
Escherichia coli (diarrheagenic)	Heat labile toxin (LT)	Activation of adenylate cyclase → cAMP in target cell → secretory diarrhea
	Heat stable toxin (ST)	↑cGMP in target cell → secretory diarrhea
	Verocytotoxin	Inhibit protein synthesis (by inhibiting ribosome)
Shigella dysenteriae type-1	Shiga toxin	
Vibrio cholerae	Cholera toxin (CT)	Activation of adenylate cyclase → cAMP in target cell → secretory diarrhea
Pseudomonas	Exotoxin-A	Inhibit protein synthesis (by inhibiting elongation factor-2)

Table 8.5: Differences between bacterial endotoxins and exotoxins

Feature	Endotoxins	Exotoxins
Nature	Lipopolysaccharides	Proteins
Source	Part of cell wall of gram-negative bacteria	Secreted both by gram-positive and negative bacteria; diffuse into surrounding medium
Released by	Cell lysis, not by secretion	Actively secreted by the bacteria
Heat stability	Highly stable	Heat labile, destroyed at 60°C
Mode of action	↑IL-1 and TNF-α	Mostly enzyme like action
Effect	Nonspecific (fever, shock, etc.)	Specific action on particular tissues
Tissue affinity	No	Specific affinity for tissues
Fatal dose	Only large doses are fatal	More potent, even smaller doses-fatal
Antigenicity	Poorly antigenic	Highly antigenic
Neutralization by antibodies	Ineffective	Neutralized by specific antibodies
Used for vaccine	No effective vaccine is available using endotoxin	Toxoid forms are used as vaccine, e.g. tetanus toxoid

EXPECTED QUESTIONS

I. Write short notes on:
- a. Reservoirs of infection.
- b. Carriers.
- c. Various modes of transmission of infection.
- d. Mechanisms of microbial pathogenicity.
- e. Differences between endotoxins and exotoxins.

II. Multiple Choice Questions (MCQs):

1. Chemical nature of endotoxin is:
a. Lipopolysaccharide b. Protein
c. Carbohydrate d. None

2. The following are exotoxins, *except*:
a. Botulinum toxin b. Anthrax toxin
c. Diphtheria toxin d. Lipid A portion of LPS

3. The following are vector borne diseases, *except*:
a. Malaria b. Filariasis
c. Rubella d. Chagas disease

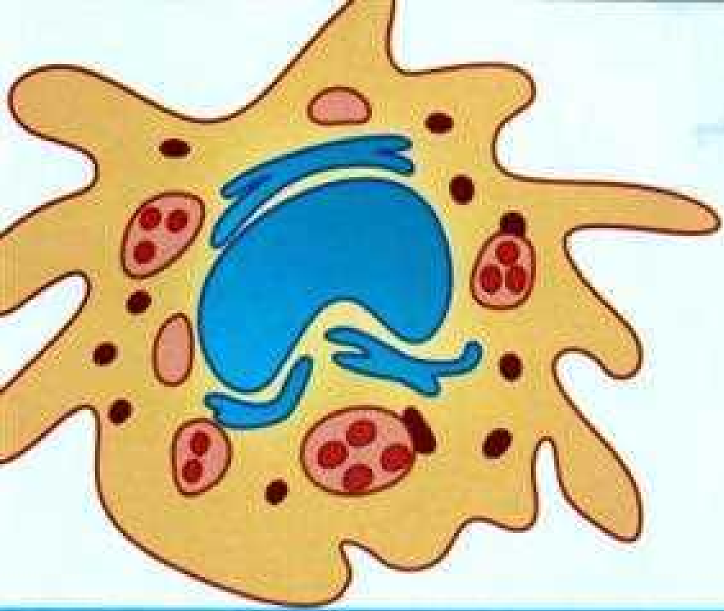
4. Obligate intracellular organisms are all, *except*:
a. *M. leprae* b. *Rickettsia*
c. *Chlamydia* d. *M. tuberculosis*

5. The following have the intracellular survival strategy of inhibition of phagolysosome fusion, *except*:
a. *Legionella* species b. *M. tuberculosis*
c. *M. leprae* d. *Chlamydia* species

6. The following bacteria require large infective dose, *except*:
a. *Escherichia coli* b. *Shigella*
c. *Salmonella* d. *Vibrio cholerae*

Answers

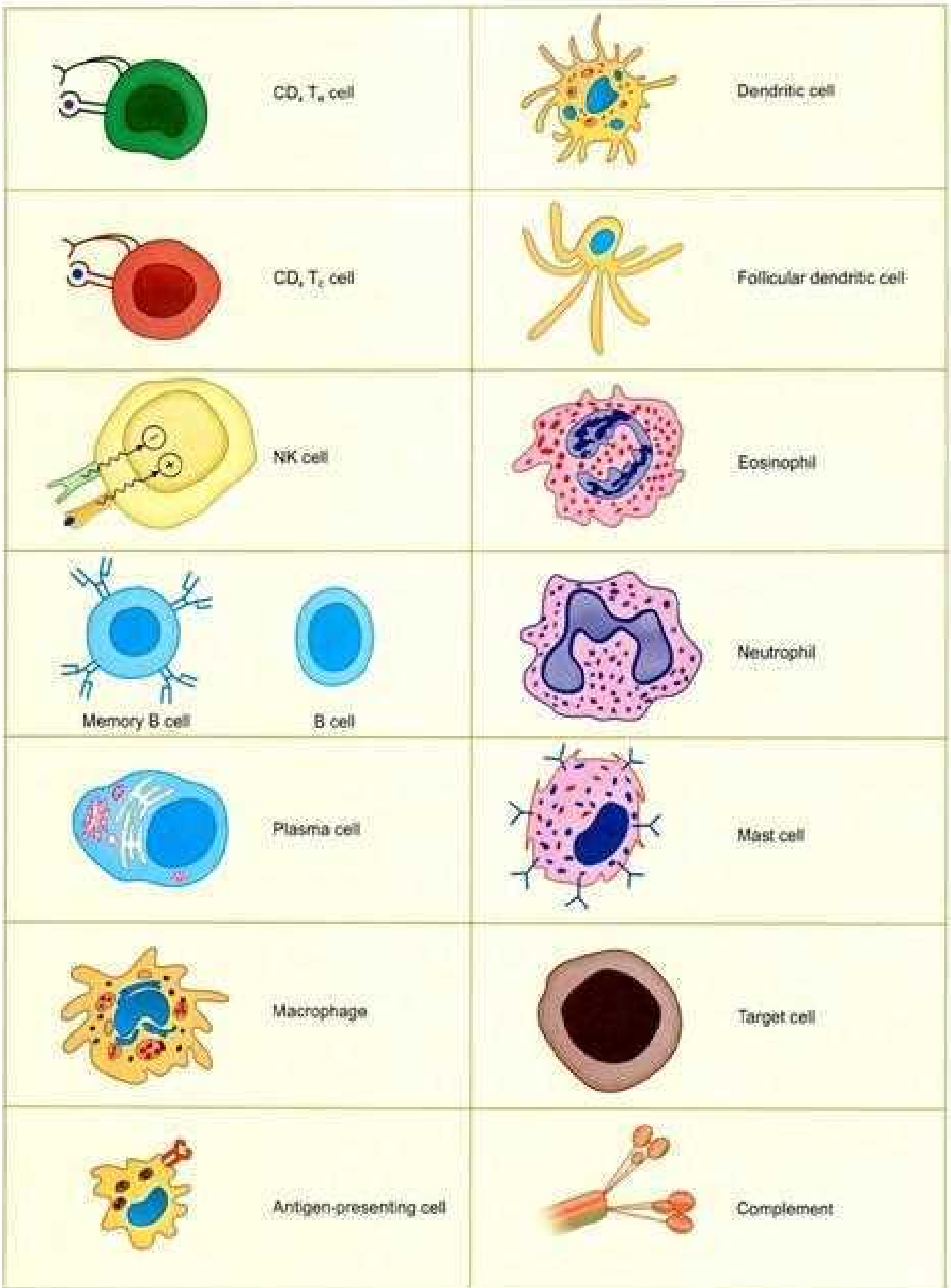
1. a **2.** d **3.** c **4.** d **5.** c **6.** b



Immunology

SECTION 2

Section Outline



CD_4 T_H cell
CD_8 T_C cell
NK cell
Memory B cell
B cell
Plasma cell
Macrophage
Antigen-presenting cell
Dendritic cell
Follicular dendritic cell
Eosinophil
Neutrophil
Mast cell
Target cell
Complement

Immunity (Innate and Acquired)

9
CHAPTER

Chapter Preview

- Innate immunity
- Acquired or adaptive immunity
  - Active immunity
    - Primary immune response
    - Secondary immune response
  - Passive immunity
- Bridges between innate and acquired immunity
- Other types of immunity
  - Local (or mucosal) immunity
  - Herd immunity
  - Adoptive immunity

The term "immunity" (Latin word "*immunitas*", means freedom from disease) is defined as the resistance offered by the host against microorganism(s) or any foreign substance(s). Immunity can be broadly classified into two types:

1. Innate immunity—present right from the birth.
2. Acquired/adaptive immunity—acquired during the course of the life.

INNATE IMMUNITY

Innate immunity is the inborn resistance against infections that an individual possesses right from the birth, due to his genetic or constitutional makeup.

Innate immunity has certain unique properties by which it can be differentiated from acquired immunity (Table 9.1).

- **Acts in minutes:** Innate immunity is the **first line of host defense** against infections; occurs immediately after the microbial entry
- **Prior microbial exposure is not required:** Innate immunity is independent of prior exposure to the microbes; present even before the first entry of the microorganism
- **Diversity is limited:** Innate immunity is active only against a limited repertoire of antigens; in contrast to acquired immunity which is more varied and involves specialized immune responses
- **Non-specific:** Cells of innate immunity are non-specific in their action; can be directed against any microbial antigen(s)
- **No memory:** Innate immunity does not have a memory component. Response to a repeat infection is identical to the primary response.

Innate immunity may be confined to a particular species, race or individual.

- **Species immunity** is the innate immunity towards a microbe exhibited by all members of a given species. For example, frogs are resistant to *Bacillus anthracis*; while toads are susceptible
- **Racial immunity:** Sometimes, innate immunity is confined to a particular race; may be absent in other communities. For example, Negroes of America are more susceptible to tuberculosis than the Whites
- **Individual immunity** refers to the antimicrobial defense mechanisms that are confined to a particular individual; may not be exhibited by others

Several factors determine the degree of innate immunity exhibited by the host, such as:

- **Age:** Certain infections are common in a particular age. For example, congenital infections like rubella is common in fetal life, chickenpox and measles occur in children, whereas urinary tract infection is common in adults
- **Hormone:** Certain hormonal disorders (e.g. diabetes mellitus) or patients on hormone therapy (e.g. corticosteroids) are at increased risk of developing various infections
- **Nutrition:** Malnutrition suppresses the host immunity, thereby predisposes to various infections.

MECHANISMS OF INNATE IMMUNITY

Receptor Interaction

Following the exposure to microorganisms, several mediators of innate immunity are recruited to the site

Table 9.1: Differences between innate and acquired immunity

Innate immunity	Acquired/Adaptive immunity
Resistance to infection that an individual possesses from birth	Resistance to infection that an individual acquires during his lifetime
Immune response occurs in minutes	Immune response occurs in days
Prior exposure to the antigen is not required	Develops following the antigenic exposure
Diversity is limited, acts through a restricted set of reactions	More varied and specialized responses
Immunological memory responses are absent	Immunological memory responses are present
Microbial antigen: Innate immunity develops against antigens that are shared by many microbes (called microbes-associated molecular patterns)	Microbial antigen: Acquired immunity develops against antigens that are specific for each microbes
Host cell receptors of innate immunity (called pattern recognition receptors) are non-specific, e.g. Toll-like receptor	Host cell receptors are specific, e.g. T cell receptors and B cell immunoglobulin receptors
Components of innate immunity • Anatomical barriers such as skin and mucosa • Physiological barriers (e.g. body temperature) • Phagocytes (neutrophils, macrophages and monocytes) • Natural killer (NK) cells • Other classes of lymphocytes: ➢ γδ T cells, NK-T cells ➢ B-1 cells and marginal-zone B cells • Mast cells and dendritic cells • Complement pathways—alternate and mannose binding pathways • Fever and inflammatory responses • Normal resident flora • Cytokines: TNF-α, interleukins (IL-1, IL-6, IL-8, IL-12, IL-16, IL-18), IFN-α, β and TGF- β • Acute phase reactant proteins (APRs)	**Components of acquired immunity** T cell B cell Classical complement pathway Antigen presenting cells Cytokines (IL-2, IL-4, IL-5, IFN-γ) **Types of acquired immunity** It can be classified in two ways: • Active and passive immunity • Artificial and natural immunity

Abbreviations: TNF, tumor necrosis factor; TGF-β, transforming growth factor-β; IFN, interferon; IL, interleukin.

of infection (Table 9.1). The first step that takes place is **attachment,** which involves binding of the surface molecules of microorganisms to the receptors of cells of innate immunity.

Microbial Surface Molecules

They are the repeating patterns of conserved molecules which are common to most microbial surfaces; called **Microbes-associated molecular patterns (MAMPs)**. Examples of MAMPs include peptidoglycan, lipopolysaccharides (LPS), teichoic acid and lipoproteins present on bacterial surface.

Pattern Recognition Receptors (PRRs)

These are the molecules present on the surface of host cells (e.g. phagocytes) that recognize MAMPs. They are generally conserved regions, encoded by germ line genes. **Toll-like receptors (TLRs)** are classical examples of pattern recognition receptors (see box below).

- Signals generated following binding of TLRs to MAMPs activate transcription factors that stimulate expression of genes encoding cytokines and enzymes, which are involved in several antimicrobial activities of cells of innate immunity
- The most important transcription factors activated by TLR signals are:
  - **Nuclear factor κβ (NF-κβ),** which promotes production of various cytokines and
  - **Interferon regulatory factors (IRFs)**, which stimulate expression of the antiviral interferons α and β.

Toll-like Receptors

They are so named because they are similar to Toll receptors present in the fruit fly *(Drosophila)*, where it is the main receptor for induction of innate immunity.

There are **13 types** of Toll-like receptors (TLR 1 to 13) recognized so far which bind to particular **MAMP** molecules on microbial surfaces. Important ones are:

- TLR-2 binds to bacterial peptidoglycan
- TLR-3 binds to dsRNA of viruses
- TLR-4 binds to LPS of gram-negative bacteria
- TLR-5 binds to flagella of bacteria
- TLR-7 and 8 bind to ssRNA of viruses
- TLR-9 binds to bacterial DNA.

Components of Innate Immunity

There are several mediators of innate immunity. They exert antimicrobial activities by various mechanisms as described below. Some of these mediators are not purely part of innate immunity; they often act as bridge between innate and acquired immunity (e.g. complements and macrophages).

Anatomical and Physiological Barriers

- Anatomical barriers such as skin and mucosal surfaces have a spectrum of antimicrobial activities (Table 9.2)
- Physiological barriers that contribute to the innate immunity are the body temperature, pH and various soluble secretory products of mucosa (Table 9.2).

Phagocytes

Phagocytes such as **neutrophils, macrophages** including **monocytes** are the main components of innate immunity. They are rapidly recruited to the infection site. Phagocytosis involves three sequential steps—(1) engulfment of microbes and subsequent hosting in phagosome, (2) fusion of lysosome with phagosome to form phagolysosome and (3) microbial killing (described in Chapter 14).

Table 9.2: Role of barriers in innate immunity

Anatomical barrier	Function
Skin barrier	
	• Mechanically prevents entry of microbes • Produces sebum containing antimicrobial peptides and fatty acids • Killing of microbes by intraepithelial lymphocytes
Mucosal barrier	
Mucous membrane	Prevents entry of microbes mechanically and by producing **mucus** which entraps microbes
Cilia	Cilia present in the lower respiratory tract propel the microbes outside
Normal flora	Intestinal and respiratory mucosa are lined by normal flora
Physiological barrier	**Function**
Temperature	Normal body temperature inhibits the growth of some microbes
Low pH	Gastric acidity inhibits most of the microbes
Secretory products of mucosa	
Saliva	Enzymes in saliva damage the cell wall and cell membrane of bacteria
Tears	Contains lysozyme that destroys the peptidoglycan layer in bacterial cell wall
Gastric juice	HCl kills microbes by its low pH
Trypsin	Hydrolyses bacterial protein
Bile salts	Interfere with bacterial cell membrane
Fatty acids	Denature the bacterial proteins
Spermine	Present in semen, inhibits growth of gram-positive bacteria
Lactoferrin	Binds to iron, thus interferes with acquisition of iron by bacteria

Natural Killer (NK) Cells

They are a class of lymphocytes that kill virus infected cells and tumor cells. NK cell mediated mechanism of killing microbes is described in Chapter 15.

Other Rare Classes of Lymphocytes

T and B lymphocytes are the chief mediators of acquired immunity. However, there are several rare types of lymphocytes that share the features of both acquired and innate immunity (Described in detail in Chapter 14), e.g.

- $\gamma\delta$ **T cells** (also called intraepithelial lymphocytes): They are present in epithelial lining of skin and mucosa
- **NK-T cells:** They are present in epithelium and lymphoid organs
- **B-1 cells:** They are found mostly in the peritoneal cavity and mucosal tissues
- **Marginal-zone B cells:** They are present at the edges of lymphoid follicles of spleen.

Mast Cells

They are present in the epithelial lining the respiratory and other mucosa.

- They are activated by microbial products binding to toll-like receptors or by IgE antibody dependent mechanism, following which;
- They release abundant cytoplasmic granules rich in histamine, prostaglandins and cytokines that initiate inflammation and proteolytic enzymes that results in killing of bacteria.

Dendritic Cells

They respond to microbes by producing numerous cytokines that initiate inflammation. They also serve as vehicle in transporting the antigen(s) from the skin and mucosal sites to lymph nodes where they present the antigen(s) to T cells. Hence, dendritic cells serve as a bridge between innate and acquired immunity.

Complement Pathways

Alternate and mannose binding pathways are the chief mediators of innate immunity.

- Alternate complement pathway is activated in response to bacterial endotoxin whereas the mannose binding pathway is stimulated by mannose carbohydrate residues on bacterial surface.

- Following activation, the complements mediate various biological functions such as (refer Chapter 13):
  - Lysis of the target microbes (by forming pores on the microbial surfaces)
  - Stimulate inflammation (by secreting inflammatory mediators)
  - Stimulate acquired immunity: Complements are another bridge between innate and acquired immunity.

Inflammatory Response

Inflammation is defined as the biological response of vascular tissues to harmful stimuli, such as microorganisms or other foreign substances. The major events that take place during an inflammatory response following a microbial entry are as follows (Fig. 9.1):

- **Vasodilation** due to release of vasoactive substances from the damaged tissues
- **Leakage** of plasma proteins through blood vessels
- **Recruitment** of phagocytes (e.g. neutrophils) to the site of inflammation—phagocytes undergo the following steps—(1) margination (adherence to the endothelium), (2) rolling on endothelium, (3) extravasation (moves out of the blood vessels), (4) chemotactic migration to the inflammation site
- **Engulfment** of microbes and dead material by the phagocytes
- **Destruction** of the microbes.

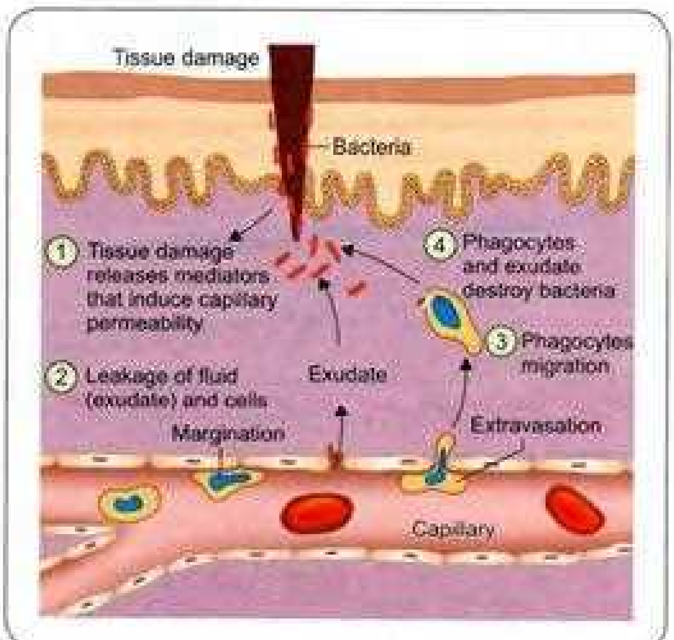


Fig. 9.1: Major events in an inflammatory response

Inflammation is not always protective in nature, sometime, it may produce injurious consequences to host tissues called **hypersensitivity reactions.**

Normal Resident Flora

Normal resident flora lining intestinal, respiratory and genital tract exert several antimicrobial activities, (described in Chapter 59)

- They compete with the pathogens for nutrition
- They produce antibacterial substances.

Cytokines

In response to the microbial antigens, dendritic cells, macrophages, and other cells secrete several cytokines that mediate many of the cellular reactions of innate immunity such as:

- Tumor necrosis factor-α (TNF-α)
- Interleukin-1 (IL-1), IL-6, IL-8, IL-12 and IL-16
- Interferons (IFN-α, β) and
- Transforming growth factor (TGF-β).

Acute Phase Reactant Proteins (APRs)

They are the proteins synthesized by liver at steady concentration, but their synthesis either increases or decreases exponentially during acute inflammatory conditions. Though liver is the primary site, APRs can also be synthesized by various other cells such as endothelial cells, fibroblasts, monocytes and adipocytes.

- **Positive APRs:** They are the proteins whose levels increase during acute inflammation. Examples include:
  - Serum amyloid A
  - C-Reactive protein
  - Complement proteins—complement factors (C1-C9), factor B, D, and properdin
  - Coagulation protein, e.g. fibrinogen, von Willebrand factor
  - Proteinase inhibitors, e.g. α1 antitrypsin
  - α1 acid glycoprotein
  - Mannose binding protein
  - Haptoglobin
  - Metal binding proteins, e.g. ceruloplasmin.
- **Negative APRs:** They are the proteins whose levels are decreased during acute inflammation; thus creating a negative feedback that stimulates the liver to produce positive APRs. Examples of negative APRs include albumin, transferrin and antithrombin
- **Role of APRs:** They have a wide range of activities that contribute to the host defense
  - APRs have various antimicrobial and anti-inflammatory activities (e.g. complement factors)
  - Metal binding proteins can chelate various metals such as iron, copper, etc. making them unavailable for the bacteria.

C-Reactive Protein (CRP)

C-reactive protein is an example of APR that rise in acute inflammatory conditions including bacterial infections. It belongs to beta globulin family.

- CRP is so named because it precipitates with C-carbohydrate (polysaccharide) antigen of pneumococcus. However, it is not an antibody against the C-carbohydrate antigen of pneumococcus; it is non-specific, can be raised in any inflammatory conditions.
- It is one of the most common markers of acute inflammation, used in most diagnostic laboratories.

CRP Level

The normal level of CRP is less than 0.2 mg/dL. However, it increases by several folds in acute inflammatory conditions.

- **Insignificant increase** of CRP (<1 mg/dL): It occurs in conditions such as heavy exercise, common cold, and pregnancy
- **Moderate increase** (1–10 mg/dL): It occurs in conditions such as bronchitis, cystitis, malignancies, pancreatitis, myocardial infarction
- **Marked increase** of CRP (>10 mg/dL): It occurs in conditions such as acute bacterial infections, major trauma and systemic vasculitis.

CRP Can be Detected by

- Precipitation method using C-carbohydrate antigen (obsolete, not in use now)
- Latex (passive) agglutination test using latex particles coated with anti-CRP antibodies
  - It is the most widely used method employed worldwide
  - Detection limit of CRP by latex agglutination test is 0.6 mg/dL.

Highly Sensitive CRP (hs-CRP) Test

Minute quantities of CRP can be detected by various methods (e.g. nephelometry, enzyme immunoassays). This is useful in assessing the risk to cardiovascular diseases.

ACQUIRED OR ADAPTIVE IMMUNITY

Acquired immunity is defined as the resistance against the infecting foreign substance that an individual acquires or adapts during the course of his life.

Acquired immunity has unique properties by which it can be differentiated from innate immunity (see Table 9.1).

- **Mediators: T cells and B cells** are the chief mediators of acquired immunity. Other mediators include:
  - Classical complement pathway
  - Antigen presenting cells
  - Cytokines (IL-2, IL-4, IL-5).
- **Response occurs in days:** Acquired immunity involves activation of T and B cells against the microbial antigens; which takes several days to weeks to develop, following the microbial entry
- **Requires prior microbial exposure:** Acquired immunity develops only after the exposure to the microbes. It is not present prior to the first contact with the microbes
- **Specific:** Acquired immunity is highly specific; directed against specific antigens that are unique to the microbes
- **Memory present:** Acquired immunity does have a memory component. A proportion of T and B cells become memory cells following primary contact of the microbe, which play an important role when the microbe is encountered subsequently
- **Diversity is wide:** Acquired immunity though takes time to develop, is active against a wide range of repertoire of antigens
- **Host cell receptors** of acquired immunity are specific for a particular microbial antigen
  - Examples include T cell receptors and B cell immunoglobulin receptors
  - They are encoded by genes produced by somatic recombination of gene segments.

Types of acquired immunity: Acquired immunity can be classified in two ways:

1. Active and passive immunity.
2. Artificial and natural immunity.

ACTIVE IMMUNITY

Active immunity is the resistance developed by an individual toward an antigenic stimulus.

- Here, the host's immune system is actively involved in response to the antigenic stimulus; leading to the production of immunologically active T cells, B cells and production of specific antibodies
- Active immunity may be induced naturally or artificially
  - **Natural active immunity** occurs following an exposure to a microbial infection (e.g. measles virus infection)
  - **Artificial active immunity** develops following an exposure to an immunogen by vaccination (e.g. measles vaccine). Vaccines are discussed in Chapter 20.
- As host's immune apparatus is actively involved, active immunity often fails to develop when the host is immunocompromized
- **Long-lasting:** Active immunity usually lasts for longer periods, but the duration varies depending on the type of pathogen
  - It may last life long, e.g. following certain viral infections such as chickenpox, measles, smallpox, mumps and rubella
  - It may last for short duration, e.g. following influenza virus infection
  - It may last for as long as the microbe is present. Once the disease is cured, the patient becomes susceptible to the microbe again. This is called

premunition or concomitant immunity. It is seen following some microbial infections like spirochetes and *Plasmodium*
- Active immunity may not be protective at all, e.g. for *Haemophilus ducreyi*, the patient may develop genital lesions following reinfection even while the original infection is active.

Types of immune response in active immunity vary depending on the microbial exposure that occurs for the first time (called primary immune response) and subsequent time (called secondary immune response).

Primary Immune Response

When the antigenic exposure occurs for the first time, the following events take place:
- **Latent or lag period:** Active immunity develops only after a latent period following the antigenic exposure, which corresponds to the time required for the host's immune apparatus to become active
- **Effector cells:** Majority of activated T and B cells against the antigenic stimulus become effector T and B cells
  - Effector T cells such as helper T cells and cytotoxic T cells
  - Effector B cells include plasma cells.
- **Memory cells:** A minor proportion of stimulated T and B cells become memory cells, which are the key cells for secondary immune response
- **Antibody surge:** Effector B cells produce antibodies (mainly IgM type). Antibodies appear in the serum in slow and sluggish manner; reach peak, maintain the level for a while and then fall down. Finally, a low titer of baseline antibodies may be maintained in the serum (Fig. 9.2).

Secondary Immune Response

When the same antigenic exposure occurs subsequently, the events which take place are as follows (Fig. 9.2).

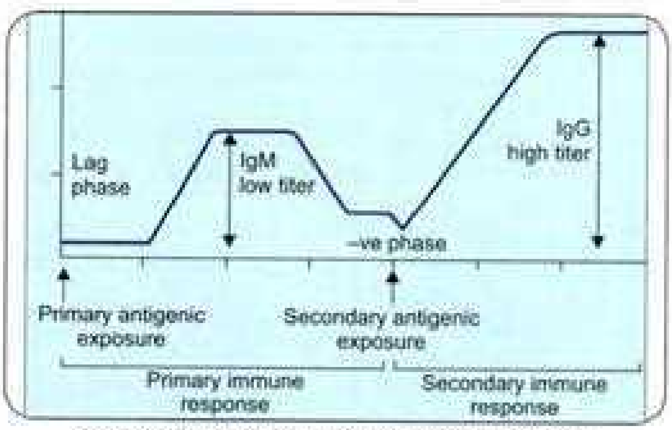


Fig. 9.2: Primary and secondary immune responses

Table 9.3: Differences between primary and secondary immune response

Primary immune response	Secondary immune response
Immune response against first antigenic challenge	Immune response against subsequent antigenic challenge
Slow, sluggish (appear late) and short lived	Prompt, powerful and prolonged (long lasting)
Lag period is longer (4–7 days)	Lag period is absent or short (1–3 days)
No negative phase	Negative phase may occur
• Antibody produced in low titer and is of IgM type	Antibody produced in high titer and is of IgG type
• Antibodies are more specific but less avid	Antibodies are less specific but more avid
Antibody producing cells—Naive B cells	Antibody producing cells—Memory B cells
Both T dependent and T independent antigens are processed	Only T dependent antigens are processed

- **Latent period** is either absent or of short duration. This is because memory cells become active soon after the antigenic exposure
- **Negative phase:** At the onset of secondary immune response, there may be a negative phase during which the antibody level may become lower than it was before the antigenic stimulus. This is because the exposed antigen combines with the pre-existing antibody and thus the antibody level in serum falls down
- **Antibody surge:** Secondary antibody response is prompt, powerful, long-lasting and mainly of IgG type. Hence, it is said that, the booster doses of vaccines are more effective than the first dose.

The differences between primary and secondary immune response are tabulated in Table 9.3.

PASSIVE IMMUNITY

Passive immunity is defined as the resistance that is transferred passively to a host in a "readymade" form without active participation of the host's immune system.
- Passive immunity can also be induced naturally or artificially
  - **Natural passive immunity** involves the IgG antibody transfer from mother to fetus across the placenta
  - **Artificial passive immunity** develops following readymade transfer of commercially prepared immunoglobulin (e.g. Rabies immunoglobulin).
- Passive immunity plays a very important role in:
  - Immunodeficient individuals (as host's immune apparatus is not effective) and;
  - Post-exposure prophylaxis; when an immediate effect is warranted

Table 9.4: Differences between active and passive immunity

Active immunity	Passive immunity
Produced actively by host immune system	Immunoglobulins received passively
Induced by: • Infection (natural) • Vaccination (artificial)	Acquired by: • Mother to fetus IgG transfer (natural) • Readymade antibody transfer (artificial)
Long lasting	Lasts for short time
Lag period present	No Lag period
Memory present	No memory
Booster doses are useful	Subsequent doses are less effective
Negative phase may occur	No negative phase
Not useful in immunodeficiency individuals	Useful in immunodeficient individuals

- Passive immunity **develops faster**; there is no lag phase or negative phase
- There is no **immunological memory** as the memory cells are not involved
- **Booster doses are not effective:**
  - As memory component is absent, the effect produced following subsequent immunoglobulin administration is same as the effect produced after the primary dose
  - Some time, the booster doses of an immunoglobulin may be less effective because of its immunological clearance, which is mediated by the antibodies produced against the first dose of immunoglobulin.

The differences between active and passive immunity are listed in Table 9.4.

BRIDGES BETWEEN INNATE AND ACQUIRED IMMUNITY

The innate and acquired immunity do not work independently; rather they function in a highly interactive and collaborative manner, increasing each other's efficiency and producing a combined response, which is more effective than either branch could produce by itself. Certain immune components play important roles in both types of immunity and are considered as bridges between innate and acquired immunity. Examples include:

- **Macrophages** and **dendritic cells** belong to innate immune system, but as antigen presenting cells, they present the antigenic peptides to T cells. More so, cytokines secreted from macrophages (interleukin-1) are also involved in T cell activation
- **ADCC** (antibody dependent cell-mediated cytotoxicity) is a type of cell-mediated immune response (CMI) described in Chapter 15, which involves both innate and adaptive components. Cells of innate immunity such as NK cell, eosinophils and neutrophils destroy the target cells which are coated with specific antibodies
- **Complements** (classical pathway) are also part of both innate and adaptive immunity. They destroy the target cells which are coated with specific antibodies. However, alternate and mannose binding pathways do not take help of antibodies (Described in Chapter 13)
- **Cytokines** secreted from cells of innate immunity can activate cells of adaptive immunity and vice versa. For example, IL-1 secreted from macrophage activates helper T cells and interferon-γ secreted by helper T cell can activate macrophage
- **Rare classes of lymphocytes** such as γδ T cells, NK-T cells, B-1 cells and marginal-zone B cells: These cells have many characteristics that place them in the border of innate and acquired immunity
  - They function in the early defense against microbes as part of innate immunity
  - Although their receptors are encoded by somatic recombination of genes (similar to that of classical T and B cells), but these receptors have limited diversity
  - They develop a memory phenotype in contrast to the property of innate immunity.

OTHER TYPES OF IMMUNITY

Local (or Mucosal) Immunity

Local or mucosal immunity is the immune response that is active at the mucosal surfaces such as intestinal or respiratory or genitourinary mucosa.

- It is usually mediated by a type of IgA antibody called secretory IgA, which prevents the entry of microbes at the local site itself
- Local immunity can only be induced by natural infection or by live vaccination (but not by killed vaccines)
- **Example:** Following administration of live oral polio vaccine (OPV) or following infection with poliovirus; secretory IgA antibodies are synthesized and coated on intestinal mucosa which prevent subsequent poliovirus infections. Such immunity does not develop following injectable killed polio vaccine (IPV).

Herd Immunity

Herd immunity is defined as the overall immunity of a community (or herd) towards a pathogen.

- Herd immunity plays a vital role in preventing epidemic diseases. If the herd immunity is good, that means large population of the community are immune towards a pathogen. Hence, epidemics are less likely to occur and eradication of the disease may be possible

- Elements that contribute to the development of a strong herd immunity are:
  - Occurrence of clinical and subclinical cases in the herd
  - On-going immunization program
  - Herd structure, i.e. type of population involved
  - Type of pathogen-Herd immunity may not be strong in a community against all the pathogens.
- Herd immunity develops following effective vaccination against some diseases like:
  - Diphtheria and pertussis vaccine
  - Measles, mumps and rubella (MMR) vaccine
  - Polio (oral polio vaccine)
  - Smallpox vaccine.

Adoptive Immunity

Adoptive immunity is a special type of cell-mediated immune response (CMI) which develops following injection of immunologically competent T-lymphocytes known as **transfer factor**. It is useful for treatment when the CMI is low, e.g. in lepromatous leprosy.

EXPECTED QUESTIONS

I. Essay:

1. Define immunity. Describe in detail about the properties and mediators of innate immunity.

II. Write short notes on:

1. Herd immunity.
2. Differences between innate and acquired immunity.
3. Differences between active and passive immunity.

III. Multiple Choice Questions (MCQs):

1. Which is not a mediator of innate immunity?
a. T cells b. NK cell
c. B-1 cell d. Neutrophil

2. Which of the following about innate immunity is wrong?
a. Immune response occurs in minutes
b. Non-specific
c. First line of defense
d. Need prior contact with the antigen

3. Which of the following about active immunity is correct?
a. No lag phase
b. Booster doses are useful
c. Useful in immunodeficient people
d. No memory cells

4. Primary immune response—the correct statement is:
a. Involves IgG
b. Antibody producing cells- Memory B cell
c. No lag period
d. Slow and sluggish

5. Innate immunity differs from acquired immunity in all, *except*:
a. Present from birth
b. Diversity is limited
c. Immunological memory absent
d. Prior antigen exposure is must

6. Toll-like receptor is a component of:
a. Innate immunity b. Acquired immunity
c. Active immunity d. Passive immunity

7. The molecules present on the surface of host cells that recognize the microbes-associated molecular patterns (MAMP) on microbial surface?
a. Pattern recognition receptors
b. Pre B cell receptor
c. B cell receptor
d. T cell receptors

8. Which of the following cells act as bridge between innate and acquired immunity?
a. γδ T cells b. NK-T cells
c. B-1 cells d. Marginal-zone B cells
e. All of the above

9. Which of the following cytokine is a component of innate immunity?
a. IL-1 b. IL-2
c. IL-4 d. IL-5

10. Components of innate immunity is:
a. Classical complement pathway
b. Antigen presenting cells
c. Mast cells
d. IFN-γ

11. Which of the following statement regarding toll-like receptor (TLR) is wrong?
a. TLR-2 binds to bacterial peptidoglycan
b. TLR-3 binds to dsRNA of viruses
c. TLR-4 binds to LPS of gram-negative bacteria
d. TLR-6 binds to flagella of bacteria

12. Acute phase reactant proteins are all, *except*:
a. Serum amyloid A b. C-reactive protein
c. Fibrinogen d. Coagulase

13. Negative acute phase reactant proteins are all, *except*:
a. Albumin b. α1 acid glycoprotein
c. Transferrin d. Antithrombin

14. Secondary immune response:
a. Lag period present
b. Negative phase may occur
c. Only T dependent antigens are processed
d. IgG type antibody produced

15. Overall immunity of a community (or herd) towards a pathogen is called as:
a. Local (or mucosal) Immunity
b. Herd immunity
c. Adoptive immunity
d. Adaptive immunity

Answers

1. a 2. d 3. b 4. d 5. d 6. a 7. a 8. e 9. a 10. c 11. d 12. d 13. b 14. a 15. b

Antigen

10
CHAPTER

Chapter Preview

- Antigen and hapten
- Antigen and host relationship
- Factors influencing immunogenicity
- Biological classes of antigens

ANTIGEN

Antigen is defined as any substance that satisfies two distinct immunologic properties—immunogenicity and antigenicity.

1. **Immunogenicity:** It is the ability of an antigen to induce immune response in the body (both humoral and/or cell mediated).
   - B cells + antigen → effector B cells (plasma cell) + memory B cells
   - T cells + antigen → effector T cells (helper T cell or cytotoxic T cell) + memory T cells.
2. **Antigenicity (immunological reactivity):** It is the ability of an antigen to combine specifically with the final products of the above two responses (i.e. antibodies and/or T cell-surface receptors).

The substance that satisfies the first property, i.e. immunogenicity (inducing specific immune response) is more appropriately called **"immunogen"** rather than using the word "antigen".

All molecules having immunogenicity property, also show antigenicity, but the reverse is not true (e.g. haptens—which are antigenic, but not immunogenic).

Epitope

Epitope or antigenic determinant is the smallest unit of antigenicity.

- It is defined as a small area present on the antigen comprising of few (four to five) amino acids or monosaccharide residues, that is capable of sensitizing T and B cells and reacting with specific site of T cell receptor or an antibody
- The specific site of an antibody that reacts with the corresponding epitope of an antigen is called **paratope**.

Epitopes may be grouped into two types:

1. **Sequential** or **linear epitope:** It presents as a single linear sequence of few amino acid residues.
2. **Conformational** or **non-sequential** epitopes are found on the flexible region of complex antigens having tertiary structures. They are formed by bringing together the surface residues from different sites of the peptide chain during its folding into tertiary structure.

In general, T cells recognize sequential epitopes, while B cells bind to the conformational epitopes.

HAPTEN

Haptens are low molecular weight molecules that **lack immunogenicity** (cannot induce immune response) but **retain antigenicity or immunological reactivity** (i.e. can bind to their specific antibody or T cell receptor). Haptens can become immunogenic when combined with a larger protein molecule called **carrier**.

The hapten-carrier complex is capable of inducing immune response in the body. It is observed that animals immunized with such a hapten-carrier conjugate produce antibodies specific for:

- Epitopes of hapten
- Unaltered epitopes on the carrier protein
- New epitopes formed by combined parts of both the hapten and carrier (Fig. 10.1).

Haptens may be classified as complex or simple:

- **Complex haptens** contain two or more epitopes; they can react with specific antibodies and the hapten-antibody complex can be visualized by various methods such as precipitation reaction
- **Simple haptens** usually contain only one epitope (univalent). Such haptens can bind to the antibodies but

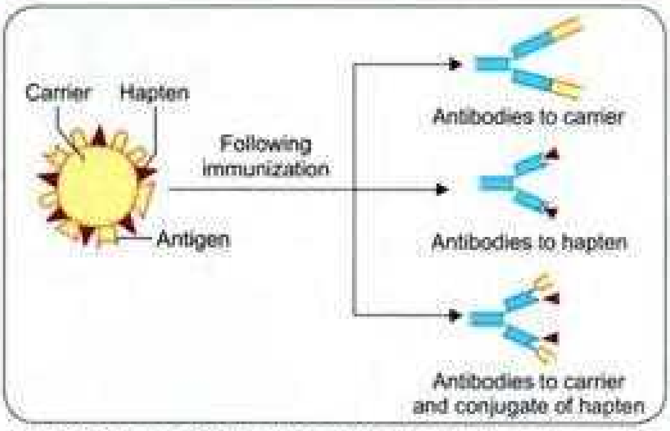


Fig. 10.1: Types of antibodies produced against a hapten-carrier conjugate

the hapten antibody complex cannot be visualized, as it is believed that precipitation to happen, it requires the antigen to have at least two or more epitopes.

ANTIGEN-HOST RELATIONSHIP

Based on the antigen-host relationship, antigens can be grouped into two groups as follows:

1. **Self or autoantigens:** They belong to the host itself; hence they are not immunogenic. Host's immune system does not react to its own antigen, which is due to exhibiting a mechanism called **immunological tolerance** (Chapter 17). However, sometimes, the self-antigens are biologically altered (e.g. as in cancer cells) and can become immunogenic
2. **Non-self or foreign antigens:** They are immunogenic and are of three types based on their phylogenetic distance to the host
   - **Alloantigens** are species specific. Tissues of all individuals in a species contain species-specific antigens
   - **Isoantigens** are type of antigens which are present only in subsets of a species, e.g. blood group antigens and histocompatibility antigens. The histocompatibility antigens are highly specific as they are unique to every individual of a species
   - **Heteroantigens:** Antigens belonging to two different species are called heteroantigens, e.g. antigens of plant or animal or microorganisms, etc. A **heterophile antigen** is a type of heteroantigen that exists in unrelated species (explained below).

Heterophile Antigens

Heterophile antigens are a type of heteroantigens that are present in two different species; but they share epitopes with each other. Antibody produced against antigen of one species can react with the other and vice versa.

Contd...

Contd...

Diagnostic Application

Heterophile antigens can be used in various serological tests. Antibody against one antigen can be detected in patient's serum by employing a different antigen which is heterophile (cross reactive) to the first antigen. For example:

- **Weil-Felix reaction** is done for typhus fever. Antibodies against rickettsial antigens are detected by using cross reacting *Proteus* antigens
- **Paul-Bunnell test** is done for infectious mononucleosis (caused by Epstein-Barr virus). Here, sheep red blood cell (RBC) antigens are used to detect cross-reacting antibodies in patient's sera
- **Cold agglutination test** and *Streptococcus* MG test are done for primary atypical pneumonia. Here, antibodies against *Mycoplasma pneumoniae* are detected by using human O blood group RBCs and *Streptococcus* MG antigens respectively.

FACTORS INFLUENCING IMMUNOGENICITY

There are various factors that influence immunogenicity of an antigen.

- **Size of the antigen:** Larger is the size; more potent is the molecule as an immunogen. It is found that molecules of more than 10,000 Dalton molecular weight only can induce immune response (e.g. hemoglobin). Substances of molecular weight between 5000–10,000 Dalton are poor immunogens (e.g. insulin)
- **Chemical nature of the antigen:** Proteins are stronger immunogens than carbohydrates followed by lipids and nucleic acids
- **Susceptibility of antigen to tissue enzymes:** Only substances that are susceptible to the action of tissue enzymes are immunogenic. Degradation of the antigen by the tissue enzymes produces several immunogenic fragments having more number of epitopes exposed. Molecules that are not susceptible to tissue enzymes such as polystyrene latex or synthetic polypeptides composed of **D-amino acids** are not antigenic; while polypeptides consisting of **L-amino acids** are antigenic as they are degradable by tissue enzymes. However, substances very rapidly broken down by tissue enzymes may not be immunogenic as that may denature the epitopes
- **Structural complexity:** Simple homopolymers made up of single amino acid lack immunogenicity. Polymers made up of two or more amino acids are immunogenic. More so, addition of aromatic amino acids increases immunogenicity. Complex proteins containing 20 amino acids and with four level of structural organization are strongly immunogenic; e.g. hemoglobin
- **Foreignness to the host:** This is one of the key factor which determines immunogenicity. Higher is the

phylogenetic distance between the antigen and the host; more is the immunogenicity
- Self-antigens are not immunogenic; whereas, hetero-antigens and alloantigens are immunogenic; the degree of immunogenicity increases with the distance
    - Plant antigens are more immunogenic than animal antigens to humans
    - Bovine serum albumin is more immunogenic to chicken than to goat.
- Isoantigens are not immunogenic to those individuals who possess these antigens; but for other individuals they are immunogenic.

❖ **Genetic factor:** Different individuals of a given species show different types of immune responses towards the same antigen. This is believed to be due to the genetic differences between the individuals
- Responders are the individuals who produce antibody faster
- Slow responders are the individuals who produce antibody slowly and may need repeated antigenic exposures
- Non-responders are the individuals who do not produce antibody in spite of repeated antigenic exposures.

❖ **Optimal dose of antigen:** An antigen is immunologically active only in the optimal dose range. A too little dose fails to elicit immune response and a too large dose leads to development of immunological tolerance (Chapter-17), a phenomenon previously designated by Felton as **immunological paralysis**

❖ **Route of antigen administration:** In general, the immune response is better induced following parenteral administration of an antigen; however it also depends on the type of antibody produced
- Immunoglobulin A (IgA) are better induced following oral administration of antigens
- Inhalation of pollen antigens induces IgE synthesis; whereas the same antigens given parenterally lead to formation of IgG antibodies
- **Site of injection** may influence immunogenicity: The hepatitis B vaccine is more immunogenic following deltoid injection than gluteal injection. This may be due to the paucity of antigen presenting cells (APCs) in gluteal fat.

❖ **Repeated doses of antigens:** Repeated doses of antigens over a period of time are needed to generate an adequate immune response. This is due to the role of memory cells in secondary immune response. However, after a certain doses of antigens, no further increase in antibody response is seen

❖ **Multiple antigens:** When two or more antigens are administered simultaneously, the effects may vary. The antibody response to one or the other antigen may be equal or diminished (due to antigenic competition) or enhanced (due to adjuvant like action, see below highlight box)

Adjuvant

The term "adjuvant" refers to any substance that enhances the immunogenicity of an antigen. They are usually added to vaccines to increase the immunogenicity of the vaccine antigen.

Examples of Adjuvant Activity

- **Alum** (aluminum hydroxide or phosphate)
- **Mineral oil** (liquid paraffin)
- **Freund's incomplete adjuvant:** It is a water-in-oil emulsion containing a protein antigen in the aqueous phase
- **Freund's complete adjuvant** is the mixture of Freund's incomplete adjuvant and suspension of killed tubercle bacilli in the oil phase
- **Lipopolysaccharide** (LPS) fraction of gram-negative bacilli, e.g. LPS of *Bordetella pertussis* acts as an excellent adjuvant for diphtheria and tetanus toxoids. This explains the reason for using combined immunization for diphtheria, pertussis and tetanus in the form of DPT vaccine
- **Other bacteria or their products:**
    - *Mycobacterium bovis*
    - Toxoid (diphtheria toxoid and tetanus toxoid act as adjuvant for *Haemophilus influenzae*—type b vaccine).
- **Nonbacterial products:** Such as silica particles, beryllium sulfate, squalene and thiomersal.

Mechanism of Adjuvant Action

Adjuvants act through the following steps:

- **Delaying the release of antigen:** Adjuvant on mixing, precipitate the antigen which is then released slowly from the site of administration, thus prolonging the antigenic exposure
- **By activating phagocytosis:** The adjuvant-antigen precipitate is of larger size, thus increases the likelihood of phagocytosis. The MDP (muramyl dipeptide) component of tubercle bacilli can activate the macrophages directly
- **By activating T_H cells:** Activated macrophages release interleukin-1 (IL-1) and express higher level of MHC-II; thus promoting helper T (T_H) cell activation which in turn activates B cells to produce specific antibodies
- **By granuloma formation:** Certain adjuvants such as Freund's complete adjuvant causes chronic inflammation and granuloma formation at the inoculation site (hence not suitable for human use). Activated phagocytes in granuloma continue to enhance T_H cell activation.

❖ **Effect of prior administration of antibody:** The immune response against a particular antigen is suppressed if its corresponding antibody was administered prior to that
- The primary immune response is more susceptible to get suppressed than the secondary immune response

- **Therapeutic application:** In Rh negative women carrying an Rh positive fetus, the anti-Rh globulin is administrated immediately following delivery (within 72 hours) which prevents the Rh sensitization in Rh negative women by a negative feedback mechanism.

BIOLOGICAL CLASSES OF ANTIGENS

Depending on the mechanisms of inducing antibody formation, antigens are classified as T cell dependent (TD) and T cell independent (TI) antigens (Table 10.1).

T-dependent (TD) Antigens

Most of the normal antigens are T cell dependent, they are processed and presented by antigen-presenting cells (APCs) to T cells which leads to T cell activation. The activated T cells secrete cytokines that in turn stimulate the B cells to produce antibodies.

T-independent (TI) Antigens

There are a few antigens such as **bacterial capsule, flagella** and **LPS** (lipopolysaccharide) that do not need the help of T cells and APCs. They directly bind to immunoglobulin receptors present on B cells and stimulate B cells polyclonally. It leads to increased secretion of non-specific antibodies (i.e. hypergammaglobulinemia).

- TI antigens can activate both mature and immature B cells. B cells can only differentiate into activated cells. There is no memory cell formation
- Activated B cells do not undergo affinity maturation and class switch over (both properties are unique to TD antigen stimulated B cells); thus such an activated B cell can produce only limited classes of antibodies such as IgM and IgG3.

Detailed mechanism of B cell activation against TD antigen is given in Chapter15.

Table 10.1: Differences between T-independent antigens and T-dependent antigen

T-independent antigen	T-dependent antigen
Structurally simple—LPS, capsular polysaccharide, flagella	Structurally complex—protein in nature
Dose dependent immunogenicity	Immunogenic over wide range of doses
No memory	Memory present
No antigen processing	Antigen processing step is needed
Slowly metabolized	Rapidly metabolized
Activate B cells polyclonally	Activate B cells monoclonally
Activate both mature and immature B cells	Activate mature B cells only
B cells stimulated against T-independent antigen do not undergo • Affinity maturation • Class switch over	B cells stimulated against T-dependent antigen undergo • Affinity maturation • Class switch over
Antibody response is restricted to IgM and IgG3	Antibodies of all classes can be produced

Abbreviations: LPS, lipopolysacchrides; Ig, immunoglobin.

Superantigens

Superantigens are the third variety of biological class of antigens, recently described in the last decade. The unique feature of superantigens is, they can activate T cells directly without being processed by antigen-presenting cells (APCs).

- The variable β region of T cell receptor (vβ of TCR) appears to be the receptor for superantigens
- They directly bridge non-specifically between major histocompatibility complex (MHC)-II of APCs and T cells (Fig. 10.2)
- Non-specific activation of T cells leads to massive release of cytokines which can activate B cell polyclonally, which leads to increased secretion of non-specific antibodies (hypergammaglobulinemia).

Table 10.2: Superantigens

Bacterial superantigen
Staphylococcal toxin: • Toxic shock syndrome toxin-1 (TSST-1) • Exfoliative toxin • Enterotoxins
Streptococcal pyrogenic exotoxin (SPE)-A and C
Mycoplasma arthritidis mitogen-I
Yersinia enterocolitica *Yersinia pseudotuberculosis*
Viral superantigen
Epstein-Barr virus associated superantigen
Cytomegalovirus associated superantigen
Rabies nucleocapsid
HIV encoded superantigen (nef- negative regulatory factor)
Fungal superantigen
Malassezia furfur

Examples of Superantigens

Various products of microorganisms behave as superantigens; the most important being staphylococcal and streptococcal toxins. (Table 10.2).

Diseases Associated with Superantigens

Superantigens can cause a number of diseases.

- Toxic shock syndrome
- Food poisoning
- Scalded skin syndrome
- Rare conditions such as—atopic dermatitis, Kawasaki syndrome, psoriasis, acute disseminated encephalomyelitis.

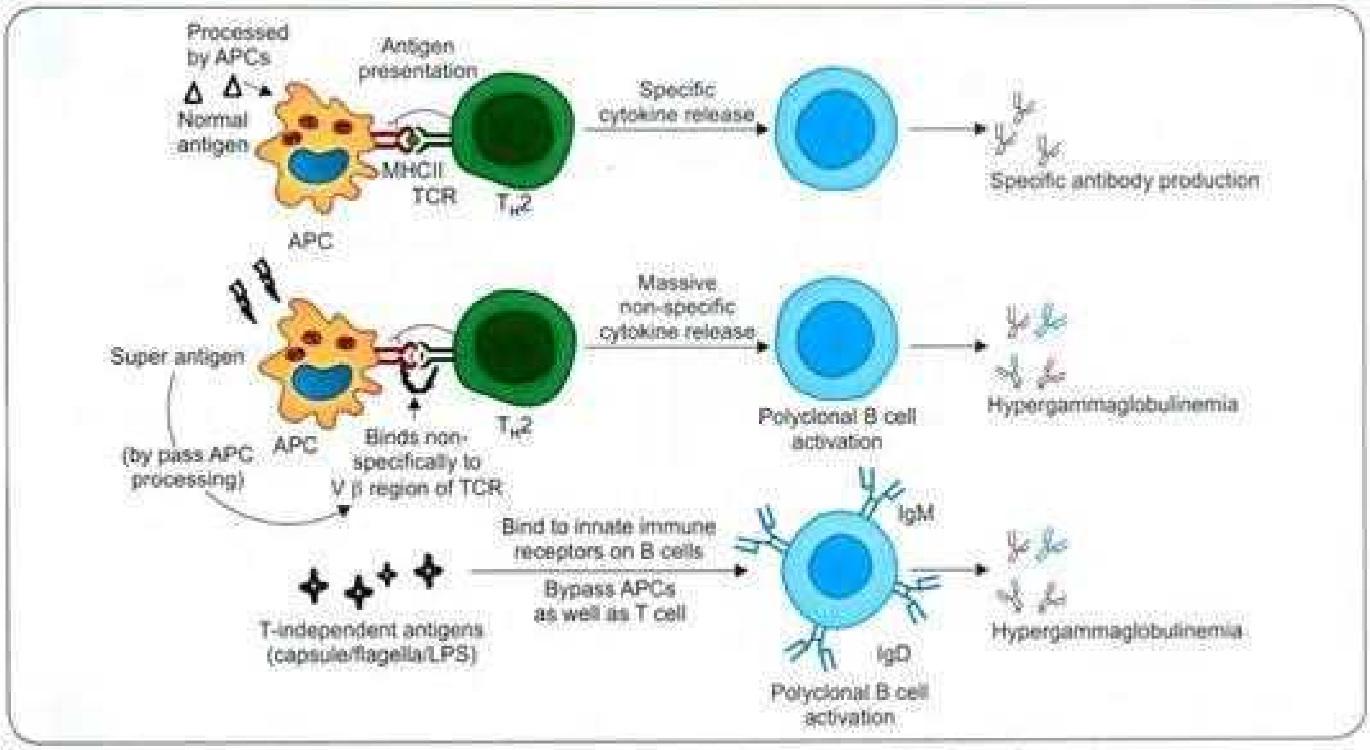


Fig. 10.2: Mechanism of action of superantigens, T-dependent and T-independent antigens

Abbreviations: APCs, antigen-presenting cells; TCR, T cell receptor; MHC, major histocompatibility complex.

EXPECTED QUESTIONS

I. Write short notes on:
1. Epitope.
2. Hapten
3. Heterophile antigens.
4. Adjuvant.
5. T independent antigen.
6. Superantigens.

II. Multiple Choice Questions (MCQs):

1. **Superantigen causes:**
   a. Enhancement of phagocytosis
   b. Polyclonal activation of B cells
   c. Antigen presentation by macrophage
   d. Activation of complement
2. **Which part of the bacteria is most antigenic?**
   a. Protein b. Carbohydrate
   c. Lipid d. Nucleic acid
3. **Which of the following statements is true about hapten?**
   a. It induces immune response
   b. It is a T-independent antigen
   c. It needs carrier to induce immune response
   d. It has no association with MHC
4. **Which is not a superantigen?**
   a. Staphylococcal enterotoxin
   b. Toxic shock syndrome toxin-1 (TSST-1)
   c. Cholera toxin
   d. Streptococcal pyrogenic exotoxin
5. **Which is the binding site of superantigen?**
   a. T cell receptor
   b. MCH II
   c. B cell receptor
   d. Variable β region of TCR
6. **Which of the following antigen is T-dependent?**
   a. Bacterial capsule
   b. Flagella
   c. Lipopolysaccharide
   d. Exotoxin
7. **All are examples of adjuvant, *except:***
   a. Mineral oil b. LPS of *H. influenzae*
   c. Diphtheria toxoid d. Thiomersal
8. **Diagnostic application of heterophile antigens include all, *except:***
   a. Weil-Felix reaction b. Paul-Bunnell test
   c. Widal test d. Cold agglutination test

Answers

1. b **2.** a **3.** c **4.** c **5.** d **6.** d **7.** b **8.** c

Antibody

11
CHAPTER

Chapter Preview

- Structure of antibody
- Immunoglobulin classes
- Antigenic determinants of immunoglobulins
- Monoclonal antibody
- Genetics of antibody production

Antibody or immunoglobulin is a specialized glycoprotein, produced from activated B cells (plasma cells) in response to an antigen, and is capable of combining with the antigen that triggered its production.

- It was found that (*A. Tiselius*,1939) when the serum is subjected to electrophoresis, the serum proteins are separated into four fragments—albumin, globulin α, β and γ (Fig. 11.1). Antibodies are located in the γ-globulin fraction. Because they immunologically react with the antigen, they were given the name as immunoglobulin
- Both the terms, immunoglobulin (Ig) and antibody are used interchangeably, representing the physiological and functional properties of the molecule respectively
- Immunoglobulin (Ig) constitutes 20–25% of total serum proteins
- There are five classes (or isotypes) of immunoglobulins recognized—IgG, IgA, IgM, IgD and IgE.

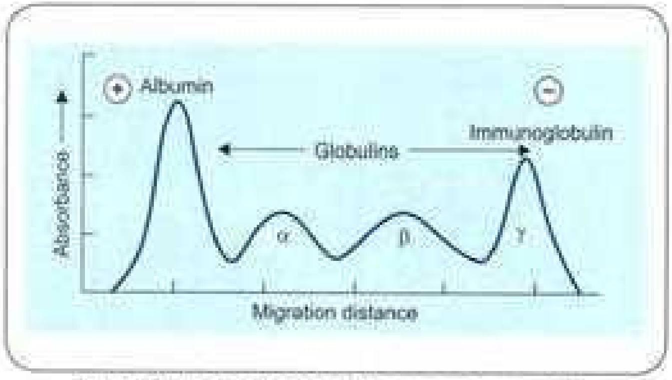


Fig. 11.1: Electrophoresis of human serum proteins

STRUCTURE OF ANTIBODY

An antibody molecule is a 'Y-shaped' heterodimer, composed of four polypeptide chains (Fig. 11.2).

- Two identical light (L) chains, of molecular weight 25,000 Da each and
- Two identical heavy (H) chains each having molecular weight 50,000 Da or more.

H and L Chain

All four H and L chains are bound to each other by disulfide bonds, and by noncovalent interactions, such as salt linkages, hydrogen bonds, and hydrophobic bonds.

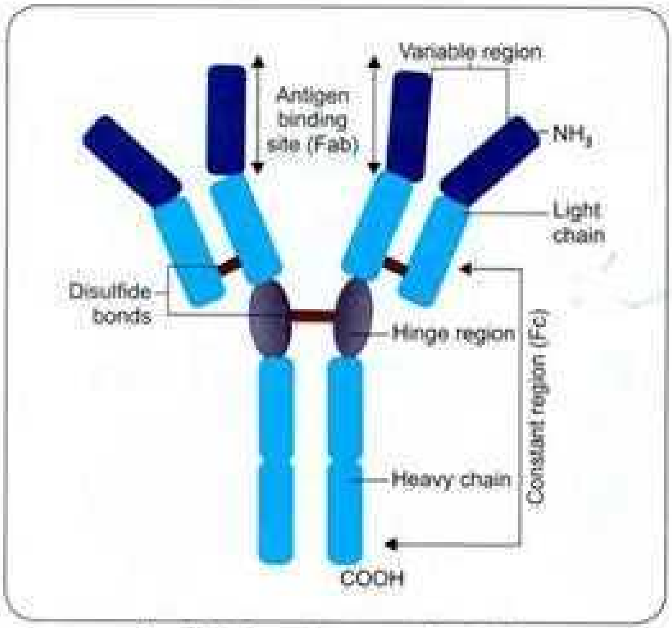


Fig. 11.2: General structure of antibody

Table. 11.1: Type of heavy chain in each immunoglobulin class

Immunoglobulin class	Heavy chain type
IgG	γ (gamma)
IgA	α (alpha)
IgM	μ (mu)
IgD	δ (delta)
IgE	ε (epsilon)

- All the chains have two ends—an amino terminal end (NH_2) and a carboxyl terminal end (COOH)
- There are five classes of H chains and two classes of light chains
- The five classes of H chains are structurally and antigenically distinct; each is designated by the Greek letters γ, α, μ, δ and ε and is present only in a particular class of Ig
- The five classes of immunoglobulins (IgG, IgA, IgM, IgD and IgE) are classified based on the amino acid sequences of the heavy chains (Table 11.1)
- The L chains are of two types—kappa (κ) and lambda (λ), named after Korngold and Lapari who originally described them
  - In humans, 60% of the light chains are kappa and 40% are lambda type (ratio 3:2)
  - Both the light chains of an antibody molecule are of same type, either κ or λ, but never both.
- L chains are composed of 214 amino acids; whereas the number of amino acids in the heavy chain varies—ranging from 446 (in α chain) to 576 (in μ chain).

Variable and Constant Regions

Each H and L chain comprises of two regions—variable and constant region, depending upon whether the amino acid sequences of the regions show variable or uniform pattern among different antibodies.

Variable Region

The first 110 amino acid residues near the amino terminal end (NH_2) of both L and H chains constitute the variable region—designated as V_L and V_H, respectively. It represents the antigen binding site of the antibody.

- **Hypervariable region:** Within the variable region, there are some zones (**hot spots**) that show relatively higher variability in the amino acid sequences. Such zones are called as hypervariable regions or complementarity determining regions (CDRs). They form the antigen—binding site. There are three hot spots in the L and four in the H chain, respectively
- **Paratope:** The site on the hypervariable regions that make actual contact with the epitope of an antigen is called as paratope.

Constant Region

It constitutes the remaining part of an Ig molecule other than that of variable region. The length of the constant regions is approximately 104 amino acids for light chain, 330 amino acids for γ, α and δ heavy chains and 440 amino acids for μ and ε heavy chains. The amino acid sequence of constant region shows uniform pattern. A single antibody molecule has two identical heavy chains and two identical light chains; H_2L_2.

Heavy and Light Chain Domains

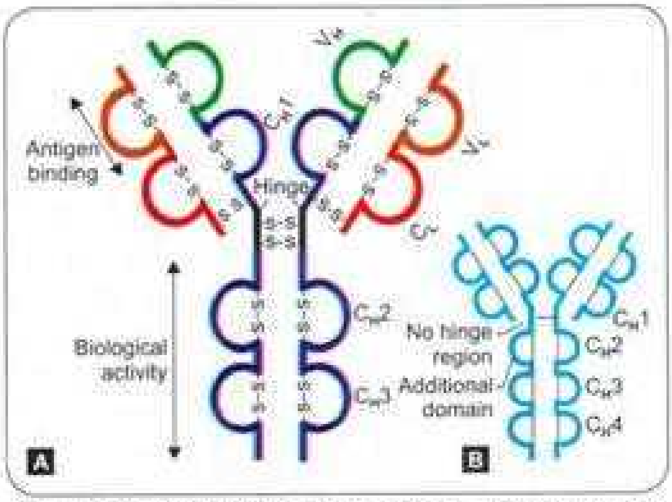
Heavy (H) and light (L) chains are further folded into domains, each containing about 110 amino acid residues. Within the domain, a loop like structure of 60 amino acids is present which is formed due to an intrachain disulfide bond. The number of domains in each chain varies:

- Light chain contains one variable domain (V_L) and one constant domain (C_L)
- Heavy chains possess one variable domain (V_H) and 3 or 4 numbers of constant domain (C_H):
  - Heavy chains γ, α and δ have three constant domains- C_H1, C_H2 and C_H3 (Fig. 11.3A)
  - Heavy chains μ and ε have four constant domains- C_H1 to C_H4 (Fig. 11.3B).

Hinge Region

In heavy chain (γ, α, and δ), the junction formed between C_H1 and C_H2 domain constitutes the hinge region (Fig. 11.3A).

- This region is rich in proline and cysteine. The hinge region is quite flexible, allowing the Ig molecule to assume different positions, thus helps the antibody in reaching towards the antigen



Figs 11.3A and B: Immunoglobulin domains. **A.** Ig with γ, δ and α heavy chains; **B.** Ig with μ and ε heavy chains

- In IgE and IgM, the ε and μ heavy chains do not have hinge region; instead, their constant region has an additional domain (C_H4) (Fig. 11.3B)
- The hinge region is sensitive to various enzymatic digestions.

Enzymatic Digestion

When an immunoglobulin molecule is subjected to enzymatic digestion, it generates various fragments (Fig. 11.4).

- **Papain digestion:** Papain cleaves the Ig molecule at a point above the disulfide bridge of hinge region; resulting in three fragments each having a sedimentation coefficient of 3.5 S:
  - **Two Fab fragments:** Soluble fragments which bind to the antigen (Fab for antigen binding fragment) and
  - **Fc fragment:** An insoluble fraction which gets crystallized in the cold (Fc for crystallizable fragment).
- **Pepsin digestion**: Pepsin cleaves the Ig molecule at a point below the disulfide bridge of hinge region; resulting in formation of:
  - **One F (ab')$_2$ fragment:** A fragment having a sedimentation coefficient of 5S; composed of two Fab subunits bound together
  - **Many smaller fragments:** Due to digestion of Fc portion by pepsin into smaller fragments.
- **Mercaptoethanol** reduction of Ig molecule—generates four fragments (two H and two L chains) as it cleaves only disulfide bonds sparing the peptide bonds.

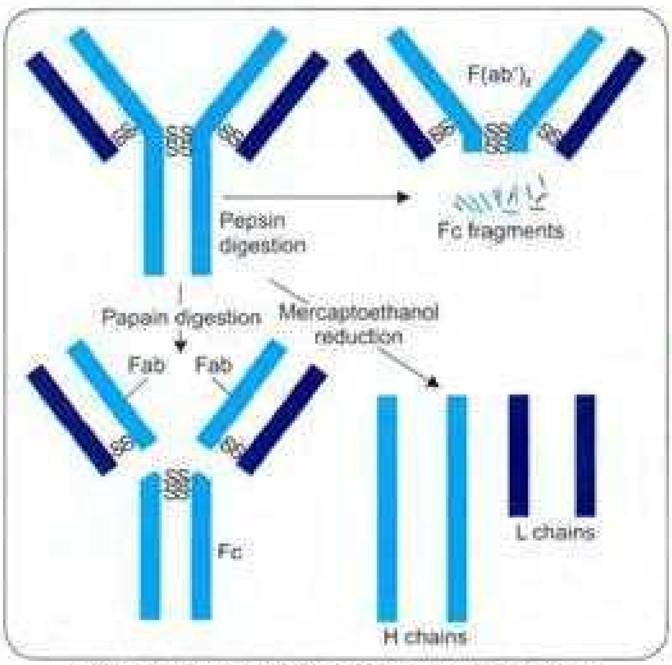


Fig. 11.4: Enzymatic digestion of immunoglobulin

FUNCTIONS OF IMMUNOGLOBULINS

Antigen Binding (by Fab Region)

Binding to the antigen is the primary function of an antibody which can result in protection of the host.

- The Fab fragment bears the variable region and is involved in interaction with the antigen
- The valency of an antibody refers to the number of Fab regions it possesses. Thus, a simple monomeric antibody molecule has a valency of two.

Effector Functions (by Fc Region)

Most of the times, the binding of an antibody to its antigen does not result in any direct biological effect. Rather, variety of secondary "effector functions" are produced; mediated by Fc region of the antibody. These effector functions include:

- **Fixation of complement:** Antibody coating the target cell binds to complement through its Fc receptor which leads to complement mediated lysis of the target cell
- **Binding to various cell types:** Phagocytes, lymphocytes, platelets, mast cells, NK cell, eosinophils and basophils bear Fc receptors (FcR) that bind to Fc region of immunoglobulins. This binding can activate the cells to perform some biological functions (described with individual immunoglobulins and also in Chapter 15). Some immunoglobulins (e.g. IgG) also bind to receptors on placental trophoblasts, which results in transfer of the IgG across the placenta.

IMMUNOGLOBULIN CLASSES

Based on five types of heavy chains, there are five classes of immunoglobulins (IgG, IgA, IgM, IgD and IgE). Each class can also exist as two types due to presence of different light chain type—kappa or lambda. IgG and IgA are further divided into subclasses (four for IgG and two for IgA) due to minor differences in amino acid sequences in constant region of heavy chains. Important properties of different Ig classes are summarized in Table 11.2.

Immunoglobulin G (IgG)

It constitutes about 70-80% of total Ig in the body.

- Among all Ig, IgG has maximum daily production, longest half-life of 23 days and highest serum concentration
- **IgG has four subclasses:** IgG1, IgG2, IgG3 and IgG4; all differ from each other in the amino acid sequences of the constant region of their γ-heavy chain
- The subclasses vary in their biological functions, length of hinge region and number of disulfide bridges. IgG3 has longest hinge region with 11 interchain disulfide bonds.

Table 11.2: Properties of various immunoglobulins

Property	IgG	IgA	IgM	IgD	IgE
Usual form	Monomer	Monomer, dimer	Monomer, pentamer	Monomer	Monomer
Valency	2	2 or 4	2 or 10	2	2
Other chains	None	J chain, secretory component	J chain	None	None
Subclasses	G1, G2, G3, G4	A1, A2	None	None	None
Molecular weight (kDa)	150	150–600	900	150	190
Serum level (mg/mL)	9.5–12.5	IgA 1–3.0 IgA 2–0.5	1.5	0.03	0.0003
% of total serum Ig	75–85%	10–15%	5–10%	0.3%	0.019%
Half-life, days	23*	6	5	3	2.5
Daily production (mg/kg)	34	24	3.3	0.4	0.0023
Intravascular distribution (%)	45	42	80	75	50
Sedimentation coefficient	7	7	19	7	8
Complement activation					
Classical	++ (IgG3>1>2)	–	+++	–	–
Alternate	–	+	–	–	–
Binds to Fc receptors of phagocytes	++	–	?**	–	–
Placental transfer	Yes (except IgG2)	–	–	–	–
Mediates coagglutination	Yes (except IgG3)	–	–	–	–
Mucosal transport	–	Yes	–	–	–
Mast cell degranulation	–	–	–	–	yes
Marker for B cells	–	–	+	+	–
Heat stability	+	+	+	+	–

*Half-life of IgG3 is 8 days; **?: Questionable

Functions of IgG

- IgG can **cross placenta**; hence provide immunity to the fetus and new born. Among subclasses, IgG2 has the poorest ability to cross placenta
- **Complement fixing:** Fc region of IgG can bind to complement factors; thus activates the classical pathway of complement system. The complement fixing ability of subclasses varies—IgG3> IgG1> IgG2. IgG4 does not fix complements
- **Phagocytosis:** IgG1 and IgG3 bind to Fc receptors present on phagocytes (macrophages, neutrophils) with high affinity and enhance the phagocytosis (opsonization) of antigen bound to them. IgG2 has an extremely low affinity for Fc receptors of phagocytes
- It mediates precipitation and neutralization reactions
- IgG plays a major role in neutralization of toxins as it can easily diffuse into extravascular space
- IgG is raised after long time following infection and represents chronic or past infection (recovery)
- **Coagglutination:** IgG subclasses (except IgG3) mediate coagglutination reaction by binding to protein-A of *S. aureus* (refer Chapters 12 and 21).

Immunoglobulin M (IgM)

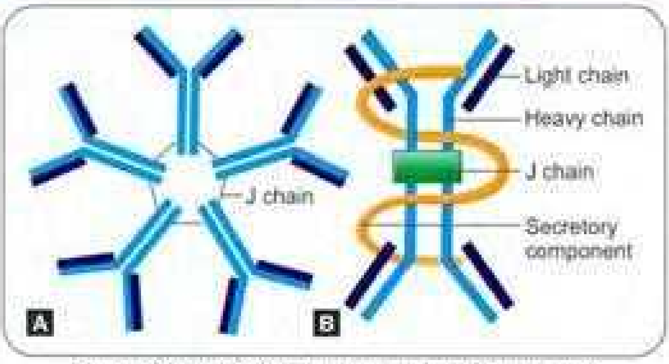
Among all immunoglobulins, IgM has highest molecular weight, and maximum sedimentation coefficient (19S). It is present only in intravascular compartment, not in body fluids or secretions.

IgM exists in both monomeric and pentameric forms:

- When present as membrane-bound antibody on B cells, it exists in monomeric form
- When present in secreted form, it is pentameric in nature; i.e. five IgM monomeric units are joined with each other (by J chain) to form a complete IgM pentamer having 10 Fab regions and 10 valencies (Fig. 11.5A).

Functions of IgM

- **Acute infection:** IgM is the first antibody to be produced following an infection; represents acute or recent infection. It is also called as primary immune response antibody
- **Complement fixing:** It is the most potent activator of classical complement pathway due to multiple complement binding sites (5 Fc regions) present in IgM pentamer



Figs 11.5A and B: A. Pentameric IgM; **B.** Dimeric IgA

- It is also present on B cell surface in monomeric form and serves as B cell receptor for antigen binding
- It acts as an **opsonin**; binds to antigen which is then easily recognized and removed. IgM is 500-1000 times more potent in opsonization than IgG
- **Fetal immunity:** It is the first antibody to be synthesized in fetal life (20 weeks); thus provides immunity to the fetus. Presence of IgM in fetus or newborn indicates intrauterine infection (as it cannot cross placenta), and its detection is useful in diagnosis of congenital infections
- **Protection against intravascular organisms:** IgM being intravascular, is responsible for protection against blood invasion by microorganisms. IgM deficiency is often associated with septicemia
- **Mediate agglutination:** IgM is about 20 times more effective in bacterial agglutination than IgG.

Immunoglobulin A (IgA)

IgA is the second most abundant class of Ig next to IgG, constituting about 10-15% of total serum Ig. It exists in both monomeric and dimeric forms (Fig. 11.5B).

Serum IgA

- IgA in serum is predominantly in monomeric form
- Functions: Serum IgA interacts with the Fc receptors expressed on immune effector cells, to initiate various functions such as antibody-dependent cell-mediated cytotoxicity (ADCC), degranulation of immune cells, etc.

Secretory IgA

Secretory IgA is dimeric in nature; the two IgA monomeric units are joined by **J chain.** In addition, there is another joining segment present between two IgA molecules called **secretory component.**

- **Location:** Secretory IgA is the predominant antibody found in body secretions like milk, saliva, tears, intestinal and respiratory tract mucosal secretions
- The secretory component is derived from poly-Ig receptor present on the serosal surfaces of the epithelial cells
- **Function:** The secretory IgA mediates **local or mucosal immunity**; provides protection against pathogens by cross-linking bigger antigens with multiple epitopes and preventing their entry through the mucosal surface
  - It is effective against bacteria like *Salmonella, Vibrio, Neisseria,* and viruses like polio and influenza
  - Breast milk is rich in secretory IgA and provides good protection to the immunologically immature infant's gut.

Secretory IgA

The dimeric secretory IgA is synthesized by plasma cells situated near mucosal epithelium. The J chain is also produced in the same cell.

Whereas, the secretory component is synthesized by the mucosal epithelial cells.

- It is derived from the poly-Ig receptor present on the basolateral surfaces of the epithelial cells
- It helps the dimeric IgA to cross the epithelial surface to reach the lumen
- It also protects IgA from denaturation by bacterial proteases produced by intestinal flora.

Subclasses of IgA

Depending upon the amino acid sequences in the constant region of heavy chain, IgA exists in two isotypes:

1. IgA1 is the dominant subclass in serum. Serum IgA comprises of ~ 90% IgA1 and 10% IgA2.
2. IgA2 is present in higher concentration in secretions than in serum (ranging from 10% to 20% in nasal and male genital secretions, 40% in saliva, to 60% in colonic and female genital secretions).
   - IgA2 lacks the disulfide bonds between the heavy and light chains
   - Polysaccharide antigens tend to induce more IgA2 synthesis than protein antigens.

Immunoglobulin E (IgE)

Among all Ig, IgE is having the lowest serum concentration, shortest half-life and minimum daily production. It is also the only heat labile antibody (inactivated at 56°C in one hour). It has affinity for the surface of tissue cells (mainly mast cells) of the same species (homocytotropism). It is mainly extravascular in distribution.

Functions of IgE

- IgE is highly potent and mediate **type I hypersensitivity** reactions by binding to the mast cells causing degranulation. IgE response is seen in various allergic conditions, such as asthma, anaphylaxis, hay fever, etc. (Described in detail in Chapter 16)
- IgE is elevated in **helminthic infections**. By coating on the surface of eosinophils, IgE stimulates the release of

the mediators on to the surface of helminths by a process known as antibody mediated cellular cytotoxicity or ADCC (Chapter 15).

Immunoglobulin D (IgD)

IgD is found as membrane Ig on the surface of B cells and acts as a B cell receptor along with IgM. It has the highest carbohydrate content among all the immunoglobulin. No other function of IgD is known so far.

ANTIGENIC DETERMINANTS OF IMMUNOGLOBULINS

Since antibodies are glycoproteins, they can themselves function as potent immunogens, having a number of antigenic determinants which can induce antibody responses in hosts other than the parent host. It is observed that the entire Ig molecule is not immunogenic, but it contains antigenic determinants at specific sites. Based on the location of antigenic determinants, the Ig molecules are divided into isotypes, idiotypes and allotypes (Fig. 11.6).

Isotypes

The five classes of Ig (IgG, IgA, IgM, IgD and IgE) and their subclasses are called as isotypes; they vary from each other in the amino acid sequences of the constant region of their heavy chains. Such variation is called as isotypic variation.

- Isotypes that are present in all members of a given species are similar in nature
- Hence, antibody against isotypes can be produced by injecting the Ig from one species into another.

Idiotypes

The unique amino acid sequence present in paratope region (in V_H and V_L regions) of one member of a species acts as antigenic determinant to other members of the same species.

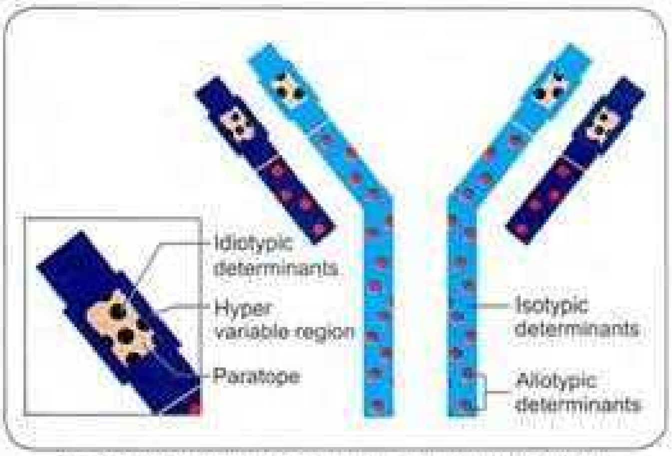


Fig. 11.6: Antigenic determinants of immunoglobulins

- Such antigenic determinants are called as idiotopes and the sum total of idiotopes on an Ig molecule constitutes its idiotypes (Fig. 11.6)
- Such variation between immunoglobulins due to differences in the amino acid sequences of the variable region is called as idiotypic variation
- Idiotypes in an individual arise continuously from mutations (somatic hypermutations) in the genes of variable region. Hence, idiotypes may act as foreign to the host itself; however, do not evoke autoimmune response because they are present in small numbers.

Allotypes

The antigenic determinants present in the isotype genes in the constant region of H and L chains, encoded by multiple alleles are called as allotypes.

- Although all members of a species inherit the same set of isotype genes, multiple alleles exist for some of the allele genes
- Hence, allotypes are present in the constant region of Ig molecules of the same class, in some, but not all, members of a species
- The sum of the individual allotypic determinants displayed by an antibody determines its allotype
- Allotypes differ in sequence of 1-4 amino acid from one another
- **Allotype systems:** To date, three systems of allotypic markers have been characterized for humans:
  - For kappa light chain (Km system)—has three Km allotypes
  - For γ heavy chain (Gm system)—has 25 Gm types
  - For α heavy chain (Am system).

Antibody to allotype determinants can be produced by injecting antibodies containing these determinants from one member to another within a given species. Anti allotype specific antibodies may also be developed following blood transfusion or by maternal passage of IgG into the fetus.

ABNORMAL IMMUNOGLOBULINS

In addition to the five classes of normal antibodies, other structurally similar proteins are seen in sera of patients and sometimes even in healthy individuals.

Bence Jones Proteins

They are produced in a neoplastic condition of plasma cells called multiple myeloma.

- This condition is also called as light chain disease as the cancerous plasma cells produce excess of light chains (Bence Jones proteins) which are accumulated in patient's serum and excreted in urine
- Such proteins have a unique property of getting coagulated at 50°C and redissolving again at 70°C.

Waldenstrom's Macroglobulinemia

It is a B cell lymphoma, producing excess IgM. It has been seen in multiple myeloma. Somatic mutations in MYD88 gene occur in over 90% of patients.

Heavy Chain Disease

It is characterized by an excessive production of heavy chains that are short and truncated. Four types of heavy chain disease have been recognized based on H chain involved:

1. α chain disease (Seligmann's disease)
2. γ chain disease (Franklin's disease)
3. μ chain disease
4. δ chain disease.

Cryoglobulinemia

It is a condition where the blood contains cryoglobulins; a type of Ig that becomes insoluble (precipitate) at low temperatures but redissolves again if the blood is heated.

- Cryoglobulins usually consist of IgM directed against the Fc region of IgG
- Cryoglobulins have been associated with multiple myeloma and hepatitis C infection.

MONOCLONAL ANTIBODY

Monoclonal antibodies (mAb) are defined as the antibodies derived from a single clone of plasma cell; all having the same antigen specificity, i.e. produced against a single epitope of an antigen.

Polyclonal vs Monoclonal Nature of Antibody

When an antigen having multiple epitopes enters the body, each epitope may stimulate one clone of B cells producing one type of antibody. Hence the resultant antibody mixture present in serum is said to be **polyclonal**, i.e. contains mixture of antibodies derived from different clones of B cells.

However, when only one clone of B cell is stimulated by a single epitope of an antigen and then is allowed to proliferate and produce antibodies; such antibodies are referred to as **monoclonal** antibodies (mAb).

Production of mAb (Hybridoma Technique)

Monoclonal antibodies are produced by Hybridoma technique, developed by G Kohler and C Milstein (1975), for which they were awarded Nobel Prize in 1984.

Principle

A clone of B cell stimulated against a single epitope of antigen is fused with an immortal cell, e.g. myeloma cell (capable of multiplying indefinitely) to produce a hybridoma cell. This hybridoma cell has two unique properties:

1. Produces monoclonal antibody of same antigen specificity (due to B cell component).
2. Multiplies indefinitely producing clone of identical cells (due to immortal myeloma cell component).

Procedure

The steps of hybridoma technique are as follows (Fig. 11.7):

- **Mouse splenic B cells:** The mouse is injected with an antigen containing the desired epitope. After an interval, the mouse splenic B cells are obtained which are activated against the epitope of the antigen injected.
- **Myeloma cells** are used as a source of immortal cells. They are cancerous plasma cells. They closely resemble

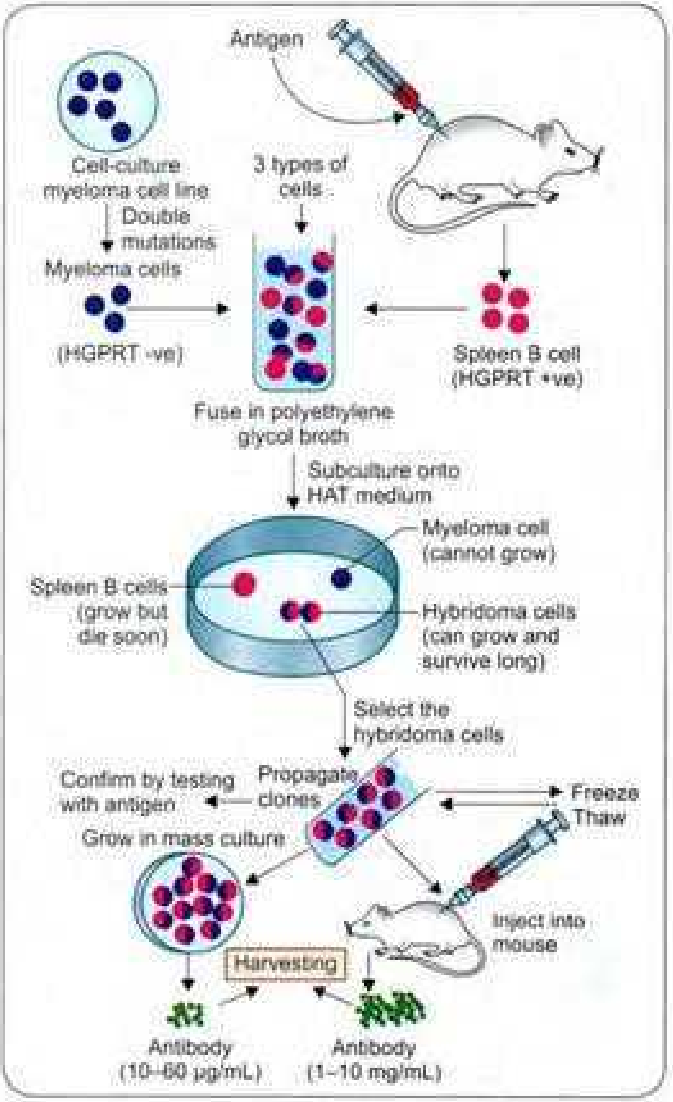


Fig. 11.7: Hybridoma technology

Abbreviation: HGPRT, hypoxanthine guanine phosphoribosyl transferase.

mouse B cells; hence are compatible for fusion. However, myeloma cells also have the capacity to produce their own antibodies. Hence myeloma cells are genetically modified with two mutations (double mutated myeloma cells), so that they lose the ability to produce their own antibody but retain immortal property

- **Fusion:** The mouse splenic B cells and mutated myeloma cells are fused in polyethylene glycol broth. In the reaction chamber, three types of cells will be generated:
  1. Unfused myeloma cells.
  2. Unfused mouse splenic B cells
  3. Fused hybridoma cells.
- **Purification** (by subculturing on HAT media): The next step is to remove the unwanted unfused cells and to propagate the clone of hybridoma cells. This is carried out by subculturing the cells in reaction chamber onto a special medium called HAT medium
- **HAT medium:** It contains hypoxanthine, aminopterin and thymidine
  - Purine synthesis in mammalian cell (e.g. splenic B cell) occurs by either *de novo* or salvage pathways
  - Aminopterin blocks the *de novo* pathway so that the cell has to perform the salvage pathway to synthesize purines for its survival
  - Salvage pathway requires two important enzymes-HGPRT (hypoxanthine guanine phosphoribosyl-transferase) and thymidine kinase
  - So any cell (e.g. myeloma cell) that lacks HGPRT cannot grow on HAT medium.
- **Fate of three types of cells on HAT media:**
  1. Unfused splenic B cells: They can grow, but do not survive long as they are not immortal.
  2. Unfused myeloma cells: They cannot grow as they lack HGPRT enzyme to perform the salvage pathway of purine synthesis.
  3. Hybridoma cells: They can grow and survive long.
- **Selection of individual hybridoma cells:** If the original antigen used has multiple epitopes, many B cells would fuse with myeloma cells to produce a mixture of hybridoma cells each having specificity for one epitope. The medium containing hybridoma cells is then diluted into multi-well plates to such an extent that each well contains only one cell. The hybridoma cells producing the desired monoclonal antibodies are selected by radioimmunoassay or enzyme-linked immunosorbent assay (ELISA) techniques using the specific antigen fragments, and are selectively proliferated
- **Maintenance of mAb:** The selected hybridoma cells can be maintained in two ways:
  1. Hybridoma cell is cultured to generate a clone of identical cells; producing pure form of monoclonal antibodies at a concentration of 10–60 μg/mL.
  2. Alternatively the desired hybridoma cell is injected into the peritoneal cavity of mouse where it can multiply and produce mAb in ascitic fluid at a concentration of 1-10 mg/mL. Such mAb obtained from mouse ascitic fluid and serum may not be in pure form, is often mixed with other antibodies; hence, it is purified by chromatography or by immunoprecipitation test.

Types of Monoclonal Antibodies

The above mentioned procedure would yield monoclonal antibodies whose 100% amino acids are mouse derived. The problem of mouse monoclonal antibody is that, the mouse proteins being foreign; can induce immune response in humans producing human anti-mouse antibodies (HAMA); that in turn eliminate the monoclonal antibodies faster from the body. Hence mouse derived monoclonal antibodies are not the best for human use. Since the discovery of hybridoma technique, various modifications have been attempted to produce monoclonal antibodies by recombining human and mouse proteins (Fig. 11.8).

- **Mouse mAb:** It contains 100% mouse derived proteins
- **Chimeric mAb:** It is prepared by recombination of 34% mouse proteins (variable region) and 66% human proteins (constant region)
- **Humanized mAb:** Here, only the antigen binding site (i.e. CDR—complementarity determining region) is mouse derived (10%) and the remaining part of mAb is human derived
- **Human mAb:** It contains 100% human derived amino acids. It is the best accepted mAb in humans.

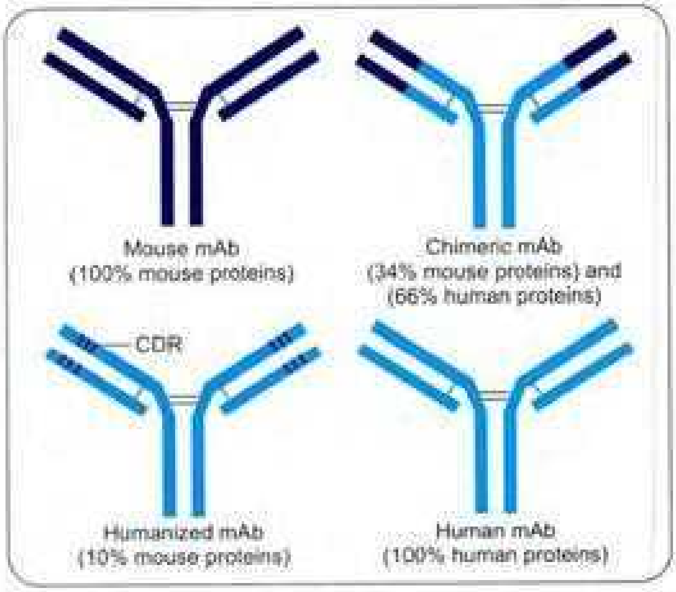


Fig. 11.8: Types of monoclonal antibodies

Applications of Monoclonal Antibodies

- **Isolation and purification:** Monoclonal antibodies can be used to purify individual molecule from a mixture when they are present in low concentration, e.g. interferon and coagulation factor VIII
- **Identification of cells and clones:** For example T_H and T_C cells are identified by using anti-CD4 and anti-CD8 mAb
- **Diagnostic reagents:** The widest application of mAb is detection of antigen. The antigen detection kits employ various mAb tagged with detection molecules, such as fluorescent dye or enzyme to detect the specific antigens in the clinical specimen. Examples include:
  - Detection of infections, such as hepatitis B, serogrouping of streptococci, etc.
  - Pregnancy detection test—by using monoclonal antibody against human chorionic gonadotropin
  - Blood grouping can be done by using anti-A and anti-B monoclonal antibodies
  - Tumor detection and imaging: By using mAb specific for tumor antigens secreted by tumor cells (e.g. prostate specific antigen)
  - Tissue typing for transplantation can be done by using anti-HLA monoclonal antibodies.
- **Monitoring** proteins and drug levels in serum
- **Passive immunity:** For post-exposure prophylaxis against various infections, mAb targeting specific antigens of the infecting organism can be administered. Examples include—immunoglobulins against hepatitis B, rabies, and tetanus
- **Therapeutic use:** Monoclonal antibodies are used in the treatment of various inflammatory and allergic diseases and cancers (Table 11.3). The mechanisms by which the mAb works as therapeutic agent are:
  - Suppresses immune system
  - Kills or inhibits malignant cells
  - Inhibits angiogenesis.
- **Used as immunotoxin:** mAb conjugated with bacterial/chemical toxins (e.g. diphtheria toxin) can be used to kill the target cells such as cancer cells. Here, mAb against surface receptors helps in binding to the target cells and the toxin helps in target cell killing
- **Used as enzymes: Abzyme** is a monoclonal antibody with catalytic activity.

Table 11.3: Therapeutic uses of monoclonal antibodies

Monoclonal antibody	Targeted against	Used in treatment of
Suppress immune system		
Adalimumab and infliximab	TNF-α	Rheumatoid arthritis Crohn's disease
Omalizumab	IgE	Asthma
Daclizumab	IL-2 receptor	Rejection of kidney transplants
Muromonab	CD3	
Anticancer		
Trastuzumab	HER-2	Breast cancer
Rituximab	CD20	Lymphoma
Inhibit angiogenesis		
Bevacizumab	VEGF (vascular endothelial growth factor)	Colorectal cancers
Abciximab	Platelet receptor GpIIb/IIIa	Coronary artery disease

Note:
- Mouse mAb ends with suffix 'umab or onab'
- Chimeric mAb ends with suffix 'ximab'
- Humanized mAb ends with suffix 'zumab'
- Human mAb ends with suffix 'mumab'

GENETICS OF ANTIBODY PRODUCTION

The mechanism of organization of Ig gene is unique and different from the classical 'one gene-one polypeptide' genetic model. In contrast, Ig polypeptide chains are coded by more than one gene. This concept was first introduced by Dreyer and Bennett. Later, it was proved by Tonegawa and Hozumi (1975) for which they were awarded Nobel Prize (1987).

Multigene Organization of Immunoglobulin

There are three basic principles of this model which are summarized below:

1. **Ig molecule is not coded by a single gene:** The two H and two L chains of an Ig molecule are coded by separate gene segments which are then joined later.
   - Heavy chain is coded by four gene segments: V (variable), D (diversity) and J (joining) and C (constant) gene segments. C_H gene region further consists of nine segments. The V_H, D_H and J_H gene segments collectively code for the variable region and the C_H gene segment codes for the constant region of H chain
   - Light chains are coded by three genes V, J and C gene segments. The D gene segment is absent. The V and J segments together code for variable region and C gene segment codes for the constant region of L chain. Kappa and lambda L chains are coded by two different sets of V, J and C genes.
2. **Ig genes are encoded in different chromosomes:**
   - H chain gene family is located on chromosome number 14
   - Kappa light chain gene family is located on chromosome number 2.

- Lambda light chain gene family is located on chromosome number 22.

3. **Multiple genes:** There are multiple genes existing for each genetic segment of Ig chain (described later under antibody diversity).

Rearrangement of Immunoglobulin Genes

The complete Ig molecule is formed by recombination between various gene segments. Gene rearrangement occurs at DNA and RNA levels.

Rearrangement at DNA Level

This involves rearrangement and splicing between the DNA segments of variable region of both H and L chains.

- H chain gene region undergoes rearrangement first followed by L chain gene region
- In H chain, V-D joining occurs first followed by VD-J joining; whereas in L chain, only V-J joining takes place. Recombination of gene segments occurs at the time of joining which is mediated by special **recombinase** enzymes, encoded by *RAG* (recombination activation genes)
- The C gene segments of both H and L chains are not joined at DNA level; but remain separate.

Rearrangement at RNA Level

The V, D, J and C gene segments are transcribed to generate primary RNA transcript. Then the C region RNA transcripts combine with variable region RNA transcripts to generate complete H and L chains.

- The whole C_H region is not transcribed simultaneously. First, the Cμ and Cδ genes are transcribed and combined with corresponding variable region RNA transcript to produce complete IgM and IgD respectively
- Based on class switch over of B cell, other C_H region genes are transcribed, to produce Ig of other classes (described later)
- **Differential Ig RNA processing:** It is an important event which occurs at post-translational level which is responsible for:
  - Directing the synthesis of immunoglobulin as membrane bound Ig or secretory Ig
  - Simultaneous expression of membrane Ig (IgM and IgD) on surface of mature B cells.

Antibody Diversity

Human immune system is capable of producing vast number of antibodies (10^8 or even more) corresponding to various epitopes of different antigens. With the understanding of genetics of Ig genes and their rearrangement; many possible mechanisms have been put forward to explain this diversity.

Mechanism of Antibody Diversity

- **Multiple genes for each segment coding for Ig chain:** Large number of different genes are known to exist for each genetic segment of Ig gene (Table 11.4). For example, there are 51 V_H genes known to exist in nature and every H chain would have been encoded by one out of 51 V_H genes
- **Many possible combinations of joining of variable region gene segments (Table 11.5 and Fig.11.9):** As at each gene segment multiple genes exist, there are several combinations possible by which joining of V/D/J genes can occur
- **Junctional flexibility:** It is postulated that V-DJ, D-J and V-J joining can take place at any level of several nucleotides present at the ends of V, D, J segments
- **Junctional diversity:** The V/D/J joining is a highly inaccurate process that results in the addition or subtraction of variable number of nucleotides and, thus, generates junctional diversity
- **Somatic hypermutation:** After formation of complete Ig molecule, still changes can occur in the nucleotide sequence of variable region. This usually occurs following antigenic stimulus to B cells in lymphoid follicles. The V region genes undergo point mutations (resulting from nucleotide substitutions) at a higher frequency (10^{-3} /bp/generation) than the normal (10^{-8} /bp/generation), hence named as hypermutation. This helps in affinity maturation of B cells.

Class Switch Over

Once B cell is stimulated by an antigen, heavy chain gene undergoes rearrangement where VDJ segment combines

Table 11.4: Multiple genes coding H and L chains of Ig

Gene segment	Number of genes		
	H chain	κ L chain	λ L chain
V	51	40	30
D	27	0	0
J	6	5	4
C	9	1	4

Table 11.5: Possible number of combinations of variable genes joining

Type of joining	Possible combinations
V-D-J combinations in heavy chain	$51V_H \times 27D_H \times 6J_H$ genes = 8262
V-J combinations in κ chain	40Vκ X 5Jκ genes = 200
V-J combinations in λ chain	30Vλ X 4Jλ genes = 120
Combinations of H and L chains	8262 X 200 X 120 = 2.64 X 10^8

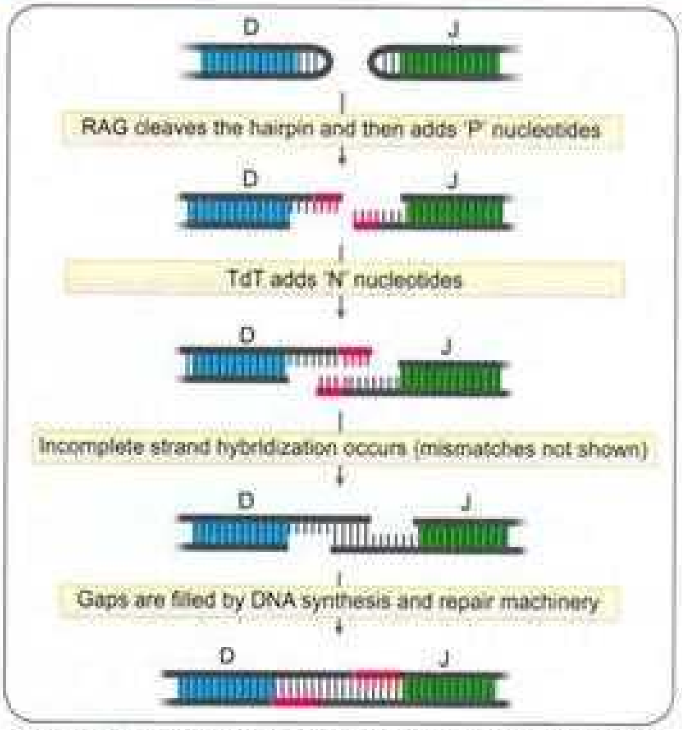


Fig. 11.9: Mechanisms of junctional diversity of gene coding for antibody

with one of the C_H gene segments; first with Cμ (forming IgM) followed by the others. This process is called class switching or isotype switching.

The classes and subclasses of Ig vary in their constant region of H chain. The C region of heavy chain contain nine segments each coding for a specific class or subclass of H chain. They are arranged in a specific sequence after the J chain. The sequence from the 5' end is as follows—5' → V → D → J → Cμ → Cδ → Cγ3 → Cγ1 → Cα1 → Cγ2 → Cγ4 → Cε → Cα2 → 3'

- Each C_H region genes except Cδ, contains a highly conserved DNA flanking sequence containing tandem repeats called as **switching site**
- It is proposed that there are class specific and subclass specific recombinase which are capable of binding to these switching sites, following which the corresponding C_H region gene and VDJ segment are joined together
- The class switch over helps the B cells to produce Ig of different class, retaining the same antigen specificity
- Class switch over is not a random phenomenon, but regulated by specific cytokines secreted by helper T cells (Described in detail in Chapter 15).

EXPECTED QUESTIONS

I. Essay:

1. Define antibody. Describe in detail about the structure and functions of various types of antibodies.

II. Write short notes on:

1. Idiotypes.
2. Monoclonal antibodies.
3. Abnormal immunoglobulins.

III. Multiple Choice Questions (MCQs):

1. **Which antibody crosses placenta?**
 a. IgA b. IgG c. IgE d. IgM
2. **Which is an example of surface Ig?**
 a. IgA b. IgG c. IgE d. IgM
3. **Ig heavy chain is encoded in which chromosome?**
 a. 2 b. 14 c. 22 d. 23
4. **What is the total valancies of IgM?**
 a. 10 b. 5 c. 2 d. 1
5. **Which antibody mediates type I hypersensitivity reaction?**
 a. IgA b. IgG c. IgE d. IgM
6. **Which antibody is elevated in parasitic infection?**
 a. IgA b. IgG c. IgE d. IgM
7. **Which antibody is called as reagin antibody?**
 a. IgA b. IgG c. IgE d. IgM
8. **Which antibody is heat labile?**
 a. IgA b. IgG c. IgE d. IgM
9. **Which is the most abundant antibody?**
 a. IgA b. IgG c. IgE d. IgM
10. **Which antibody is largely intravascular?**
 a. IgA b. IgG c. IgE d. IgM
11. **Which antibody is elevated in acute infection?**
 a. IgA b. IgG c. IgE d. IgM
12. **Which antibody is elevated in chronic infection?**
 a. IgA b. IgG c. IgE d. IgM
13. **Which antibody elevated in past infection?**
 a. IgA b. IgG c. IgE d. IgM
14. **Which antibody mediates precipitation reaction?**
 a. IgA b. IgG c. IgE d. IgM
15. **Which antibody mediates agglutination reaction?**
 a. IgA b. IgG c. IgE d. IgM
16. **Which antibody mediates ADCC (antibody-dependent cell mediated cytotoxicity)?**
 a. IgA b. IgG c. IgE d. IgM
17. **Which antibody mediates co-agglutination reaction?**
 a. IgA b. IgG c. IgE d. IgM
18. **Which antibody mediates mucosal immunity?**
 a. IgA b. IgG c. IgE d. IgM
19. **Which antibody is dimeric?**
 a. IgA b. IgG c. IgE d. IgM
20. **Secretory component is present in?**
 a. IgA b. IgG c. IgE d. IgM

Answers

1. b **2.** d **3.** b **4.** a **5.** c **6.** c **7.** c **8.** c **9.** b **10.** d **11.** d **12.** b **13.** b **14.** b **15.** d **16.** b **17.** b **18.** a **19.** a **20.** a

Antigen–Antibody Reaction

12

CHAPTER

Chapter Preview

- General properties of antigen–antibody reactions
- Types of antigen–antibody reactions
- Conventional immunoassays
  - Precipitation reaction
  - Agglutination reaction
  - Complement fixation test
  - Neutralization test
- Newer techniques
  - Enzyme-linked immunosorbent assay (ELISA)
  - Enzyme-linked fluorescent assay (ELFA)
  - Immunofluorescence assay (IFA)
  - Radioimmunoassay (RIA)
  - Chemiluminescence-linked immunoassay (CLIA)
  - Western blot
  - Rapid tests
  - Techniques using electron microscope

The antigen–antibody reaction is a bimolecular association where the antigen and antibody combine with each other specifically and in an observable manner similar to an enzyme-substrate interaction, the only difference is, it does not lead to an irreversible alteration in either antibody or in antigen.

GENERAL PROPERTIES OF ANTIGEN–ANTIBODY REACTIONS

Antigen (Ag)–antibody (Ab) reactions are characterized by the following general properties:

Specific

Ag-Ab reaction involves specific interaction of epitope of an antigen with the corresponding paratope of its homologous antibody. Exception is the cross reactions which may occur due to sharing of epitopes among different antigens. In such cases, antibody against one antigen can cross react with a similar epitope of a different antigen.

Noncovalent Interactions

The union of antigen and antibody requires formation of large number of non-covalent interactions between them such as:

- Hydrogen bonds
- Electrostatic interactions
- Hydrophobic interactions
- Van der Waals forces.

Strength

The strength or the firmness of the association is influenced by the affinity and avidity of the antigen–antibody interaction.

Affinity

It refers to the sum total of noncovalent interactions between a single epitope of an antigen with its corresponding paratope present on antibody. Affinity can be measured by two methods: (1) by equilibrium dialysis and (2) by surface plasmon resonance method.

Avidity

It is a term used to describe the affinities of all the binding sites when multivalent antibody reacts with a complex antigen carrying multiple epitopes.

- The total strength (i.e. avidity) would be much higher than the individual affinity at each binding site, but lower than the sum of all affinities. This difference is primarily due to geometry of Ag-Ab binding
- The geometry of the multivalent antibody gets stretched when it reacts with a complex antigen, as it has to reach and accommodate all the epitopes, thus resulting in less optimal binding interactions
- Avidity is a better indicator of strength of an antigen-antibody reaction. Avidity of an antibody can compensate for its low affinity. For example, IgM has a low affinity than IgG, but it is multivalent (10 valencies), therefore has a much higher avidity. Hence, it can bind to an antigen more effectively than IgG

- Avidity increases with time: Though IgG has a low avidity initially, IgG produced in later part of infection will have stronger avidity. This property is used in IgG avidity test; described later in this chapter.

Diagnostic Use

Because Ag-Ab reactions are specific and observable, they are extensively used in the laboratories for the diagnosis of infectious diseases. The diagnostic tests based on Ag-Ab reactions are called as immunoassays. Most immunoassays are also called serological tests as they are performed in serum samples. However, other samples can also be used such as urine, CSF, etc. Immunoassays can be broadly categorized into two types:

1. **Antigen detection assays:** Detect antigens in patient's sample by employing specific antibody.
2. **Antibody detection assays:** Detect antibodies in patient's sample by employing specific antigen.

Qualitative vs Quantitative Immunoassays

Immunoassays can be performed by both qualitative and quantitative methods.

Qualitative Assays

Here, the undiluted specimen containing the antibody is directly mixed with the suspension of antigen or vice versa. The result is read as 'positive' or 'negative' based on presence or absence of antigen or antibody in the clinical specimen. The exact amount of antigen or antibody present in the specimen will not be known.

Quantitative Assays

When the qualitative test turns positive, the exact amount of antibody in serum can be known by serial dilution of the patient's serum and mixing each dilution of the serum with a known quantity of antigen. The measurement of antibody is expressed in terms of titer.

- The **antibody titer** of a serum is the highest dilution that shows an observable reaction with the antigen
- Antigen titer can also be measured in the sera in similar fashion by testing the series of diluted sera against known quantity of antibody.

The problem with qualitative test is that if the number of antigen or antibody molecules in the reaction are disproportionate to each other and if either antigen or antibody are present in higher quantity, then the antigen antibody reaction does not take place optimally and often the result turns negative (false-negative). To rule out a false negative result, it is ideal to test a series of diluted sera (quantitative test), instead of just testing once the undiluted serum. Quantitative tests are more reliable as they can differentiate between true negative and false-negative results. This can be explained by Marrack's Lattice hypothesis.

Marrack's Lattice Hypothesis

When the sera containing antibody is serially diluted (in normal saline), gradually the antibody level decreases. When a fixed quantity of antigen is added to such a set of test tubes containing serially diluted sera, then it is observed that the Ag-Ab reaction occurs at its best only in the middle test tubes where the amount of antigen and antibody are equivalent to each other (*zone of equivalence*). The Ag-Ab reaction is weak or fails to occur when the number of antigen and antibodies are not proportionate to each other (Figs 12.1A to C).

- In the earlier test tubes, *antibodies are excess*, hence the Ag-Ab reaction does not occur: This is called as **prozone phenomenon**
- In the later test tubes, *antigen is excess*, hence the Ag-Ab reaction fails to occur: This is called as **postzone phenomenon**.

Marrack (1934) proposed the **lattice hypothesis** to explain this mechanism. According to this concept the multivalent antigens combine with bivalent antibodies in varying proportions, depending on the antigen antibody ratio in the reacting mixture (Figs 12.1A to C).

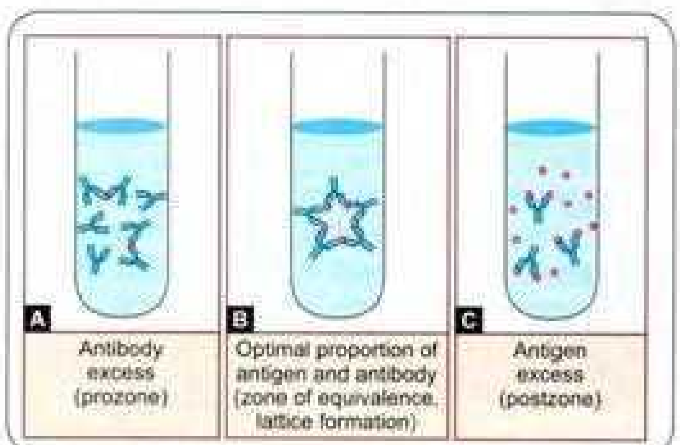
- Ag-Ab reaction optimally occurs when a large lattice is formed consisting of alternating antigen and antibody molecules. This is possible only in the zone of equivalence
- In the zones of antibody or antigen excess (prozone/post zone), the lattice does not enlarge, due to inhibition of lattice formation by the excess antibody or antigen respectively.

Lattice hypothesis was described first for precipitation reaction, but it also holds true for agglutination and other techniques of Ag-Ab reactions.

The prozone phenomenon is of great importance in clinical serology, as sera rich in high titer of antibody may sometimes give a false-negative result, unless serial dilutions of sera are tested.

Evaluation of Immunoassays

Evaluation of the performance of any diagnostic test including immunoassays can be done by calculating various statistical measures. Among all, sensitivity and specificity are the two most important statistical parameters.



Figs 12.1A to C: A. Prozone; **B.** Zone of equivalence; **C.** Postzone

Table 12.1: Types of antigen-antibody reactions

Conventional techniques
• Precipitation reaction
• Agglutination reaction
• Complement fixation test
• Neutralization test
Newer techniques
• Enzyme-linked immunosorbent assay (ELISA)
• Enzyme-linked fluorescent assay (ELFA)
• Immunofluorescence assay (IFA)
• Radioimmunoassay (RIA)
• Chemiluminescence-linked immunoassay (CLIA)
• Immunohistochemistry
• Rapid tests ➢ Lateral flow assay (Immunochromatographic test) ➢ Flow through assay
• Western blot
• Immunoassays using electron microscope

Sensitivity is defined as ability of a test to identify correctly all those who have the disease, i.e. true-positives.

$$\text{Sensitivity is calculated as} = \frac{\text{True positives}}{\text{True positives + False negatives}}$$

Specificity is defined as ability of a test to identify correctly all those who do not have disease, i.e. true negatives.

$$\text{Specificity is calculated as} = \frac{\text{True negatives}}{\text{True negatives + False positives}}$$

TYPES OF ANTIGEN-ANTIBODY REACTIONS

The antigen-antibody reactions used in diagnostic laboratories are based on various techniques which are broadly classified as conventional or old techniques and newer techniques (Table 12.1).

CONVENTIONAL IMMUNOASSAYS

PRECIPITATION REACTION

Definition

When a **soluble antigen** reacts with its antibody in the presence of optimal temperature, pH and electrolytes (NaCl), it leads to formation of the antigen-antibody complex in the form of:
- ❖ Insoluble precipitate band when gel containing medium is used or
- ❖ Insoluble floccules or precipitate ring when liquid medium is used.

Precipitation in Liquid Medium

- ❖ **Ring test:** In a narrow tube (e.g. capillary tube), antigen solution is layered over an antiserum; a precipitate ring appears at the junction of the two liquids. Examples of ring tests are streptococcal grouping by Lancefield technique, and Ascoli's thermoprecipitin test done for anthrax
- ❖ **Flocculation test:** When a drop of antigen is mixed with a drop of patient's serum, then the precipitate formed remains suspended as floccules. This test can be done on a slide or in a tube
  - ▪ Examples of slide flocculation test: VDRL (Venereal Disease Research Laboratory) and RPR (Rapid Plasma Reagin) tests used for diagnosis of syphilis
  - ▪ Examples of tube flocculation test: Kahn test used previously for syphilis.

Precipitation in Gel (Immunodiffusion)

Incorporation of 1% soft agarose gel in precipitation reaction has many advantages over liquid medium:
- ❖ It results in formation of clearly visible **bands** instead of floccules, which can be preserved for longer time
- ❖ It can differentiate individual antigens from a mixture, as each antigen forms a separate band after reacting with specific antibody.

Immunodiffusion tests are based on two principles:
1. Whether only Ag diffuses (single diffusion) or both Ag and Ab diffuse (double diffusion).
2. Whether Ag or Ab diffuses in *one dimension* (i.e. vertical diffusion when test is done in a tube layered with gel) or **two dimensions** (i.e. diffusion in both X and Y axis, which occurs when the test is done on a slide or a petri dish layered with gel).

There are four types of immunodiffusions in gel:
1. **Single diffusion in one dimension (Oudin procedure):** When antigen solution is poured over a layer of gel containing antibody in a test tube, only the antigen diffuses in one direction towards antibody to form a band (Fig. 12.2).
2. **Double diffusions in one dimension (Oakley-Fulthorpe procedure):** In the above test, if a column of plain agar is placed between the antigen layer and

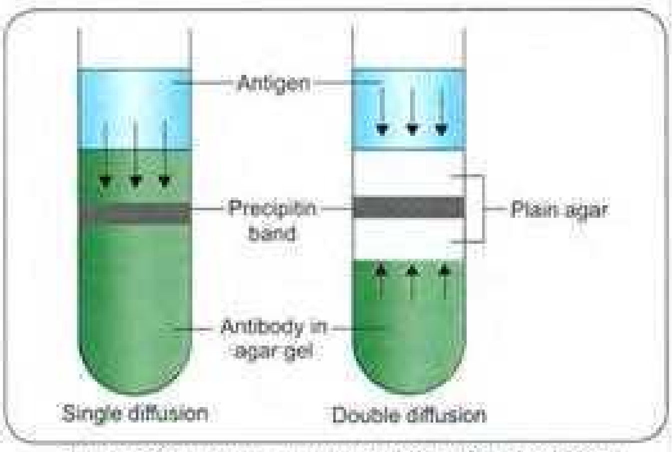


Fig. 12.2: Single and double diffusion in one dimension

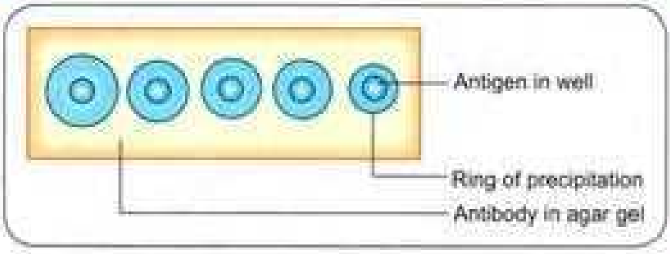


Fig. 12.3: Single diffusion in two dimensions

the layer of gel incorporated with antibody; then both antigen and antibody move towards each other in opposite directions and the precipitate band is formed at the line they meet in the plain agar (Fig. 12.2).

3. **Single diffusion in two dimensions (Radial immunodiffusion):** Gel incorporated with antibody is placed on a slide and several wells are cut. When drops of antigen are placed in the wells, they diffuse radially in all directions to meet with antibody and ring-shaped bands are formed around the well (Fig. 12.3). The diameter of the ring is directly proportional to the concentration of the antigen in the well.
4. **Double diffusions in two dimensions (Ouchterlony procedure):** On a slide poured with agar gel, antibody is placed in a central well and the surrounding wells are filled with different antigens. Both antigen and antibody move towards each other in all the directions and form bands (Fig. 12.4).

   The adjacent precipitate bands interact in 3 ways:
   - They may **fuse** with each other (indicates both antigens are identical)
   - They may **cross** one another (indicates antigens are unrelated)
   - They may cause **spur** formation (indicates cross reaction or partial identity) (Fig. 12.4).

Examples of double diffusions in two dimensions include—Elek's test for detecting toxin of *Corynebacterium diphtheriae* and Eiken test to detect toxin of *Escherichia coli*.

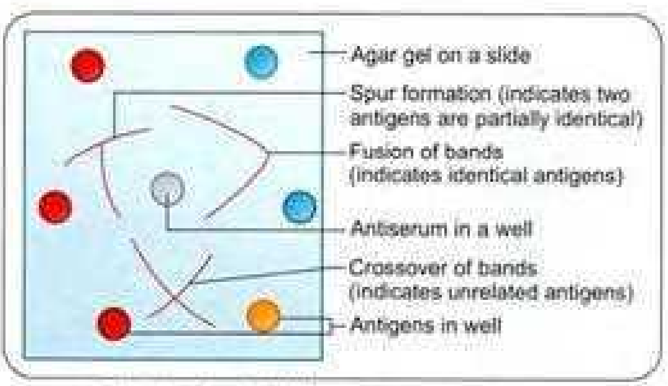


Fig. 12.4: Double diffusion in two dimensions

Precipitation in Gel in Presence of Electric Current

The problem with immunodiffusion in gel is that, antigen and antibody move very slowly in a gel, hence it takes several days to finish the procedure. Their movement can be made faster if immunodiffusion is carried out in presence of electric current.

Electroimmunodiffusion (EID)

When electric current is applied to a slide layered with gel, the serum proteins (antigen mixture) placed in a well are separated into individual antigen components. Antiserum present in a trough moves towards the antigen components resulting in formation of separate precipitin lines in 18–24 hrs, each indicating reaction between individual proteins with its antibody (Fig. 12.5). This test helps in identification and approximate quantitation of various proteins present in the serum.

Countercurrent Immunoelectrophoresis (CIEP)

This test is even faster (takes 30 minutes) and more sensitive than EID because it involves simultaneous electrophoresis of the antigen and the antibody in gel in opposite directions resulting in band formation (Fig. 12.6). This test was very popular in the past, for detecting various antigens such as alpha fetoprotein in serum and capsular antigens of *Cryptococcus* and meningococcus in the cerebrospinal fluid.

Rocket Electrophoresis

This is an one-dimensional single electroimmunodiffusion test, mainly done in the past for quantitative estimation of antigens (Fig. 12.7).

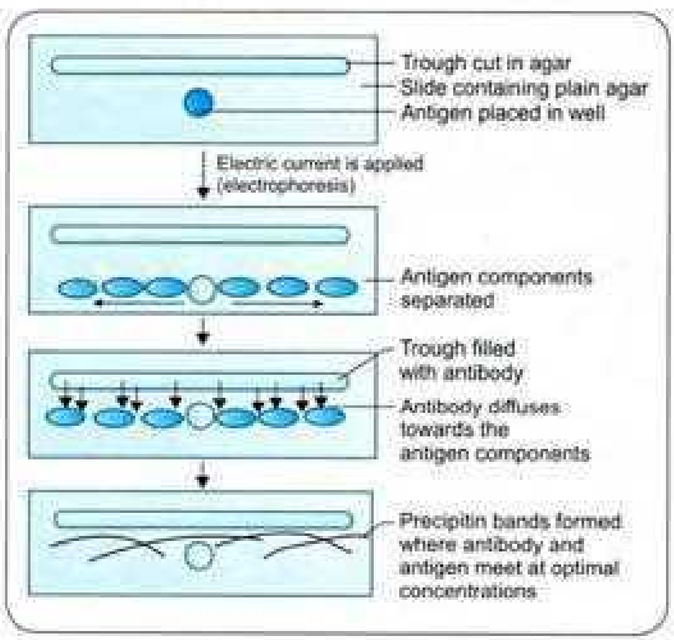


Fig. 12.5: Electroimmunodiffusion (EID)

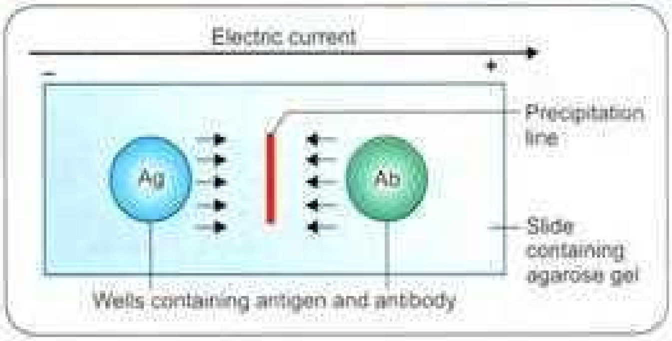


Fig. 12.6: Countercurrent immunoelectrophoresis

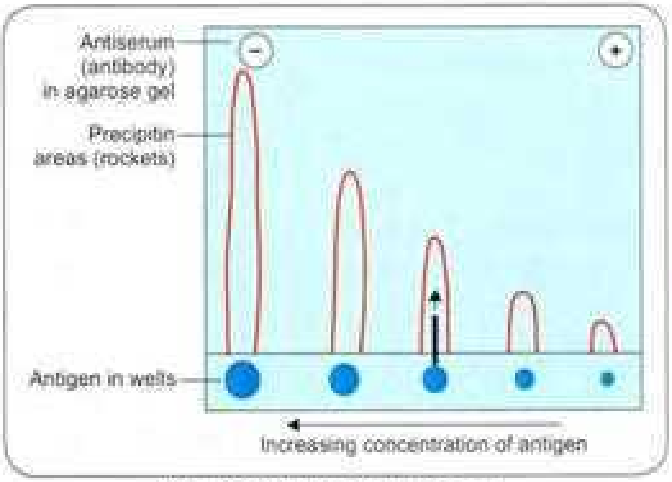


Fig. 12.7: Rocket electrophoresis

Once upon a time precipitation reactions were one of the widely used serological tests; however with the advent of simple and rapid newer techniques their application is greatly reduced, except the VDRL test which is still used for the diagnosis of syphilis.

AGGLUTINATION REACTION

Definition

When a **particulate** or **insoluble** antigen is mixed with its antibody in the presence of electrolytes at a suitable temperature and pH, the particles are clumped or agglutinated.

- **Advantage:** Agglutination is more sensitive than precipitation test and the clumps are better visualized and interpreted as compared to bands or floccules. Hence, agglutination tests are widely used even in today's modern era of diagnosis
- **Applications:** Agglutination reactions are classified as direct, indirect (passive) and reverse passive agglutination reactions. All these agglutination tests are performed either on a slide, or in tube or in card or some time in microtiter plates.

Direct Agglutination Test

Here, the antigen directly agglutinates with the antibody.

Slide Agglutination

It is usually performed to confirm the identification and serotyping of bacterial colonies grown in culture. It is also the method used for blood grouping and cross matching.

Bacterial colony is mixed with a drop of saline on a slide to form a uniform smooth milky white suspension

↓

To this, a drop of the antiserum (serum containing appropriate antibody) is added and the slide is shaken thoroughly (manually or by rotator) for few seconds

↓

A positive result is indicated by visible clumping with clearing of the suspension (Fig. 12.8)

or

If the milky white suspension remains unchanged, indicates a negative result (Fig. 12.8)

Tube Agglutination

This is a quantitative test done for estimating antibody in serum. The **antibody titer** can be estimated as the highest dilution of the serum which produces a visible agglutination.

A fixed volume of a particulate antigen suspension is added to an equal volume of serial dilutions of a serum sample (containing appropriate antibody) in test tubes

↓

Positive test indicates agglutination (clump formation at the bottom of the tube with clearing of the supernatant)

or

Negative test indicates agglutination has not occurred (Ag suspension forms button at the bottom of the tube)

Tube agglutination is routinely used for the serological diagnosis of various diseases, such as:

Fig. 12.8: Slide agglutination test

Source: Department of Microbiology, JIPMER, Puducherry (with permission).

- Typhoid fever (Widal test): It detects antibodies against both H (flagellar) and O (somatic) antigens of *Salmonella* Typhi
  - H antigen-antibody clumps appear as loose fluffy clumps
  - O antigen-antibody clumps appear as chalky white granular dense deposits
- Acute brucellosis (Standard agglutination test)
- Coombs antiglobulin test (explained later in this chapter)
- Heterophile agglutination tests:
  - Typhus fever (Weil Felix reaction)
  - Infectious mononucleosis (Paul Bunnell test)
  - *Mycoplasma* pneumonia (Cold agglutination test).

Microscopic Agglutination

Here, the agglutination test is performed on a microtiter plate and the result is read under a microscope. The classical example is microscopic agglutination test (MAT) done for leptospirosis.

Indirect or Passive Agglutination Test (for Antibody Detection)

As agglutination test is more sensitive and better interpreted than precipitation test, attempt has been made to convert a precipitation reaction into an agglutination reaction. This is possible by coating the soluble antigen on the surface of a carrier molecule (e.g. RBC, latex or bentonite), so that the antibody binds to the coated antigen and agglutination takes place on the surface of the carrier molecule.

Indirect Hemagglutination Test (IHA)

It is a passive agglutination test where RBCs are used as carrier molecules. IHA was used widely in the past, but is less popular at present (Refer author's first edition for detail).

Latex Agglutination Test (LAT) for Antibody Detection

Here, polystyrene latex particles (0.8–1 μm in diameter) are used as carrier molecules which are capable of adsorbing several types of antigens. For better interpretation of result, the test is performed on a black color card.

- Drop of patient's serum (containing antibody) is added to a drop of latex solution coated with the antigen and the card is rotated for uniform mixing
- Positive result is indicated by formation of visible clumps (Fig. 12.9). LAT is one of the most widely used tests at present as it is very simple and rapid
- It is used for detection of ASO (antistreptolysin O antibody).

Reverse Passive Agglutination Test (for Antigen Detection)

In this test, the antibody is coated on a carrier molecule which detects antigen in the patient's serum.

- **Reverse passive hemagglutination assay (RPHA):** Here, the RBCs are used as carrier molecules. RPHA was used in the past for detection of hepatitis B surface antigen (HBsAg); now obsolete
- **Latex agglutination test for antigen detection:** It is used widely for detection of CRP (C reactive protein), RA (rheumatoid arthritis factor), capsular antigen detection in CSF (for pneumococcus, meningococcus and *Cryptococcus*) and streptococcal grouping
- **Coagglutination test:** Here, *Staphylococcus aureus* (protein A) acts as carrier molecule. This test was used in past to detect antigen from clinical specimens; now obsolete (Refer author's first edition for detail).

Hemagglutination Test

It refers to the agglutination tests that use RBCs as source of antigen. Hemagglutination tests are of two types: direct (described below) and indirect (or IHA, obsolete now).

Direct Hemagglutination Test

Serum antibodies directly agglutinate with surface antigens of RBCs to produce a matt. Examples include:

- **Paul Bunnell test:** It employs sheep RBCs as antigens to detect Epstein-Barr virus antibodies in serum. The test is performed in tubes
- **Cold agglutination test:** It uses human RBCs as antigens to detect *Mycoplasma* antibodies in serum. Test is performed in tubes
- **Blood grouping** (ABO and Rh grouping)
- **Coombs test** or **antiglobulin test:** It is performed to diagnose Rh incompatibility by detecting Rh antibody from mother's and baby's serum.
  - Rh incompatibility is a condition when an Rh negative mother (Rh Ag -ve and Rh Ab -ve) delivers a Rh positive baby (Rh Ag +ve and Rh Ab -ve). During birth, some Rh Ag +ve RBCs may pass from fetus to the maternal circulation and may induce Rh Ab formation in the mother and affect future Rh positive pregnancies

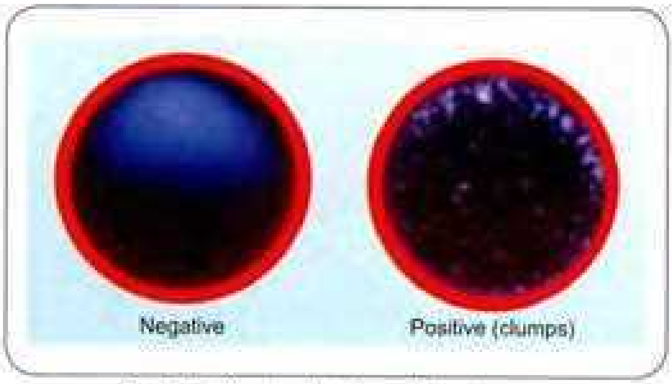


Fig. 12.9: Passive (latex) agglutination test

Source: Department of Microbiology, JIPMER, Puducherry (with permission).

- **Rh antibodies** are **incomplete** or **blocking** antibodies of IgG type. They can cross placenta and bind to Rh Ag on fetal RBCs. However, that does not result in agglutination; instead, they block the sites on fetal RBCs
- Such reaction can be visualized by adding **Coombs reagent** (antiglobulin or antibody to human IgG) which can bind to Fc portion of Rh Ab bound on RBCs, resulting in visible agglutination
- Coombs test may be of two types (Fig. 12.10):
    1. **Direct Coombs test:** It detects Rh antibodies bound to RBCs *in vivo*, present in fetus/baby's serum by directly adding Coombs reagent.
    2. **Indirect Coombs test:** It detects free Rh antibodies present in maternal serum after the first delivery of an Rh +ve fetus/baby. The free Rh Abs are mixed with the reagent containing Rh +ve RBCs (*in vitro*), following which the Coombs reagent is added.

Viral Hemagglutination Test

In strict sense, it is not an antigen-antibody reaction. The hemagglutinin antigens (HA) present on surface of some viruses (hemagglutinating viruses, e.g. influenza virus) can agglutinate with the receptors present on the surface of RBCs.

Technical Issues in Agglutination Reactions

Two main problems pertaining to agglutination are prozone phenomenon and blocking antibody; both can cause false-negative agglutination test.

- **Prozone phenomenon:** Serum containing excess antibodies may fail to agglutinate with its antigen. This can be obviated by serial dilution of the serum and testing the antigen with each dilution of the serum sample
- **Blocking antibodies:** They are incomplete IgG antibodies. When they bind to antigens, they themselves cannot produce a visible agglutination; however, they can block the sites on antigens, thus prevent binding of any other antibodies to the antigens. Such blocking antibodies may be detected by performing the test in hypertonic (4%) saline or more reliably by adding antiglobulin or Coombs reagent.

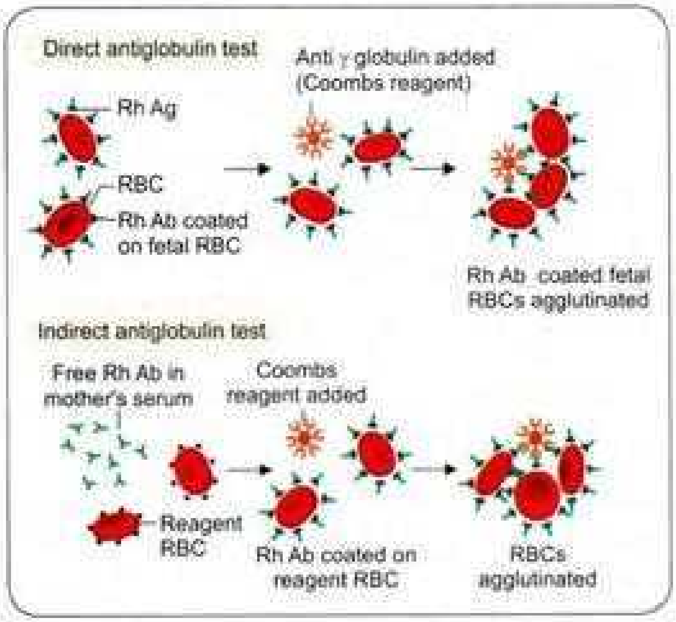


Fig. 12.10: Coombs or antiglobulin test

COMPLEMENT FIXATION TEST

Complement fixation test (CFT) detects the antibodies in patient's serum that are capable of fixing with complements. It was once very popular, now is almost obsolete (Refer author's first edition for detail).

Applications

- **Wasserman test** was the most popular CFT, used for the diagnosis of syphilis
- In addition, CFT was also widely used for detection of complement fixing antibodies in *Rickettsia*, *Chlamydia*, *Brucella*, *Mycoplasma* infections and some viral infections, such as arboviruses, rabies, etc.
- Complements are also used for various serological tests other than CFT, such as:
    - *Treponema pallidum* immobilization test
    - Sabin-feldman dye test for *Toxoplasma*
    - Vibriocidal antibody test.

NEUTRALIZATION TEST

Neutralization tests are also less commonly used in modern days. Various examples are as follows:

- **Viral neutralization test:** It detects the presence of neutralizing antibody in patient's serum. When the serum is mixed with a live viral suspension and poured onto a cell line, specific serum antibody neutralizes the surface antigen, making the virus unable to infect a cell line
- **Plaque inhibition test:** This is done for bacteriophages
- **Toxin-antitoxin neutralization test:** Examples include
    - **Schick test:** It is a diphtheria toxin-antitoxin neutralization test
    - **Nagler's reaction:** It is used for detection of α-toxin of *Clostridium perfringens*. (Chapter 26)
    - **ASO test:** Antistreptolysin O antibody was detected before by neutralization method; however, it is now replaced by latex agglutination.
- **Hemagglutination inhibition (HAI) test:** Antibodies in patient's sera can agglutinate with the hemagglutinin antigens present on the surfaces of some viruses. This test was used in past for the diagnosis of various viral

diseases, e.g. influenza. The test principle is described in Chapter 44 of author's first edition.

NEWER TECHNIQUES

The newer techniques use a detector molecule to label antibody or antigen which in turn detects the corresponding antigen or the antibody in the sample by producing a visible effect. Most of the newer techniques use the same principle, but they differ from each other by the type of labeled molecule used and the type of visible effect produced (Table 12.2).

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Enzyme immunoassay (EIA) is a term used to describe all the tests that detect either antigen or antibodies or haptens in the specimen, by using enzyme-substrate system for detection. They can be classified as below:

- **Homogenous EIA:** All reagents are added at one step. These tests are used for detection of haptens such as drugs (e.g. opiates, cocaine), but not for detection of microbial antigens and antibodies
- **Heterogeneous EIA:** It involves multiple steps with different reagents being added at every step. They are used for detection of antigens and antibodies. ELISA is a classical example.

Principle of ELISA

ELISA is so named because of its two components:

- **Immunosorbent:** Here, an absorbing material is used (e.g. polystyrene, polyvinyl) that specifically absorbs the antigen or antibody present in serum

Table 12.3: Enzymes used in ELISA and their substrate-chromogen system

Enzyme	Substrate	Chromogen
Horseradish Peroxidase	Hydrogen peroxide	Tetramethyl benzidine (TMB)
Urease	Urea	Bromocresol
β-Galactosidase	ONPG*	ONPG*
Alkaline Phosphatase	pNPP**	pNPP**

*ONPG (o-nitrophenyl-D-galactopyranoside) is cleaved by the enzyme β-galactosidase into galactose and o-nitrophenol.

**para-Nitrophenyl phosphate is a chromogenic substrate, converted to para-Nitrophenol (color product).

- **Enzyme** is used to label one of the components of immunoassay (i.e. antigen or antibody).

Substrate-chromogen system: A substrate-chromogen system is added at the final step of ELISA.

- The enzyme reacts with the substrate, which in turn activates the chromogen to produce a color
- Sometime, the substrate is chromogenic in nature (e.g. pNPP). On reaction with the enzyme, it changes its color (Table 12.3)
- The color change is detected by spectrophotometry in an ELISA reader. Intensity of the color is directly proportional to the amount of the detection molecule (Ag or Ab) present in test serum.

(Ag-Ab complex)-enzyme + substrate → activates the chromogen → color change → detected by spectrophotometry (ELISA reader, Fig. 12.11A)

ELISA is usually performed on a microtiter plate containing 96 wells (micro-ELISA) (Fig. 12.13) or less commonly performed in tubes (macro-ELISA). The

Table 12.2: Immunoassays and the types of molecule used for labeling

Abbreviation	Immunoassay method	Molecules used for labeling	Type of visible effect
ELISA	Enzyme-linked immunosorbent assay	Enzyme-substrate-chromogen complex	Color change is detected by spectrophotometer
ELFA	Enzyme-linked fluorescent assay	Enzyme-substrate	Fluorometric detection
IFA	Immunofluorescence assay	Fluorescent dye	Emits light, detected by fluorescence microscope
RIA	Radioimmunoassay	Radioactive isotope	Emits β and γ radiations, detected by β and γ counters
CLIA	Chemiluminescence-linked immunoassay	Chemiluminescent compounds	Emits light, detected by luminometer
IHC	Immunohistochemistry	Enzyme or fluorescent dye	Color change (naked eye) or fluorescence microscope
WB	Western blot	Enzyme	Color band (naked eye)
Rapid tests	Immunochromatographic test	Colloidal gold or silver	Color band (naked eye)
	Flow-through assay	Protein A conjugate	Color band (naked eye)
IEM	Immunoferritin electron microscopy	Electron dense molecules (e.g. ferritin)	Appears as black dot under electron microscope

Figs 12.11A and B: A. ELISA reader (Biorad); **B.** ELISA washer
Source: Biorad Pvt. Ltd (*with permission*).

microtiter plate or the tubes are made up of polystyrene, polyvinyl or polycarbonate material.

ELISA kits are commercially available; contain all the necessary reagents (such as enzyme conjugate, dilution buffer, substrate/chromogen etc). The procedure involves a series of steps done sequentially; at each step, a reagent is being added, and then incubated followed by washing of the wells [manually or by an automated ELISA washer (Fig. 12.11B)].

Automated ELISA systems are also available commercially where all the steps are carried out automatically by the instrument, e.g. EVOLIS (Biorad)(Fig. 12.12).

Types of ELISA

There are several types of ELISA, which differ from each other in their principles (Table 12.4).

Direct ELISA

It is used for detection of antigen in test serum. Here, the primary antibody (targeted against the serum antigen) is labeled with the enzyme.

- **Step 1:** Wells of microtiter plate are empty, not precoated with Ag or Ab

Table 12.4: Principles of different ELISA types

ELISA type	Used for detection of	Enzyme is labeled with
Direct ELISA	Antigen	Primary antibody
Indirect ELISA	Antibody or antigen	Secondary antibody
Sandwich ELISA	Antigen	Primary antibody in direct sandwich ELISA Secondary antibody in indirect sandwich ELISA
Competitive ELISA	Antigen or antibody	Secondary antibody
ELISPOT	Cells producing antibody or cytokine	Primary antibody

Note: Primary antibody is directed against the antigen, secondary antibody is an antihuman (or other species) Ig directed against Fc region of any human/other species Ig.

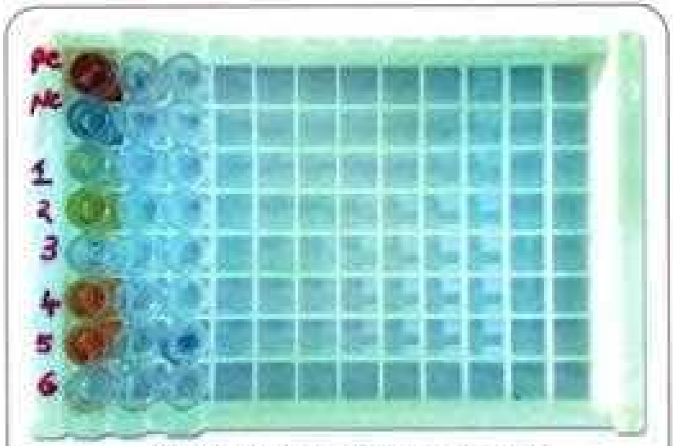
- **Step 2:** Test serum (containing antigen) is added into the wells. Antigen becomes attached to the solid phase by passive adsorption
- **Step 3:** After washing, the enzyme-labeled primary antibodies (raised in rabbits) are added
- **Step 4:** After washing, a substrate-chromogen system is added and color is measured.

Well + Ag (test serum) + primary Ab-Enzyme + substrate-chromogen → Color change (Fig. 12.14)

Indirect ELISA

It is used for detection of antibody or less commonly antigen in serum. It differs from the direct ELISA in that the secondary antibody is labeled with enzyme instead of primary antibody. The secondary antibody is an anti-species antibody, e.g. anti-human Ig (an antibody targeted to Fc region of any human Ig).

Fig. 12.12: Automated ELISA (EVOLIS System, Biorad)
Source: Department of Microbiology, JIPMER, Puducherry (with permission).



PC: Positive control; NC: Negative control
Interpretation: Sample No. 2, 4 & 5: Positive and Sample No: 1, 3 and 6: Negative

Fig. 12.13: ELISA for HBsAg
Source: Department of Microbiology, Pondicherry Institute of Medical Sciences (with permission).

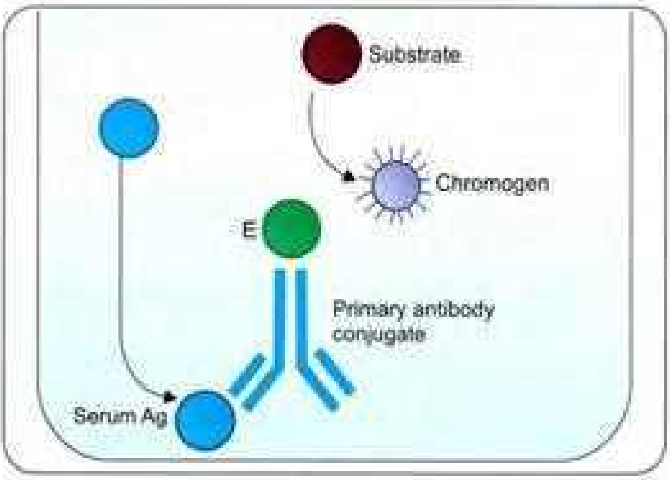


Fig. 12.14: Direct ELISA

Indirect ELISA for antibody detection

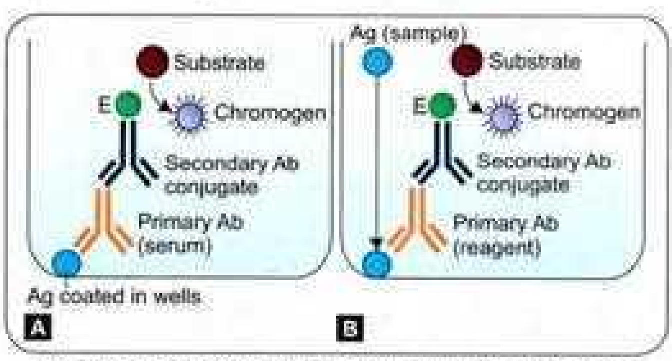
- **Step 1:** The solid phase of the wells of microtiter plates are precoated with the Ag
- **Step 2:** Test serum (containing primary Ab specific to the Ag) is added to the wells. Ab gets attached to the Ag coated on the well
- **Step 3:** After washing, enzyme-labeled secondary Ab (anti-human immunoglobulin) is added
- **Step 4:** After washing, a substrate-chromogen system is added and color is developed.

Wells are coated with Ag + primary Ab (test serum) + secondary Ab-Enzyme + substrate- chromogen → development of color (Fig. 12.15A)

Indirect ELISA for antigen detection: Here, the wells are empty, not precoated with Ag or Ab (Fig. 12.15B).

- **Step 1:** The test antigen (serum) is added to the well. The Ag gets absorbed onto the well
- **Step 2:** The primary antibody raised in rabbits (reagent) is added. The Ag binds to the primary antibody
- **Step 3:** After washing, enzyme-labeled secondary Ab (anti-rabbit Ab) is added
- **Step 4:** After washing, a substrate-chromogen system is added and color is developed.

Ag (test serum) added, gets adsorbed to well + primary Ab + secondary Ab-Enzyme + substrate- chromogen → development of color (Fig. 12.15B)



Figs 12.15A and B: Indirect ELISA. **A.** For antibody detection; **B.** For antigen detection

Sandwich ELISA

It detects the antigen in test serum. It is so named because the antigen gets sandwiched between a capture antibody and a detector antibody. There are two types of sandwich ELISA-direct and indirect, depending upon whether the detector antibody is a primary antibody (direct) or secondary antibody (indirect).

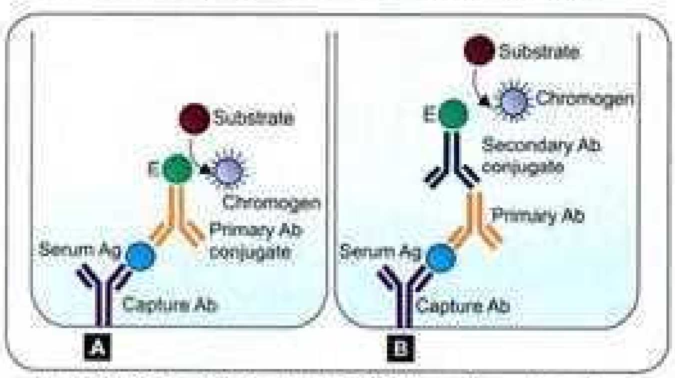
Direct Sandwich ELISA

- **Step 1:** The microtiter well is precoated with the capture antibody (monoclonal Ab raised in rabbit) targeted against the test antigen
- **Step 2:** The test serum (containing antigen) is added to the wells. Ag gets attached to the capture antibody coated on the well
- **Step 3:** After washing, an enzyme labeled primary 'detector antibody' specific for the antigen is added. The detector antibody can be same as the capture antibody
- **Step 4:** After washing, a substrate-chromogen system is added and color is developed.

Wells coated with capture Ab + Ag (test serum) + primary Ab-enzyme + substrate–chromogen → color (Fig. 12.16A)

Indirect Sandwich ELISA

The primary antibody and the capture antibody belong to different species. More so, the primary antibody is not labeled with enzyme. Another enzyme-labeled secondary antibody targeted against the primary antibody is added. Thus, it is more specific than direct sandwich ELISA.



Figs 12.16A and B: Sandwich ELISA (for antigen detection). **A.** Direct sandwich; **B.** Indirect sandwich

Wells coated with capture Ab + Ag (test serum) + primary Ab + secondary Ab- enzyme + substrate- chromogen → color (Fig. 12.16B)

Competitive ELISA

Competitive ELISA is so named because, antigen in test serum competes with another antigen of same type coated on well to bind to the primary antibody.

- **Step 1:** Primary antibody is first incubated in a solution with a serum sample containing the test antigen
- **Step 2:** This antigen-antibody mixture is then added to the microtiter well precoated with the same type of antigen
- **Step 3:** The free antibodies bind to the antigen coated on the well. More the test antigens present in the sample, lesser free antibodies will be available to bind to the antigens coated onto well
- **Step 4:** After washing (to remove free antibodies and antigens), enzyme-conjugated secondary antibody is added
- **Step 5:** After washing, a substrate-chromogen system is added and color is developed. Intensity of the color is inversely proportional to the amount of antigen present in the test serum (Fig. 12.17).

The competitive ELISA can also be used for the detection of antibody in serum. More so, different formats of competitive ELISA are available such as direct, indirect and sandwich formats. The example given above is an indirect competitive ELISA format used for antigen detection (Fig. 12.17).

ELISPOT Test

It is modification of ELISA that allows the quantitative detection of cells producing antibodies (plasma cells) or cytokines (e.g. macrophage). ELISPOT used for quantitating the cytokine producing cells is described below (Fig. 12.18).

- Microtiter well is coated with the capture antibody specific for the cytokine
- A suspension of the cell population under investigation is then added to the coated wells and incubated

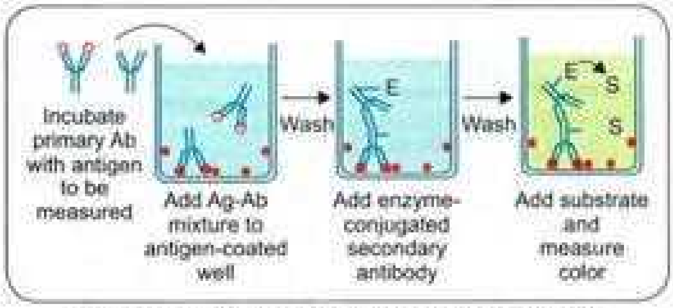


Fig. 12.17: Competitive ELISA for antigen detection

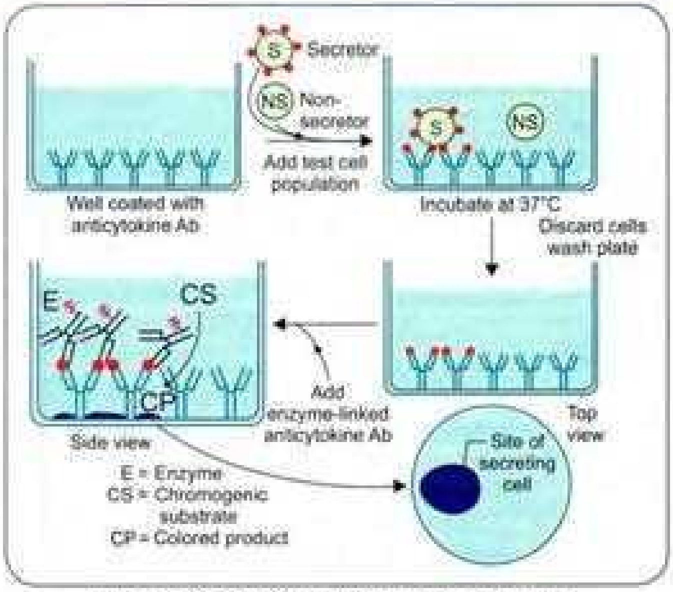


Fig. 12.18: ELISPOT test (for cytokine detection)

- The sensitized cells capable of producing the cytokines settle onto the surface of the well
- The released cytokines are bound by the capture antibodies in the vicinity of the secreting cells, producing a ring of antigen-antibody complexes around each cell that is producing the cytokine of interest
- After the incubation period, the well is washed and an enzyme-labeled anticytokine antibody is added
- After washing away unbound antibody, a chromogenic substrate that forms an insoluble colored product is added. The colored product (purple) precipitates and forms a spot only on the areas of the well where cytokine-secreting cells have been deposited
- **Quantitation:** The number of colored spots corresponds to the number of cytokine producing cells present in the added cell suspension
- ELISPOT test is currently used in IGRA (interferon gamma assay), for diagnosis of latent tuberculosis.

For quantitative detection of antibody producing cells, the procedure is similar except that the wells are coated with capture antigen which would bind to the antibodies produced by the cells.

IgG Avidity ELISA

Principle: Avidity of IgG indicates how firmly the IgG antibody is bound with its antigen. Avidity reflects the maturity of the antibodies; which usually increases with time.

- Low avidity antibodies are synthetized during the primary infection. It also occurs in immunosuppression
- High avidity antibodies are produced by memory B-cells during the secondary infection, or reactivation, recovery or in vaccinated people.

Contd...

Contd...

Procedure: ELISA is performed in two parallel wells of microtiter plate; one well with untreated patient's serum and a second well with serum sample after treating with dissociating agents such as urea. Low avidity antibodies dissociate from complexes and are washed away during the washing step of ELISA; whereas high avidity antibodies withstand urea treatment and remain bound to the antigens.

Result: Avidity (%) is expressed as absorbance of the well with urea to absorbance of the well without urea.

- Avidity <40%—interpreted as low avidity
- Avidity >60%—interpreted as high avidity
- Avidity 40–60%—interpreted as grey zone (inconclusive result); test has to be repeated.

Uses: IgG avidity ELISA is useful in:

- Differentiating recent and past infection- in situations where IgM disappears very soon in primary infection; or IgM persists for long time even after resolution of primary infection
- Diagnosing atypical or sub clinical course of the disease
- For diagnosing congenital infection.

Applications: IgG avidity test is available for the following infections: rubella, CMV, VZV, toxoplasmosis, EBV, HIV, viral hepatitis and West Nile virus infection.

Advantages of ELISA

ELISA is the method of choice for detection of antigens/antibodies in serum in modern days, especially in big laboratories as large number of samples can be tested together using the 96 well microtiter plate.

- It is economical, takes 2–3 hours for performing the assay
- ELISA is the most sensitive immunoassay, i.e. why, it is commonly used for performing screening test at blood banks and tertiary care sites
- Its specificity used to be low. But now, with use of more purified recombinant and synthetic antigens, and monoclonal antibodies, ELISA has become more specific.

Disadvantages of ELISA

- In small laboratories having less sample load, ELISA is less preferred than rapid tests as the latter can be done on individual samples
- It takes more time (2–3 hours) compared to rapid tests which take 10–20 minutes
- It needs expensive equipment such as ELISA washer and reader.

Applications of ELISA

ELISA can be used both for antigen and antibody detection.

- ELISA used for antigen detection: Hepatitis B [hepatitis B surface antigen (HBsAg) and precore antigen (HBeAg)], NS1 antigen for dengue, etc.
- ELISA can also be used for antibody detection against hepatitis B, hepatitis C, HIV, dengue, EBV, HSV, toxoplasmosis, leishmaniasis, etc.

ENZYME-LINKED FLUORESCENT ASSAY (ELFA)

It is an modification of ELISA, differs from ELISA in two ways: (i) automated system, all steps are performed by the instrument by itself. (ii) Ag-Ab-enzyme complex is detected by fluorometric method. VIDAS and miniVIDAS (bioMérieux) are commercially available systems based on ELFA technology (Fig. 12.19).

- **Procedure:** The solid phase receptacle (Fig. 12.19) present in reagent strip (equivalent to wells in microtiter plate in ELISA) serves as the solid phase; which is either coated with capture antigen (for antibody detection) or antibody (for antigen detection)
  - The conjugate used here is either a derivative of the analyte or an antibody labeled with enzyme alkaline phosphatase and the substrate used is 4-methyl-umbelliferyl phosphate
  - Following Ag-Ab-enzyme conjugate complex formation; the excess of enzyme conjugate are washed out
  - Then the conjugate enzyme catalyzes the hydrolysis of this substrate into a fluorescent product (4-methyl-umbelliferone), the fluorescence of which is measured at 450 nm
  - The intensity of the fluorescence depends on the concentration of alkaline phosphatase present which in turn depends upon the amount of analytate (target Ag or Ab) in the sample.

Fig. 12.19: miniVIDAS system and reagent strip (first well is solid phase receptacle coated with Ag or Ab and other wells contain various reagents)

Source: Department of Microbiology, JIPMER, Puducherry (with permission).

- **Advantages:** It has many advantages over ELISA: (i) an automated system, (ii) easy to perform and user friendly, (iii) less contamination chance, (iv) gives quantitative results and (v) more sensitive and specific
- **Disadvantages:** (i) Expensive, (ii) can run only 12–24 number of tests at a time, (iii) can run 2–4 types of tests at a time
- **Use:** It can be used to detect more than 100 parameters
  - *Infectious diseases:* Markers of hepatitis viruses and HIV (Ag and Ab), Ab to TORCH infection, measles, mumps, varicella, *H. pylori* and antigen to *C. difficile*, Rotavirus, etc.
  - *Other use:* Biomarkers (e.g. procalcitonin), hormones (e.g. thyroid), tumor markers, cardiac markers and screening for allergy.

IMMUNOFLUORESCENCE ASSAY (IFA)

It is a technique similar to ELISA, but differs by some important features:

- Fluorescent dye is used instead of enzyme for labeling of antibody
- It detects cell surface antigens. It is also used to detect antibodies bound to cell surface antigens, unlike ELISA which detects free antigen or antibody.

Principle

Fluorescence refers to absorbing high energy-shorter wavelength ultraviolet light rays by the fluorescent compounds and in turn emitting visible light rays with a low energy-longer wavelength.

- The fluorescent dye is used to conjugate the antibody and such labeled antibody can be used to detect the antigens or antigen-antibody complex on the cell surface
- The fluorescent compounds commonly used-fluorescein isothiocyanate (FITC).

Types

Direct Immunofluorescence Assay

- **Step 1:** Sample containing cells carrying surface antigens is smeared on a slide
- **Step 2:** Primary antibody specific to the antigen, tagged with fluorescent dye is added
- **Step 3:** Slide is washed to remove the unbound antibodies and then viewed under a fluorescence microscope (Fig. 12.20A).

Indirect Immunofluorescence Assay

This detects antibodies in sample. Slides smeared with cells carrying known antigens are commercially available.

- **Step 1:** Test serum containing primary antibody is added to the slide

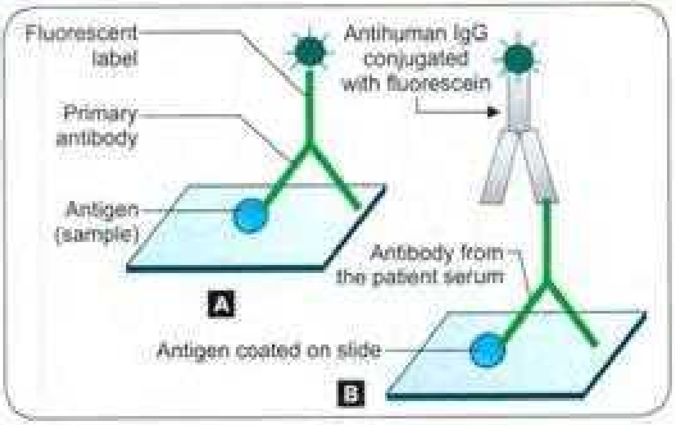


Fig. 12.20: Immunofluorescence assay. **A.** Direct; **B.** Indirect

- **Step 2:** Slide is washed to remove the unbound antibodies. A secondary antibody (antihuman antibody conjugated with fluorescent dye) is added
- **Step 3:** Slide is washed and then viewed under a fluorescence microscope (Fig. 12.20B).

Applications: Immunofluorescence assay has various applications, such as:

- Detection of autoantibodies (e.g. antinuclear antibody) in autoimmune diseases
- Detecting microbial antigens, e.g. rabies antigen in corneal smear
- Detection of viral antigens in cell lines inoculated with the specimens.

Flow Cytometry

Flow cytometry is a laser-based technology that quantitatively analyses and separates the cells as they pass through the laser beam. Its advanced and upgraded form is called as fluorescence-activated cell sorting (FACS).

Principle (Fig. 12.21): Cells (e.g. CD4 T cells) coated with specific fluorescent tagged antibodies (e.g. anti-CD4) are mixed with sheathed fluid and are passed through a special instrument called ultrasonic nozzle vibrator.

- The sheathed fluid aligns the cells so that they pass through the nozzle in a single row
- The nozzle vibrator has a unique property of allowing only one cell to exit at a time
- The cells after exiting from the nozzle pass through a beam of laser which excites the fluorescent dye on them resulting in emission of light
- The cells based on their electrical charge are then passed through the electromagnetic deflection plates so that different cell types are collected in separate containers.

In flow cytometry, four important properties can be determined during the flow of cells—

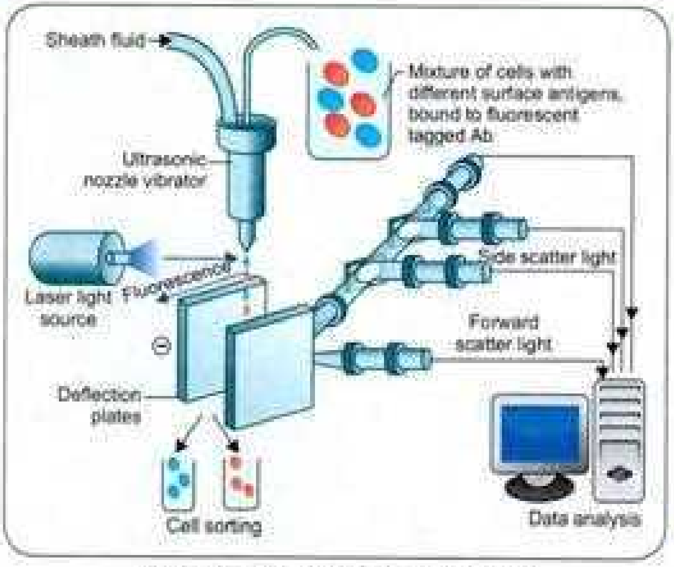


Fig. 12.21: Principle of flow cytometry

1. **Cell counting:** Cells exit from the nozzle at a rate of more than thousand cells per second. Each time, a cell crosses the laser beam, fluorescent light is emitted which indicates the exit of a single cell from the nozzle. This property is exploited to count the number of cells that pass through the laser beam, e.g. CD4 T cell count in blood.
2. **Differentiating between two cells:** Two fluorochrome dyes (fluorescein and rhodamine) can be used in flow cytometry that label two different antibodies targeting two type of cells (e.g. CD4 and CD8 T cells). Each generates a different color light while passing through laser beam. Fluorescein coated cells emit green color and rhodamine coated cells emit red color light.
3. **Scattering:** The laser beam gets deflected each time a cell crosses it. The deflection may happen towards forward direction or lateral direction.
   - Forward scatter is proportional to size of the cell; larger cells have greater forward scatter
   - Side scatter is proportional to intracellular complexity; cells with more granules (e.g. neutrophils) have more side scatter than cells with a simple cytoplasm (lymphocytes).
4. **Sorting out of cells:** The electromagnetic deflection plates help in separation of cells so that different cell types are collected in separate containers.

Applications: Flow cytometry can be used to analyse multiple parameters of cells (e.g. leukocytes) such as cell counting, cell sorting, analysis of size, shape, granularity, DNA or RNA content of a cell, etc. Important applications include:

- CD4 T cell count in HIV infected patients
- Detection of leukocyte with specific markers for the diagnosis of various lymphomas.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry refers to the process of detecting antigens (e.g. proteins) in cells of a tissue section by exploiting the principle of using labeled antibodies binding specifically to the antigens in biological tissues.

- It can be based on principles of ELISA or IFA:
  - The antibody (directed against tissue antigen) is conjugated to an enzyme, such as peroxidase, that can catalyse a color-producing reaction (immunoperoxidase staining)
  - Alternatively, the antibody can also be tagged to a fluorescent dye.
- It is widely used in the diagnosis of abnormal cells (e.g. tumor cells).

RADIOIMMUNOASSAY (RIA)

Radioimmunoassay (RIA) is a very sensitive and specific technique that is used for quantitative detection of antigens such as hormones, proteins, drugs, vitamins and microbial antigens (e.g. HBsAg) at a concentration of <0.01 µg/mL. It was developed by Berson and Yalow (1960), for which they were awarded Nobel Prize in 1977. The principle of RIA is similar to that of competitive ELISA for antigen detection, except that in RIA, the radioactive molecules are used for labeling and the test is done in a liquid medium. Because of the radiohazard associated, the use of RIA is reduced.

CHEMILUMINESCENCE-LINKED IMMUNOASSAY (CLIA)

Chemiluminescence refers to the emission of light (luminescence), as a result of a chemical reaction. The principle of CLIA is similar to that of ELISA; however, the chromogenic substance is replaced by chemiluminescent compounds (e.g. luminol and acridinium ester) that generate light during a chemical reaction (luxogenic). The light (photons) can be detected by a photomultiplier, also called as luminometer (Fig. 12.22).

(Ag-Ab complex)-enzyme (e.g. HRP) + chemiluminescent substrate (e.g. luminol and acridinium ester) → product+ light (photons) → detected by luminometer or photomultiplier.

Advantages of CLIA

CLIA claims to be 10 times more sensitive than ELISA.

- CLIA can be further modified by using an enhancer that potentiates the chemical reaction. This gives CLIA an overall improvement of 200 folds over ELISA
- Most samples have no 'background' signal, i.e. luminol compounds do not themselves emit light

Fig. 12.22: Chemiluminescence system (CLIA) and its principle
Source: Department of Microbiology, JIPMER, Puducherry (with permission).

- Measurement of chemiluminescence is not a ratio unlike the measurement of fluorescence (IFA) and or color (ELISA)
- Individual specimens can be tested in CLIA in contrast to ELISA which is preferred for testing multiple samples.

Applications

CLIA has limited applications in diagnostic microbiology compared to ELISA. Currently, it is available for detection of antigens or antibodies against various infections such as hepatitis viruses, HIV, TORCH infections and biomarkers such as procalcitonin.

WESTERN BLOT

Western blot detects specific proteins (antibodies) in a sample containing mixture of antibodies each targeted against different antigens of same microbe.

- It is so named for its similarity to Southern blot (detects DNA fragments) and Northern blot (detects mRNAs)
- **Eastern blot** is the latest addition to the list; it is a modification of Western blot, which detects the carbohydrate epitopes present on proteins or lipids.

Procedure

Western blot comprises of three basic components as follows (Fig. 12.23):

SDS PAGE: This is a method which separates complex protein antigen mixture into individual fragments. It has two steps:

1. **SDS:** The complex protein (antigen) mixture is treated with a strong denaturing detergent called SDS (*sodium dodecyl sulfate*).
2. **PAGE:** Then the mixture is subjected to polyacrylamide gel electrophoresis (PAGE) which separates the antigenic components according to their molecular weight; lower molecular weight components migrate farther than higher molecular weight ones.

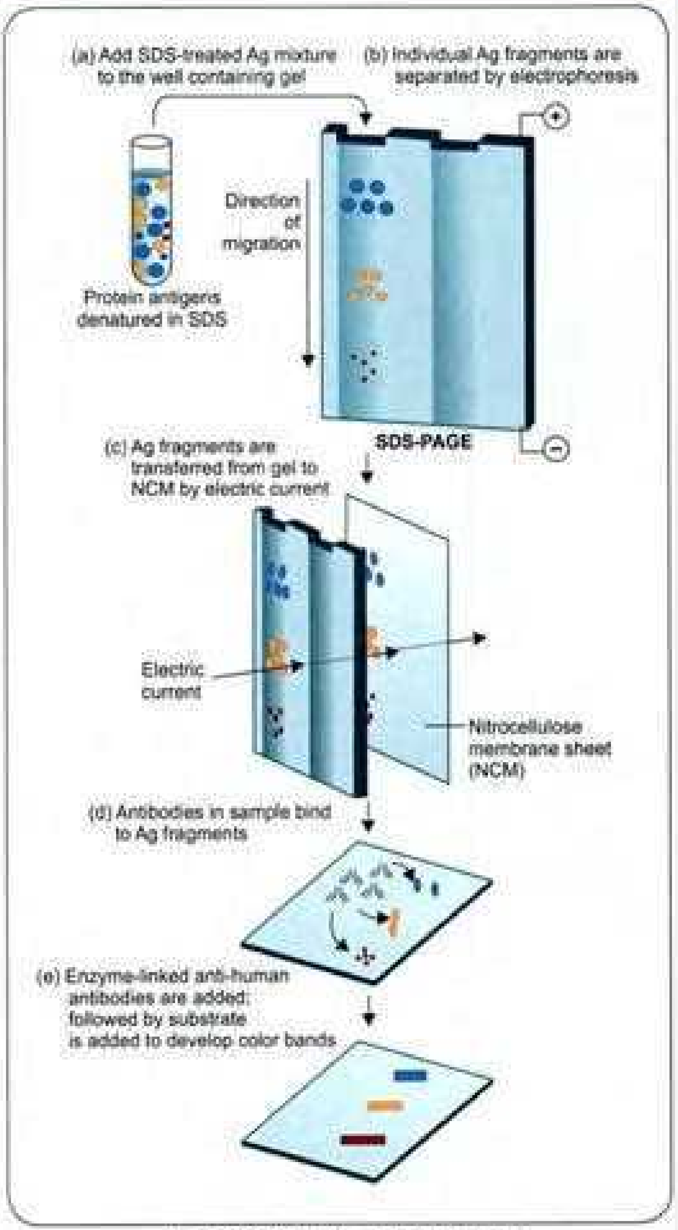


Fig. 12.23: Western blot (principle)

Nitrocellulose membrane (NCM) blotting: The gel is removed from the electrophoresis apparatus and placed over a protein-binding sheet such as nitrocellulose or nylon and the antigen fragments in the gel are transferred (blotted) to the NCM sheet by the passage of an electric current.

Enzyme immunoassay (to detect the antibodies):

- NCM strips containing protein antigen fragments are treated with patient's sample containing antibodies. Individual serum antibodies would bind to the respective antigen fragments, thus making western blot highly specific

- Addition of enzyme-linked antihuman Ig, detects the individual serum antibodies bound to antigen fragments. Substrate/chromogen is added for development of colored bands.

Applications

Western blot has an excellent specificity. Hence, it is often used as a supplementary test to confirm the result of ELISA or other immunoassays having higher sensitivity. Western blot formats are available to detect antibody in various diseases such as HIV, Lyme's disease, Herpes simplex virus infection, cysticercosis, hydatid disease and toxoplasmosis.

RAPID TESTS

Rapid tests are revolutionary in the diagnosis of infectious diseases. They are very simple to perform (one step method), rapid (takes 10-20 minutes), require minimal training, do not need any sophisticated instruments.

- These tests are also called **Point-of-care** (POC) tests, because unlike ELISA and other immunoassays, the POC tests can be performed independent of laboratory equipment and deliver instant results
- Two principles of rapid tests are available—lateral flow assay and flow through assay
- Both the formats are available for the diagnosis of various diseases such as malaria, hepatitis B, hepatitis C, HIV, leptospirosis, *Helicobacter pylori*, syphilis, etc.

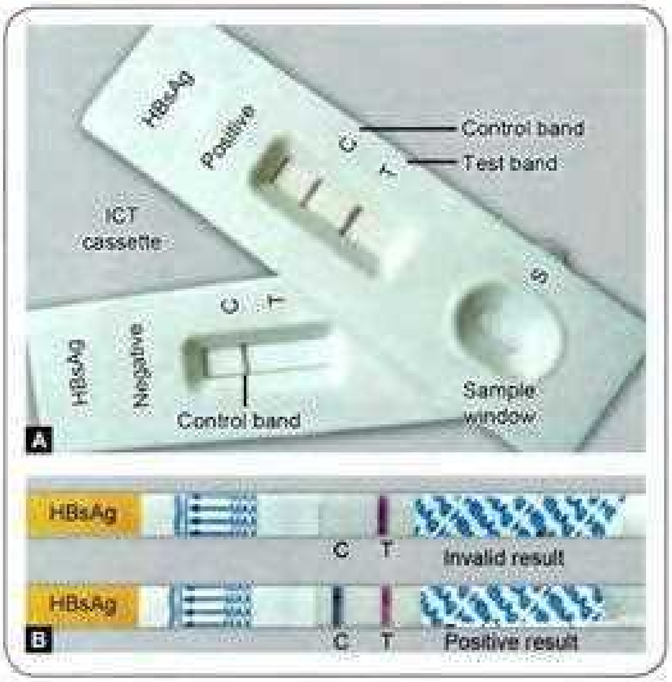
Immunochromatographic Test (Lateral Flow Assay)

Immunochromatographic test (ICT) is based on lateral flow technique. It is widely used in diagnostic laboratories because of its simplicity, economy and rapidity. It can be used for both antigen and antibody detection in sample. Principle of antigen detection method is described below.

Principle of ICT (Antigen Detection)

The test system consists of a nitrocellulose membrane (NCM) and an absorbent pad. Two formats are available: cassette or strip (Figs 12.24A and B). The NCM is coated at two places in the form of lines—a test line, coated with monoclonal antibody targeted against the test antigen and a control line, coated with anti-species immunoglobulin. Specific Ab against the target Ag labelled with chromogenic marker (specific Ab tagged with *colloidal gold or silver*, a visually detectable marker) is infiltrated in the absorbent pad lining the sample window.

- The sample (serum) containing the test antigen is added to sample well; it reacts with antibody labeled with chromogenic marker (*colloidal gold* or *silver*, a visually detectable marker)



Figs 12.24A and B: ICT for HBsAg detection. **A.** Cassette format; **B.** Strip format

Source: Department of Microbiology, Pondicherry Institute of Medical sciences (with permission).

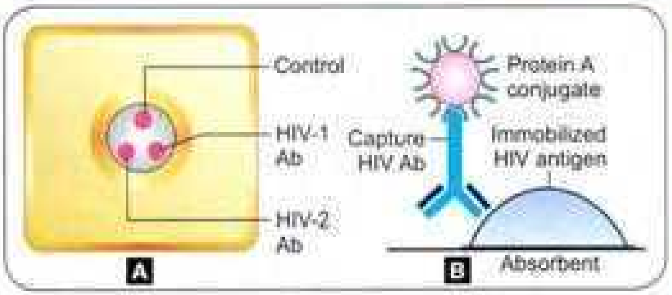
- Both 'Ag-specific Ab-colloidal gold complex' as well as the 'free colloidal gold labeled Ab' move laterally along the nitrocellulose membrane
- **Test band:** At the test line, the Ag-labeled Ab complex is immobilized by binding to the monoclonal Ab in the test line to form a colored band (Fig. 12.24)
- **Control band:** The free colloidal gold labeled Ab can move further and binds to the anti-human Ig to form a color control band. If the control band is not formed, then the test is considered invalid irrespective of whether the test band is formed or not (Fig. 12.24).

Flow-through Assay

Flow-through tests are another type of rapid diagnostic assays which differ from ICT in two aspects: (1) protein A is used for labeling antibody instead of gold conjugate and (2) the sample flows vertically through the nitrocellulose membrane (NCM) as compared to lateral flow in ICT.

Flow-through tests can be used for both antigen and antibody detection. HIV TRIDOT test is a classical example (described below, Fig. 12.25A). It detects antibodies to HIV-1 and 2 separately in patient's serum.

- The test system is in a cassette format, consisting of a NCM and an absorbent pad. The NCM is coated at three regions- two test regions coated with HIV-1 and 2 antigens and a third control region coated with antihuman Ig



Figs 12.25A and B: Flow-through assays. **A.** HIV TRI-DOT assay for HIV 1 and 2 antibodies detection; **B.** Principle of HIV TRI-DOT

- Sample and buffer reagents are added sequentially from the top following which they pass through the membrane and excess fluid is absorbed into the underlying absorbent pad
- As the patient's sample passes through the membrane, HIV antibodies, if present bind to the immobilized antigens (Fig. 12.25B)
- **Test dots:** Protein-A conjugate (present in buffer) binds to the Fc portion of the HIV antibodies to give distinct pinkish purple DOT(s), separately for HIV-1 and 2 antibodies
- **Control dot:** Irrespective of whether the HIV antibodies are present or not, protein-A can bind to any IgG present in serum and the IgG-protein A complex can further bind to the antihuman Ig at the control line to give a pinkish purple DOT.

TECHNIQUES USING ELECTRON MICROSCOPE

Immunoferritin Test

In electron microscopy, the electron dense areas appear darker than other areas. Electron dense molecules such as ferritin particle can be used to label an antibody which can bind to surface antigens of cells and tissues. When visualized under electron microscope, the electron dense label absorbs more electrons and appears as small black dot, thus confirming the presence of antigen.

Immunoelectron Microscopy

Viral particles appear to be clumped when mixed with specific antisera and observed under the electron microscope. This is used for finding hepatitis A virus and rotavirus particles from stool specimen.

EXPECTED QUESTIONS

I. Essay:

1. Enumerate the properties and types of antigen-antibody reactions. Describe in detail about the principle, types, and applications of ELISA?
2. Describe in detail about the principle, types, and applications of agglutination reaction?

II. Write short notes on:

1. Indirect immunofluorescence assay
2. Flow cytometry
3. Western blot
4. Immunochromatographic test
5. Chemiluminescence immunoassay (CLIA)
6. Prozone phenomena

III. Multiple Choice Questions (MCQs):

1. Nagler reaction is example of:
a. Precipitation b. CFT
c. Agglutination d. Neutralization

2. Paul Bunnell test is example of:
a. Agglutination b. Precipitation
c. Neutralization d. CFT

3. All are precipitation reactions, *except*:
a. VDRL test b. Kahn test
c. Widal test d. Ascoli's test

4. The following methods of diagnosis utilize labeled antibodies, *except*:
a. ELISA (enzyme-linked immunosorbent assay)
b. Radioimmunoassay
c. Hemagglutination inhibition test
d. Immunofluorescence

5. Prozone phenomenon is due to:
a. Excess antigen
b. Excess antibody
c. Hyperimmune reaction
d. Both antigen and antibody excess

6. All of the following interactions exist between antigen and antibody, *except*:
a. Hydrogen bonds
b. Covalent interaction
c. Electrostatic interactions
d. Van der Waals forces

7. Which of the following statement is wrong regarding affinity and avidity?
a. Avidity refers to affinities of all the binding sites when multivalent antibody reacts with a complex antigen carrying multiple epitopes
b. Avidity is higher than the sum of the individual affinity at each binding site.
c. IgM has better avidity than IgG
d. IgG has a higher affinity than IgM

8. Radial immunodiffusion is an example of:
a. Single diffusion in one dimension
b. Single diffusion in two dimension
c. Double diffusions in one dimension
d. Double diffusions in two dimensions

Answers

1. d **2.** a **3.** c **4.** c **5.** b **6.** b **7.** b **8.** b

Complement

13
CHAPTER

Chapter Preview

- General properties
- Complement pathways
  - Effector functions of complement
- Regulation of complement pathways
- Complements deficiencies

GENERAL PROPERTIES

The term 'complement' (C) represents a group of proteins normally found in serum in inactive form, but when activated they augment the immune responses. They constitute about 5% of normal serum proteins and their level does not increase following either infection or vaccination. Complements have the following general properties:

- **Bind to Fc region of antibody:** The effector function of complement is mediated by binding with Fc portion of antibody. The binding of complement to an antibody is described by various terms as, **fixing** or **consumption** (as it disappears from serum following binding)
- **Role of antigen:** The classical pathway of complements do not bind to free antibodies but they can only fix to those antibodies which are bound with antigens. However fixation of complement is not influenced by the nature of antigens, but only by the class of antibody
- **Species nonspecific:** Complements are present in the sera of all mammals, birds, amphibians and fish. Complements from one species can react with antibodies from other species, though the efficiency decreases with increase in taxonomic distance
- **Heat labile:** Complements get denatured by heating the serum at 56°C for 30 minutes. Such serum with lost complement activity is called **inactivated serum**.

History

The role of complement in mediating lysis of target cells (e.g. hemolysis and bacteriolysis), was described since nineteenth century.

- Pfeiffer (1894) discovered the complement mediated lysis of *Vibrio cholerae* in immunized guinea pigs. Bacteriolysis in *vivo* was known as Pfeiffer's phenomenon
- Jules Bordet named it as alexine which was later called complement by Ehrlich.

Complement Components

The complement system comprises of about 30 serum proteins grouped into complement components, the properdin system and the regulatory proteins.

- The complement components are named by numerals. There are nine components; C1 to C9. C1 has three subunits—C1q, C1r and C1s
- The properdin system and the regulatory proteins are named by letter symbols, e.g. factor-B.

Synthesis

Liver is the major site of synthesis of complement proteins. Other minor sites include blood monocytes, tissue macrophages, and epithelial cells of GIT and genitourinary tract.

Complement Activation

All the complement proteins are synthesized in inactive form (e.g. zymogens) and are activated by proteolysis.

- Complements have two unequal fragments (large and small fragment)
- The larger fragments are usually designated as 'b' (e.g. C3b) and the smaller fragments are designated as 'a' (e.g. C3a). An exception is C2a which is larger fragment
- During proteolysis, the smaller fragment is removed exposing the active site of the larger fragment
- The larger fragment participates in the cascade reaction of complement pathway and the smaller fragment diffuses away to mediate other functions
- **Cascade reaction:** The fragments of complements interact in a definite sequential manner with a cascade

like effect, which leads to formation of complex. Such complex having enzymatic activity is designated by putting a bar over the number or symbol (e.g. $\overline{CbBb}$).

COMPLEMENT PATHWAYS

There are three pathways of complement activation:

1. **Classical pathway:** This is an antibody dependent pathway. Pathway is triggered by the antigen-antibody complex formation.
2. **Alternative pathway:** This is an antibody independent pathway, triggered by the antigen directly.
3. **Lectin pathway:** This is a recently described pathway. It resembles classical pathway, but it is antibody independent.

Stages of complement activation

There are four main stages in the activation of any of the complement pathways.

1. Initiation of the pathway
2. Formation of C3 convertase
3. Formation of C5 convertase
4. Formation of membrane attack complex (MAC).

All the three pathways (Fig. 13.1) differ from each other in their initiation till formation of C3 convertase. Then, the remaining stages are identical in all the pathways.

CLASSICAL PATHWAY

Classical pathway is antibody dependent. However, not all antibodies can bind to complements of classical pathway. Decreasing order of ability of antibodies to fix complement is—IgM (most potent) > IgG3 > IgG1 > IgG2. The other classes of antibodies do not fix complements. C_H2 domain on IgG, C_H4 on IgM participate in complement binding. The classical pathway begins with activation of C1 and binding to antigen-antibody complex.

Initiation

The first step is the binding of C1 to the antigen-antibody complex (Fig. 13.1).

- The first binding portion of C1 is C1q, which reacts with the Fc portion of IgM or IgG bound to antigen
- C1q is a hexamer having six globular heads each acting as a combining site
- Effective activation of classical pathway begins only when C1q is attached to the Fc portion of antibody by at least two of its globular binding sites
    - IgM being pentameric, has five Fc regions, hence one molecule of IgM can initiate the pathway
    - Whereas IgG is monomeric, therefore two IgG molecules are needed to initiate the process. Hence, IgM is much efficient stimulator of classical pathway.

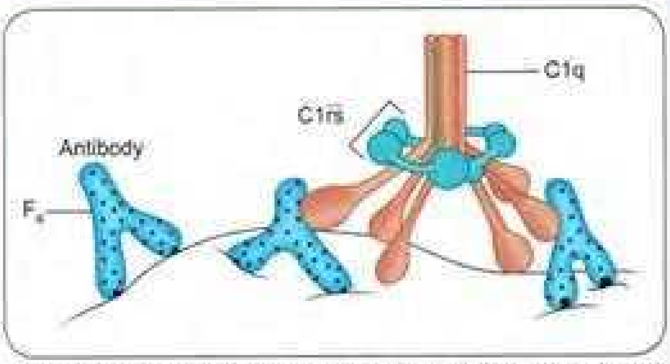


Fig. 13.1: Activation of C1 (initiation step of classical complement pathway)

- C1q binding in the presence of calcium ions, in turn activates sequentially C1r followed by C1s.

Formation of C3 Convertase

Activated $\overline{C1s}$ acts as an esterase (C1s esterase), which can cleave C4 to produce C4a (an anaphylatoxin), and C4b which binds to C1 and participates further in complement cascade.

- $\overline{C14b}$ in the presence of magnesium ions cleaves C2 into C2a, which remains linked to complement complex, and C2b (has kinin like activity), which is released outside
- $\overline{C14b2a}$ is referred to as C3 convertase of the classical pathway.

Formation of C5 Convertase

C3 convertase hydrolyses many C3 molecules into two fragments: C3a (an anaphylatoxin) and C3b which remains attached to $\overline{C14b2a}$ to form $\overline{C14b2a3b}$ complex, which acts as C5 convertase of classical pathway.

Formation of Membrane Attack Complex

This phase begins with C5 convertase cleaving C5 into C5a (an anaphylatoxin, released into the medium) and C5b, which continues with the cascade.

- $\overline{C5b}$ is extremely labile, gets stabilized by binding soon with C6 and C7 to form $\overline{C5b67}$ followed by addition of C8
- The hydrophobic regions on C7 and C8 help in penetration into the target cell membrane
- This inserted membrane complex ($\overline{C5b678}$) has a catalytic property to bind to C9 molecule and then it polymerizes the C9 into a tubular channel of 10 nm diameter
- Penetration of C9 causes formation of channels or pores on the target cell membrane
- Each tubular channel behaves hydrophobic outside, but hydrophilic inside; thus allowing free passage of ions and water into the cell leading to cellular swelling and lysis

- Because $\overline{C5b6789}$ destroys the target cell by attacking the cell membrane; it is called membrane attack complex (MAC) and the process of cytolysis is referred to as complement-mediated cytotoxicity (Figs 13.2 and 13.3).

ALTERNATIVE PATHWAY

Alternative pathway (Fig. 13.3) is independent of antibody; hence is considered as a part of innate immunity. It also

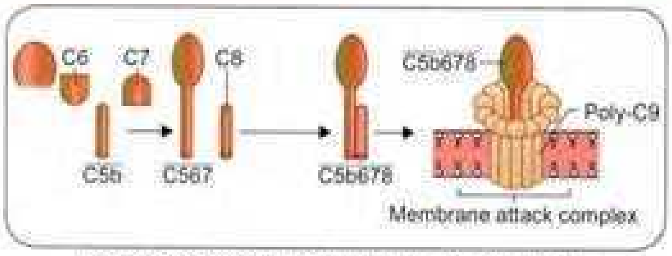


Fig. 13.2: Formation of membrane attack complex

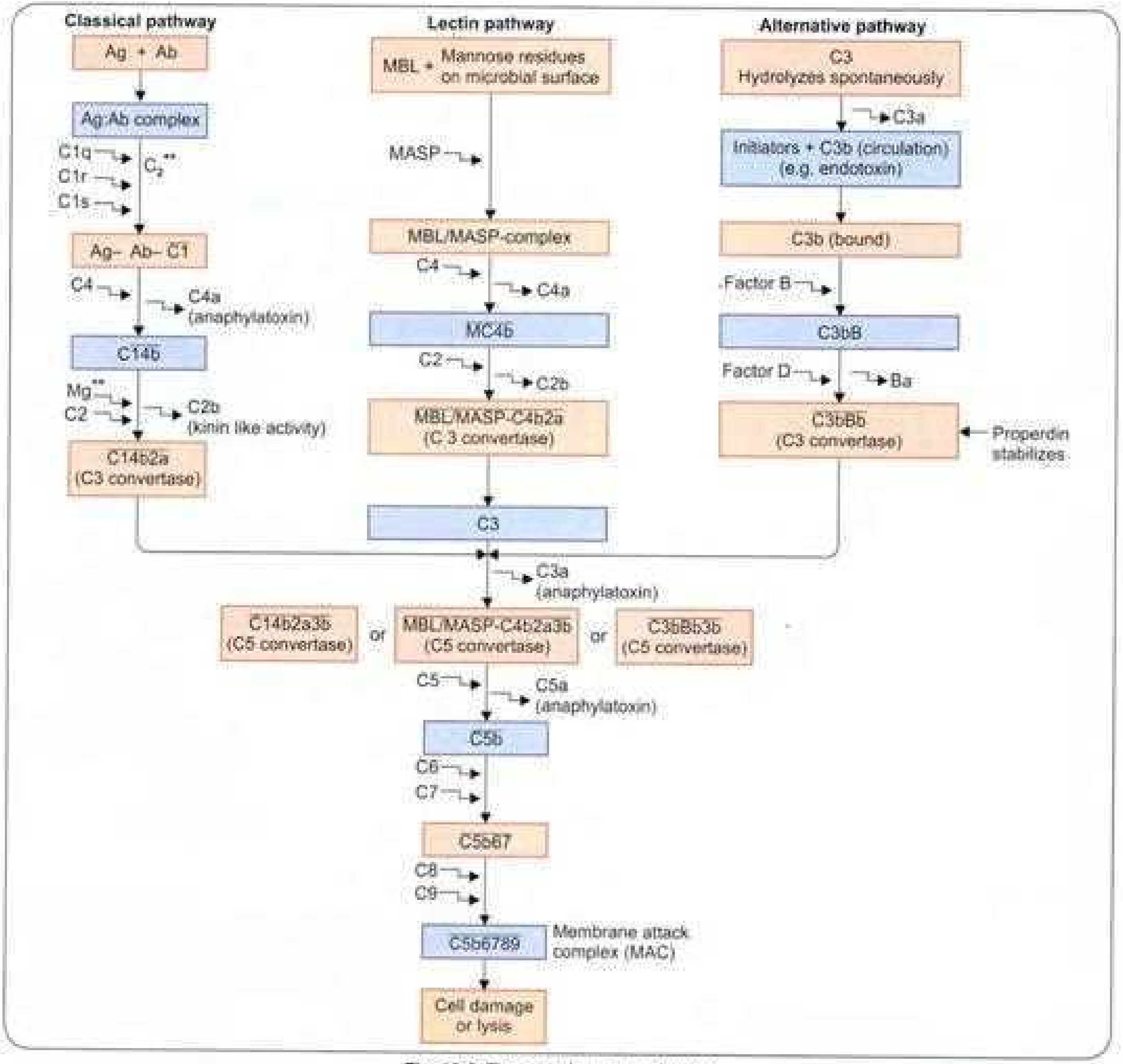


Fig. 13.3: The complement pathways

Abbreviations: MBL, mannose binding lectins; MASP, MBL-associated serine protease.

Table 13.1: Initiators for alternative pathway	
Antigens from pathogen	**Nonmicrobial initiators**
Endotoxin or LPS (lipopolysaccharide) from gram-negative bacteria	Human antibodies in complexes—IgA, IgD
Teichoic acid from gram-positive bacteria	Tumor cells
Fungal cells	Cobra venom factor
Yeast cells	Heterologous RBCs from mouse, rabbit and chicken
Parasites like trypanosomes	Anion polymer like dextran sulfate
Virus-infected cells	Pure carbohydrates like agar and inulin

goes through the four stages; but differs from the classical pathway in first two stages. Unlike the classical pathway which involves all complement components from C1 to C9; in alternative pathway three complement components C1, C4 and C2 are not involved. Instead, it requires three other complement proteins present in serum named factor B, factor D and properdin.

Initiation

The alternative complement cascade is initiated by various cell surface constituents that are foreign to the host, e.g. bacterial endotoxin (Table 13.1).

The first complement component to be involved in alternative pathway is free C3 in the serum. C3 hydrolyzes spontaneously, to generate:

- C3a which diffuses out and
- C3b fragment which attaches to foreign cell surface antigen.

Formation of C3 Convertase

- In the next step, **Factor B** binds to C3b coated foreign cells
- **Factor D**, another alternative pathway complement factor, acts on factor B, and cleaves it into Ba (diffuses out) and Bb (remains attached)
- $\overline{C3bBb}$ is also called C3 convertase of alternative pathway
- $\overline{C3bBb}$ has a very short half-life of 5 minutes. If it is stabilized by another complement protein called properdin its half-life is increased to 30 minutes.

The remaining two stages, i.e. formation of C5 convertase and formation of membrane attack complex are identical to that of classical pathway.

LECTIN PATHWAY

Lectin pathway is another complement pathway of innate immunity described recently, that works independent of antibody.

- It is mediated through lectin proteins of the host that interact with mannose residues present on microbial surface; hence the name lectin pathway
- Among the four stages, the first stage differs from classical pathway
- Lectin pathway involves all complement components used for classical pathways except C1 (i.e. from C2 to C9); Instead of C1, host lectin protein called mannose binding lectins mediate the first 'initiation' stage (see Fig. 13.3).

Initiation

Antigens that activate lectin pathway are the mannose carbohydrate residues of glycoproteins present on microbial surfaces.

- A specific host lectin protein called mannose binding lectins (MBL) bind to mannose residues on microbial surface
- MBL is an acute phase reactant protein, similar to C1q in structure
- After binding of MBL to microbial surface, another host protein called MBL-associated serine protease (MASP) gets complexed with MBL
- MASP is similar or C1r and C1s and mimics their functions
- The remaining three stages are similar to the classical pathway
- The MBL-MASP complex cleaves C4 which in turn splits C2 and the $\overline{\text{MBL/MASP-C4b2a}}$ acts as C3 convertase.

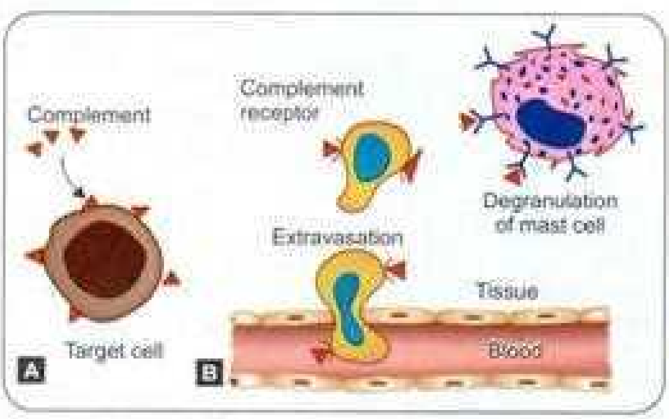
Important differences between the three complement pathways are summarized in Table 13.2.

Table 13.2: Differences between the three complement pathways			
Features	**Classical pathway**	**Alternative pathway**	**Lectin pathway**
Activator (initiator)	Antigen-antibody complex	Endotoxin IgA, IgD, cobra venom, Nephritic factor	Carbohydrate residue of bacterial cell wall (mannose binding protein) that binds to host lectin antigen
First complement activated	C1	C3b	C4
C3 convertase	$\overline{C14b2a}$	$\overline{C3bBb}$	$\overline{\text{MBL/MASP-C4b2a}}$
C5 convertase (C3 convertase + 3b)	$\overline{C14b2a3b}$	$\overline{C3bBb3b}$	$\overline{\text{MBL/MASP-C4b2a3b}}$
Complement levels in the serum	All C1-C9: Low	• C1,C4,C2: Normal • Others: Low	• C1: Normal • Others: Low
Immunity	Acquired	Innate	Innate

EFFECTOR FUNCTIONS OF COMPLEMENT

The membrane attack complex (MAC) and other complement by-products produced during the activation of complement pathways augment the immune response in many ways; which are collectively called as the effector functions of complement products. The functions are as follows:

1. **Target cell lysis by MAC:** As already explained, the MAC makes pores or channels in the target cell membrane; thereby allows the free passage of various ions and water into the cell leading to cell swelling, lysis and death. Bacteria, enveloped viruses, damaged cells, tumor cells, etc. are killed by this mechanism, commonly referred to as complement-mediated cell lysis (Fig. 13.4A).
2. **Inflammatory response:** Complement by-products such as C3a, C4a and C5a are called anaphylatoxins. They bind to surface receptors of mast cells and induce their degranulation leading to release of histamine and other inflammatory mediators. They cause vasoconstriction, and increased vascular permeability (Fig. 13.4B).
3. **Opsonization:** C3b and C4b act as major opsonins that coat the immune complexes and particulate antigens. Phagocytic cells express complement receptors (CR1, CR3 and CR4) for complement components (C3b, C4b), and are able to bind to complement coated antigens and enhance phagocytosis (Fig 13.5). C5a augments this process by enhancing the CR1 expression on phagocytes by 10 folds.
4. **Removing the immune complexes from blood:** C3b plays an important role in removing immune complexes from the blood. C3b bound immune complexes are recognized by complement receptor CR1 present on RBCs. Immune complexes bound to RBCs are taken to liver and spleen where they are phagocytosed after being separated from the RBCs (Fig. 13.6).



Figs 13.4A and B: A. Complement mediated cell lysis; **B.** Activation of inflammatory response

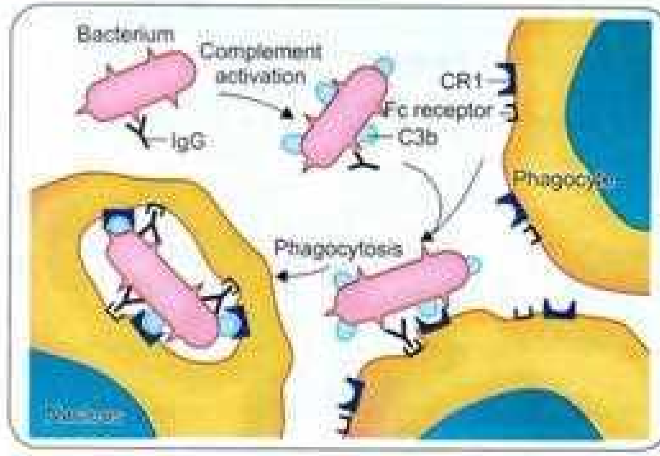


Fig. 13.5: Complement-mediated opsonization

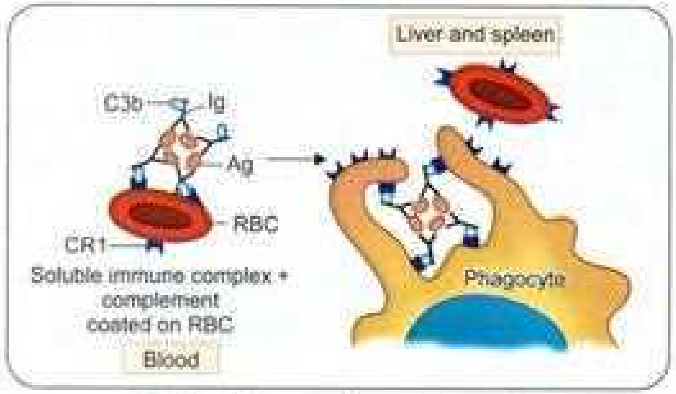


Fig. 13.6: Clearance of immune complexes

5. **Viral neutralization:** Complements play a crucial role in neutralization of the viruses; which occurs by many ways—
   - Complements coated on virus surfaces neutralize the viral infectivity by blocking their attachment sites
   - C3b mediated opsonization of viral particles
   - Lysis of the enveloped viruses either by activation of classical pathway (most viruses) or some time by alternative or lectin pathways (by some viruses like Epstein-Barr virus, rubella virus, etc.)

COMPLEMENT RECEPTORS

Complement receptors (CRs) play an important role in mediating the activities of complement products as well as in regulating their activities. They are distributed on various cell types and bind to specific ligands to mediate specific function (Table 13.3).

EVASION OF COMPLEMENT SYSTEM BY MICROORGANISMS

In order to escape from the complement mediated effector mechanisms, microorganisms can develop various counter mechanisms to evade the complement system. (Table 13.4).

Table 13.3: Complement receptors—distribution, their ligands and functions

Complement receptors	Ligands	Distribution	Function
CR1 (CD35)	C3b, C4b	RBCs, phagocytes All blood cells	• Regulates complement pathway by inhibiting C3 convertase • Helps in removal of immune complexes
CR2 (CD21)	C3d, C3dg	B cells, T cells, Follicular dendritic cells	• Forms a part of B cell co-receptor involved in humoral responses • Acts as EBV receptor
CR3, CR4	iC3b	Phagocytes	• Opsonization • Binding and extravasation of neutrophils
CR3a, CR4a, CR5a	C3a, C4a, C5a	Mast cells, Basophils	Degranulation of mast cells and basophils

Abbreviations: CR, complement receptors; EBV, Epstein-Barr virus; iC3b, inactivated C3b.

Note: C3d and C3dg are generated during break down of C3b.

Table 13.4: Mechanisms of microbial evasion of complement system

Mechanisms	Examples
Shown by gram-negative bacteria	
Long polysaccharide side chain of bacteria can prevent membrane attack complex (MAC) insertion	*Escherichia coli* *Salmonella*
Noncovalent interactions between bacterial cell wall components can prevent MAC insertion	*Neisseria gonorrhoeae*
Elastases destroy C3a and C5a	*Pseudomonas*
Shown by gram-positive bacteria	
Thick peptidoglycan cell wall prevents MAC insertion	*Staphylococcus* *Streptococcus*
Bacterial capsule forms a physical barrier between C3b and CR1 interaction	*Streptococcus pneumoniae*
Shown by other microbes	
Proteins mimicking complement regulatory proteins	Vaccinia virus, Herpes simplex virus, Epstein-Barr virus, *Trypanosoma cruzi*, *Candida albicans*

REGULATION OF COMPLEMENT PATHWAYS

Complement system are antigen non-specific; capable of attacking microorganisms as well as host cells. Hence, several regulatory mechanisms have evolved to restrict complement activity only to the designated target cells. There are a series of regulatory proteins, which inactivate various complement components at different stages (Table 13.5).

COMPLEMENT DEFICIENCIES

Complement deficiency associated diseases fall into two categories; diseases associated with—(1) complement protein deficiencies and (2) complement regulator protein deficiencies (Table 13.6).

Table 13.5: Regulation of complement system

Regulatory proteins	Pathway affected	Type of protein	Regulatory function
C1 regulator			
C1 inhibitor (C1 inhibitor, or C1 esterase inhibitor)	Classical only	Soluble	It is a glycoprotein, inhibits the action of C1q by splitting C1qrs into C1rs and C1q. Thus, the whole classical pathway is inhibited
C3 convertase regulators			
C4b-binding protein (C4bBP)	Classical and lectin	Soluble	• It blocks formation of C3 convertase by binding C4b • It acts as cofactor for cleavage of C4b by factor I
CR-1 (Complement-receptor-1)	All three pathways	Membrane bound	Blocks formation of C3 convertase by binding C3b or C4b
MCP (Membrane-cofactor protein)			
Factor H	Alternative only		
DAF (Decay accelerating factor) or CD55	All three pathways	Membrane bound	Accelerates dissociation of C3 convertase
Factor-I	All three pathways	Soluble	Cleaves C4b or C3b by using C4b-binding protein
MAC formation regulators			
S protein		Soluble	Binds to soluble C5b67 and prevents its insertion into cell membrane
Membrane inhibitor of reactive lysis (MIRL or CD59)	All three pathways	Membrane bound	Inhibit MAC formation by blocking C9 binding
Homologous restriction factor		Membrane bound	Inhibit MAC formation by blocking C9 binding

Table 13.6: Complement deficiency diseases

Complement deficiencies	Pathway(s) involved	Disease/pathology
Complement protein deficiencies		
C1, C2, C3, C4	C1, C2, C4—classical pathway C3—common deficiency	Systemic lupus erythematosus (SLE), glomerulonephritis and pyogenic infections
Properdin, Factor D	Alternative pathway	*Neisseria* and pyogenic infection
Membrane attack complex (C5-C9)	Common deficiency	Disseminated *Neisseria* infection
Complement regulatory protein deficiencies		
C1 esterase inhibitor	Overactive classical pathway	Hereditary angioneurotic edema
DAF (Decay accelerating factor) and CD59	Deregulated C3 convertase Increased RBC lysis	PNH (Paroxysmal nocturnal hemoglobinuria)
Factor I	Deregulated classical pathway with over consumption of C3	Immune complex disease; recurrent pyogenic infections
Factor H	Deregulated alternative pathway with increased C3 convertase activity	Immune complex disease; pyogenic infection

EXPECTED QUESTIONS

I. Essay:

1. What is complement? Explain in detail about classical complement pathway. List various effector functions of complement.

II. Write short notes on:

1. Alternative complement pathway.
2. Lectin complement pathway.
3. Various mechanisms of microbial evasion of complement system.
4. Complement deficiency diseases.

III. Multiple Choice Questions (MCQs):

1. C-3 convertase in alternative complement pathway is:
a. C14b2a
b. C3bBb
c. MBL/MASP-C4b2a
d. C3b

2. Which of the following acts as an anaphylatoxin?
a. C3a b. C3b
c. C4b d. C2a

3. Endotoxin acts by:
a. Classical pathway
b. Lectin pathway
c. Alternative pathway
d. None

4. Disseminated *Neisseria* infection is commonly associated with deficiency of:
a. Properdin
b. Factor D
c. C1 inhibitor deficiency
d. Membrane attack complex (MAC)

5. Complement (classical pathway) is best fixed by:
a. IgA b. IgD
c. IgE d. IgM

6. Decreasing order of IgG in complement fixation:
a. IgG1>IgG2>IgG3>IgG4
b. IgG4>IgG3>IgG2>IgG1
c. IgG3>IgG1>IgG2>IgG4
d. IgG2>IgG1>IgG3>IgG4

7. Initiators for alternative pathway are all, *except:*
a. Teichoic acid
b. LPS
c. Cobra venom factor
d. Carbohydrate residue of bacterial cell wall

8. C5 convertase in classical complement pathway is:
a. C14b2a3b b. C14a2a3b
c. C14b2b3b d. C14b2a3a

9. C5 convertase in complement pathway is -
a. C3 convertase + 3b
b. C3 convertase + 3a
c. C1 convertase + 3b
d. C1 convertase + 3a

10. C3 convertase in lectin complement pathway is:
a. C14b2a
b. C3bBb
c. MBL/MASP-C4b2a
d. C3b

11. Early complement deficiency is a predisposing factor for all, *except:*
a. Systemic lupus erythematosus (SLE)
b. Disseminated *Neisseria* infection
c. Glomerulonephritis
d. Pyogenic infections

12. C1 esterase inhibitor deficiency leads to:
a. Paroxysmal nocturnal hemoglobinuria
b. Hereditary angioneurotic edema
c. Immune complex disease
d. Recurrent pyogenic infections

Answers

1. b **2.** a **3.** c **4.** d **5.** d **6.** c **7.** d **8.** a **9.** a **10.** c **11.** b **12.** b

Structure of Immune System

14
CHAPTER

Chapter Preview

- Lymphoid organs—central and peripheral lymphoid organs
- Lymphoid cells—T cells, B cells and NK cells
- Other cells of immune system
- Major histocompatibility complex
- Cytokines

Immune system comprises of lymphoid organs, cells of immune system (lymphoid cells and other cells) and their soluble products called cytokines (Table 14.1).

CENTRAL LYMPHOID ORGANS

Bone Marrow

Almost all the cells in blood have originated from pluripotent hematopoietic stem cells of bone marrow and the process is called hematopoiesis.

- In early fetal life, hematopoiesis occurs in liver; gradually the stem cells migrate to bone marrow. By birth, the stem cells occupy most of the bone marrow space of large bones
- As the individual ages, hematopoietic activity in large bones decreases and after puberty hematopoiesis is mostly confined to axial bones such as pelvis, vertebrae, sternum, skull and ribs

Table 14.1: Structure of immune system

- **Lymphoid organs:** Consist of central and peripheral lymphoid organs
  - **Central or primary lymphoid organs,** e.g. thymus and bone marrow: They host the development of immune cells (hematopoiesis)
  - **Peripheral or secondary lymphoid organs,** e.g. lymph node, spleen, and mucosa-associated lymphoid tissue (MALT)
- **Lymphoid cells:** Consist of lymphocytes such as T cells, B cells and NK cells
- **Other cells of immune system:** Include phagocytes, such as macrophage and microphages (neutrophil, eosinophil and basophil), dendritic cells, mast cells and platelets
- **Cytokines:** They are the soluble products secreted from various cells of immune system. They include interleukins, interferons, tumor necrosis factors, colony-stimulating factors, etc.

- The progenitor T and B cells originate in bone marrow. Further development of B cells occurs in bone marrow itself, whereas the progenitor T cells migrate to thymus for further proliferation.

Thymus

Thymus is the site of proliferation and maturation of T cells.

Development

Thymus is developed in the embryonic life (third month) from third/fourth pharyngeal pouch. It is highly active at birth, continues to grow for many years, reaches its peak size at puberty, and then it degenerates.

Structure

Thymus has two lobes surrounded by a fibrous capsule. Septa arising from capsule divide thymus into lobules, and each lobule is differentiated into an outer cortex and an inner medulla (Fig. 14.1).

Cortex is densely populated and contains:

- **Thymocytes:** Lymphocytes of thymus are called as thymocytes. The cortical thymocytes are immature and many in number
- Cortical epithelial cells and
- Nurse cells (specialized epithelial cells with long membrane extensions that surround many thymocytes).

Medulla is sparsely populated and contains:

- **Thymocytes:** Medullary thymocytes are relatively more mature and fewer in number
- Medullary epithelial cells
- Interdigitating dendritic cells and
- **Hassall's corpuscles:** They are concentric layers of degenerating epithelial cells.

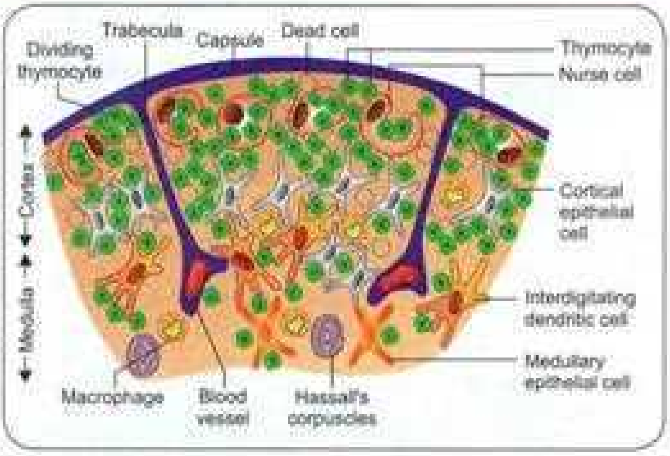


Fig. 14.1: Thymus (cross-section of a portion, schematic)

Thymic Hormones

Several thymic hormones such as thymulin, thymopoietin and thymosin are produced from the epithelial cells of thymus. They are believed to attract the precursor T cells (progenitor T cells) from bone marrow.

Maturation of T Cells

The cell-to-cell interaction between thymocytes and thymic stromal cells (including epithelial cells, dendritic cells and macrophages) and the effect of thymic hormones help in maturation of T cells in thymus. (Maturation of T cells is described in detail later in this chapter).

Central Tolerance

One very interesting fact is that only 2-5% of the developing T cells become mature and released out from thymus; remaining T cells are destroyed as they are either not capable of recognizing major histocompatibility complex (MHC) or are believed to be self-reacting in nature.

- Destruction of such self-reacting T cells prevents development of autoimmunity (immune response against self-antigens)
- Such tolerance to self-antigens mediated by thymus that occurs in embryonic life is called as central tolerance.

Defect in Thymus

Any defect in thymus leads to defect in maturation of T lymphocytes that in turn results in severe life-threatening cell-mediated immunodeficiency disorders.

- **DiGeorge syndrome:** It is an immunodeficiency disorder in man, characterized by congenital aplasia of thymus (refer Chapter 18)
- **Nude mice:** Mice with congenital absence of thymus are called as nude mice.

PERIPHERAL LYMPHOID ORGANS

Lymph Node

Lymph nodes are small bean-shaped organs; they occur in clusters or in chains, distributed along the length of lymphatic vessels. They act as physiological barriers; filter the microbial antigens carried to lymph node by activating the T and B cells.

Structure

Lymph node is divided into three parts—(1) cortex (2) medulla (both are B cell areas) and (3) paracortex (T cell area). It bears the lymphatic and blood vessels. Cortex is surrounded by a capsule and intervened by trabeculae (Fig. 14.2).

- **Cortex:** It contains lymphoid follicles that are composed of mainly B cells and few special type of dendritic cells (called follicular dendritic cells). Lymphoid follicles are mainly of two types
  1. **Primary lymphoid follicles:** They are found before the antigenic stimulus. They are smaller in size and mainly contain the resting B cells.
  2. **Secondary lymphoid follicles:** Following contact with an antigen, the resting B cells start dividing and become activated. The activated B cells differentiate rapidly into plasma cells (which produce antibodies) and memory B cells (which become activated on subsequent antigenic exposure). Follicles become larger in size and called secondary lymphoid follicles. It has two areas:
     - The central area called **germinal center;** contains dividing B cells of various stages. It has two zones—light and dark zones. It is the site where activation of B cells takes place (described in detail in Chapter 15)
     - The peripheral zone called **mantle area;** contains activated B cells.
- **Paracortical area:** It is present in between cortex and medulla. It is the **T cell area** of lymph node; rich in naive

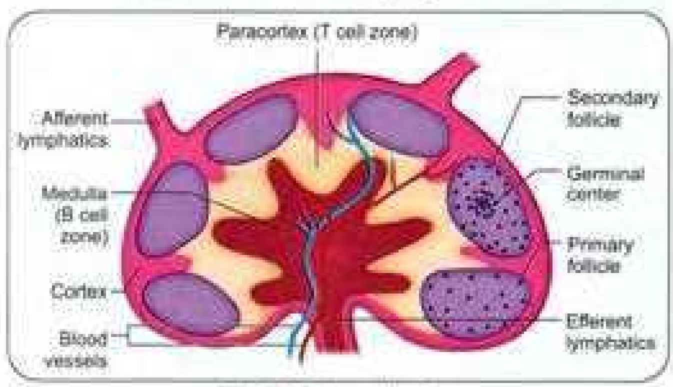


Fig. 14.2: Lymph node

T cells. In addition, it also contains macrophages and interdigitating dendritic cells, which trap the antigens and present to T cells
- **Medulla:** It is the innermost area of lymph node, rich in B-lymphocytes; mainly plasma cells.

Spleen

Spleen is the largest secondary lymphoid organ. It acts as physiological barrier similar to lymph node in clearing the microbial antigens through the stimulation of T and B cells.

Structure

Spleen is situated below the diaphragm on left side of the abdomen. Adult spleen measures about 5-inch in length and weighs 150 g. It is divided into two compartments—(1) central white pulp and (2) outer red pulp, surrounded by capsule and intervened by trabeculae (Fig. 14.3).

1. **White pulp:** It is the central densely populated area, which contains T cells and B cells. It has two parts:
   i. **Periarteriolar lymphoid sheath (PALS):** It is T cell area, rich in T cells; surrounds the branches of the splenic artery.
   ii. **Marginal zone:** It is located peripheral to the PALS and is populated by B cell lymphoid follicles (primary and secondary) and macrophages.
2. **Red pulp:** It is the area that surrounds the sinusoids. It is filled with red blood cells (RBCs). The older and defective RBCs are destroyed here.

Defect in Spleen

As spleen is the site of destruction of most of the microbes, functional or structural abnormalities of spleen or splenectomy, especially in children, often leads to an increased incidence of bacterial sepsis caused primarily by capsulated bacteria such as *Streptococcus pneumoniae, Neisseria meningitidis,* and *Haemophilus influenzae.*

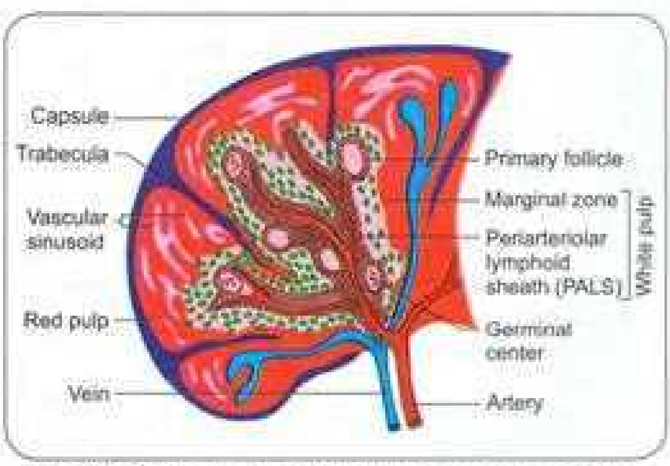


Fig. 14.3: Spleen (cross-section showing red pulp and white pulp area)

Mucosa-associated Lymphoid Tissue (MALT)

The mucous membranes lining the intestine, respiratory, and urogenital tract (total surface area of about 400 m^2) are the major sites of entry for most pathogens. Hence, defense mechanisms are needed in the mucosal sites to prevent the microbial entry.

- Group of lymphoid tissues lining these mucosal sites are collectively known as MALT
- Structurally, MALT may be arranged in two types:
  1. Loose clusters of lymphoid cells (usually found in the lamina propria of intestinal villi).
  2. Lymphoid tissues arranged as organized structures such as tonsils, appendix and Peyer's patches).

MALT in Intestinal Mucosa

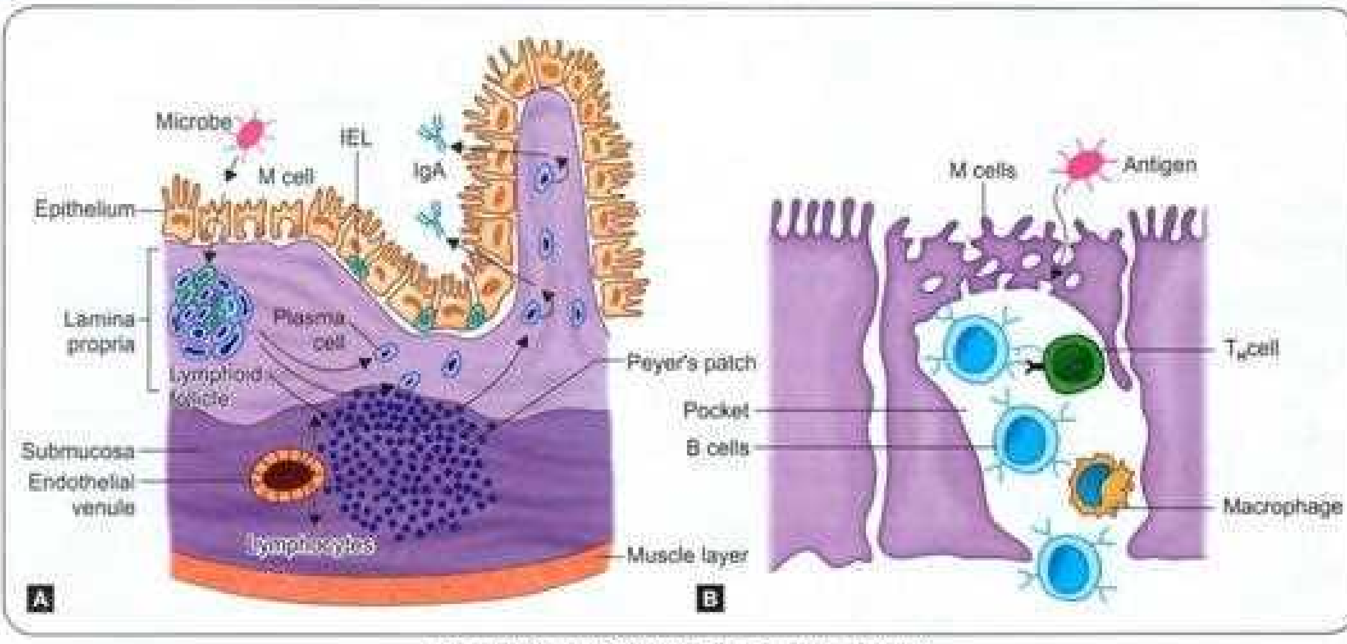
Lymphoid tissues lining the intestinal mucosa are the best studied MALT. They are present in different layers of intestinal wall.

- **Submucosa** contains Peyer's patches. Peyer's patch is a nodule of 30–40 lymphoid follicles (both primary and secondary follicles similar to that of lymph node)
- **Lamina propria** contains loose clusters of lymphocytes (B cells, plasma cells, T helper cells) and macrophages
- **Epithelial layer** contains few specialized lymphocytes called intraepithelial lymphocytes (IELs) and modified epithelial cells (called M cells)
  - **Intraepithelial lymphocytes (IELs)** are the $\gamma\delta$ T cells. The actual function of such T cells is not known, they may encounter the lipid antigens that enter through the intestinal mucosa
  - **M cells:** Described in the box below
  - **Secretory IgA:** These are the dimeric IgA antibodies that are present in the submucosa as well as in the lining epithelium. They prevent the microbial entry at the mucosal sites (local immunity).

M Cells

They are specialized flattened epithelial cells that do not have microvilli; instead they bear deep invaginations or pockets in the basolateral side that contain B cells, T cells and macrophages (Fig. 14.4B).

- M cells act as the portal of entry of a number of microbes such as *Salmonella, Shigella, Vibrio* and poliovirus
- Invading microbes are taken-up by M cells (by endocytosis), then transported in a vesicle and are delivered to the basolateral pockets
- T cells, B cells and macrophages in the underlying lymphoid follicles are activated following contact with the microbe
- B lymphocytes in MALT once activated at a site by antigenic exposure, migrate to other parts of the intestine, secrete the dimeric IgA, and thus extend the local immunity (Fig. 14.4A).



Figs 14.4A and B: A. MALT; **B.** Structure of M cell

Cutaneous-associated Lymphoid Tissue

Similar to MALT, skin also contains a few loose lymphocytes and specialized antigen presenting cells in epidermis called **Langerhans cells**.

LYMPHOID CELLS

Cells of immune system comprise of lymphoid cells or lymphocytes and other cells such as phagocytes (e.g. macrophages and granulocytes), etc.

Lymphocytes are the major components of cells of immune system. There are approximately 10^{11} lymphocytes in the body, accounting for 20–40% of the total white blood cells (WBCs) in blood and 99% of the cells in the lymph (Table 14.2).

CD molecules

Cluster of differentiation (CD) molecules are cell surface markers useful for the identification of cells of immune system. They have numerous functions, often act as surface receptors or some CD proteins may help in cell adhesion. As of 2015, CD molecules of humans are numbered up to 364.

Table 14.2: Distribution of lymphocytes (%) in organs/blood

Tissues	T cell	B cell	NK cell
Bone marrow	5–10	80–90	5–10
Thymus	99	<1	<1
Lymph node	70–80	20–30	<1
Spleen	30–40	50–60	1–5
Peripheral blood	70–80	10–15	10–15

TYPES OF LYMPHOCYTES

Based on function and cell membrane structure, lymphocytes can be of three types—(1) T lymphocytes, (2) B lymphocytes and (3) natural killer (NK) cells.

The T and B lymphocytes can also be classified into naive lymphocytes and lymphoblasts.

Naive Lymphocytes

They are resting B and T lymphocytes that have not interacted with any antigen (unprimed lymphocytes).

- They are also known as small lymphocytes, as they are small in size (6 µm); having thin rim of cytoplasm, larger nucleus with dense chromatin; fewer mitochondria, ribosomes, and lysosomes
- They generally have a short-life span (1-3 months).

Lymphoblasts

When the naive cells interact with antigen in the presence of certain cytokines (e.g. interleukin-7), become activated and transform into lymphoblasts, which eventually differentiate into effector cells or memory cells.

- **Effector cells** function in various ways to eliminate antigen
  - They have short-life span (few days to few weeks)
  - They are large lymphocytes (15 µm in size), having wider rim of cytoplasm with more organelles

Table 14.3: Differences between naive, effector and memory cells

	Naive cell	Effector cell	Memory cell
Location (present mostly in)	Secondary lymphoid organs, e.g. lymph nodes, spleen	Inflamed tissues and mucosal surfaces	Both the locations of naive and effector cell
Cell cycle	Dormant (G0 phase)	Active	Dormant (G0 phase)
Morphology	Small lymphocyte	Large lymphocyte	Small lymphocyte
Life span	Short	Short	Long
Function	Transforms to effector cell on primary exposure to antigen, occurs slow due to lag period	Eliminate antigen	Transforms to effector cell on secondary exposure to antigen, occurs fast without lag period
Surface markers			
CD127 (IL-7R)	High	Low	High
CD45 isoform	CD45RA	CD45RO	CD45RO
CD25 (IL-2R α) on T cells	No	Yes	Yes
CD27 on B cells	No	Yes	Yes
B cells producing Ig types and their affinity	IgM and IgD Low affinity	IgG, IgA, IgE High affinity	IgG, IgA, IgE, IgM and IgD High affinity

- Antibody producing plasma cells are classical example of effector B cells; whereas effector T cells include helper T cells and cytotoxic T cells.

❖ **Memory cells:** They remain dormant like naive cells but are capable of transforming into effector cells rapidly on subsequent antigenic challenge
- They have a longer life span; providing long-term immunity against many pathogens
- They look like small lymphocytes but can be distinguished from naive cells by the presence or absence of certain surface markers (Table 14.3).

T LYMPHOCYTES

T cells constitute 70–80% of blood lymphocytes. Unlike B cells, they do not have microvilli on their surface. They bear specialized surface receptors called T cell receptors (TCR).

T Cell Receptor

The T cell receptors (TCR) of T cells are equivalent to the surface immunoglobulins (B cell receptors) of the B cells. Their main function is antigen recognition. Unlike B cell receptor which binds to antigen directly, TCR does not recognize antigen by itself. It can only respond to an antigen which is processed and presented by the antigen presenting cells, such as macrophages.

TCR–CD3 Complex

Most T cell receptors (95%) comprise of two chains (α and β) which in turn have three regions—(1) extracellular domain, (2) transmembrane domain, and (3) cytoplasmic tail. The extracellular domain of each polypeptide chain has 2 regions (variable and constant region). About 5% of TCRs do not have α/β chains, instead they bear γ/δ chains. TCR is active only when both the chains (α and β) complex with CD3 molecule (Fig. 14.5).

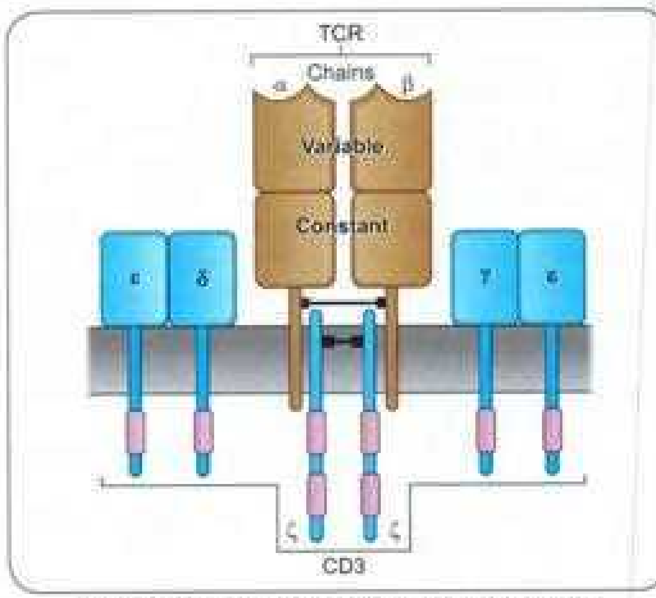


Fig. 14.5: Structure of T cell receptor - CD3 complex

❖ The **variable region** of α and β chains of TCR bind to the presented antigens. They are polymorphic in nature. Rearrangement of α and β genes during T cell development can produce large number of different combinations of TCRs. Each TCR is capable of recognizing a particular epitope of an antigen

❖ The **CD3 complex** consists of three pairs of polypeptide chains—ξξ (zeta-homodimer), δε (delta-epsilon heterodimer) and γε (gamma-epsilon heterodimer)

- Following binding of antigen to α and β chains of TCR, a signal is generated that is transmitted through the CD3 complex leading to activation of T cells.

T Cell Development

The major events of T cell maturation take place in thymus, in contrast to bone marrow for B cells.

- The progenitor T cells are originated from the bone marrow (or liver in fetal life) and then migrate to thymus through bloodstream
- Developing T cells in the thymus (collectively called as thymocytes) pass through series of stages that are marked by characteristic changes in their cell surface markers
- Most of the development events take place in the cortex of thymus, under the influence of thymic stromal cells which secrete thymic hormones and lymphopoietic growth factor IL-7.

The sequence of events of T cell development is as follows (Fig. 14.6):

- **Double negative (DN) T cells:** T cell precursors after entering into the thymus transform into double negative T cells ($CD4^- CD8^-$). These cells are so called because, they do not express the surface markers of mature T cells, i.e. CD4 (marker of helper T cells) and CD8 (marker of cytotoxic T cells). They are further subdivided into four subsets. DN1 T cells are developed first, then → DN2 → DN3 → DN4 T cells
    - As the DN1 cells develop into DN2 cells, genes for γ, δ and β chains of TCR begin to rearrange and the CD3 molecules are then expressed
    - At the late DN2 stage, T cell precursors fully commit to any one of the T cell lineages:
        - Five percent carrying TCR γδ develop into mature γδ T cells and
        - The remaining (95%) of the cells develop further to DN3 T cells.
    - As the DN3 cells differentiate into DN4 cells, the β chain of TCR is complexed with CD3 molecules to form pre-TCR
    - In DN4 stage, rearrangement of α chain takes place and then it combines with β chain to form complete αβ TCR. The αβ T cells develop further to express CD4 and CD8 molecules and transform to double positive (DP) T cells.
- **Double positive (DP) T cells ($CD4^+ CD8^+$):** They are immature T cells, carrying both CD4 and CD8 molecules on their surface. They further undergo the one of the following fate:
    - **Positive selection:** The 5% of DP T cells, whose αβ receptors are capable of recognizing their MHC molecules are positively selected. This results in MHC restriction

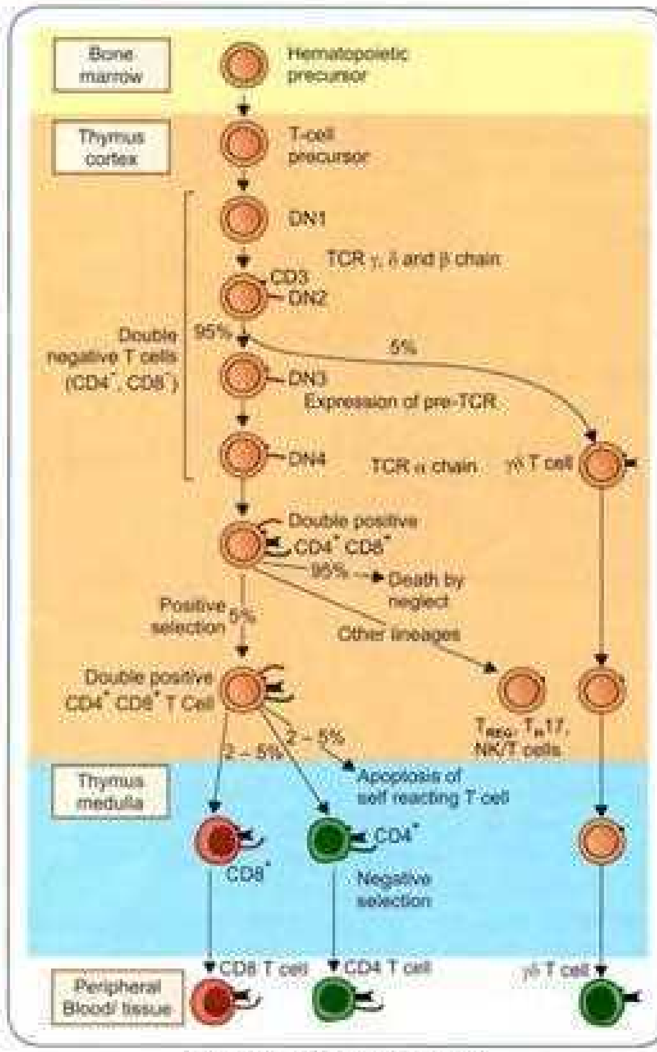


Fig. 14.6: T cell development

    - **Death by neglect:** Majority of DP cells (95%) fail positive selection because they do not specifically recognize their MHC molecules
    - **Negative selection:** The survived cells that undergo positive selection (5%) are MHC restricted. However, some of these surviving cells (2-5%) react to the self-antigens and therefore, they are selected to be killed by apoptosis and removed (negatively selection)
    - The remaining double positive T cells (2-5%) having αβ type TCR selectively shut off the expression of either CD4 or CD8 molecules and eventually become single positive mature T cells ($CD4^+/CD8^-$ or $CD4^-/CD8^+$).
- **Mature T cells** (e.g. $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells) acquire thymus specific antigens and then are released into the circulation and migrate to the peripheral lymphoid organs where they respond to the antigenic stimulus.

Types of T Cells

Effector T Cells

There are two types of effector T cells—(1) $CD4^+$ helper T cells and (2) $CD8^+$ cytotoxic T cell.

- **Helper T cells:** Helper T cells (T_H) possess CD4 molecules as surface receptors. They recognize the antigenic peptides that are processed by antigen presenting cells and presented along with MHC-II molecules (major histocompatibility complex)
  - Following antigenic stimulus, the helper T cells differentiate into either of the two types of cells—(1) T_H1 and (2) T_H2 subset; each secrete specific cytokines which modulate the cellular and humoral immune responses respectively (for detail, refer Chapter 15)
  - **T_H17 cells:** Recently a third subset of T helper cells called T_H17 cell, has been discovered and characterized. It produces IL-17 and IL-22, and is primarily involved in recruiting neutrophils which in turn kill the microbes as well as induce inflammation. They contribute to the pathogenesis of many autoimmune inflammatory diseases such as psoriasis, inflammatory bowel disease, rheumatoid arthritis, and multiple sclerosis.
- **Cytotoxic T cells:** In contrast to T_H cells, cytotoxic T cells (T_C) possess CD8 molecules and recognize the intracellular antigens (e.g. viral antigens or tumor antigens) that are processed by any nucleated cells and presented along with MHC-I. In general, T_C cells are involved in destruction of virus infected cells and tumor cells (for detail, refer Chapter 15).

Rare Subtypes of T Cells

- **Regulatory T cells (T_{REG} cells):** The T_{REG} cells (formerly known as suppressor T cells) are a subpopulation of T cells which regulate the immune system
  - They provide tolerance to self-antigens (known as peripheral tolerance), and thus prevent the development of autoimmune disease
  - Surface markers: T_{REG} cells possess surface markers such as CD4, CD25 and Foxp3 (a forkhead family transcription factor)
  - Deficiency of Foxp 3 receptors leads to a severe form of autoimmune disease known as **I**mmune dysregulation, **P**olyendocrinopathy, **E**nteropathy **X**-linked (**IPEX**) **syndrome**.
- **γδ T cells:** γδ T cells represent a small subset of T cells (5%) that possess a distinct TCR composed of γ and δ chains; instead of α/β chains. They lack both CD4 and CD8 molecules
  - They differ from the conventional αβ T cells by the fact that they do not require antigen processing and MHC presentation of peptides
  - They are part of innate immunity as the γδ receptors exhibit limited diversity for the antigen
  - They are usually found in the gut mucosa, within a population of lymphocytes known as IELs
  - The function of γδ T cells is not known, they may encounter the lipid antigens that enter through the intestinal mucosa.

B LYMPHOCYTES

B lymphocytes are the mediators of humoral immunity; constitutes 10-15% of blood lymphocytes. They are named after their site of maturation (**b**ursa of Fabricius in birds and **b**one marrow in humans and other mammals). B cells proliferate through various stages, first in bone marrow, then in peripheral lymphoid organs (Fig. 14.7).

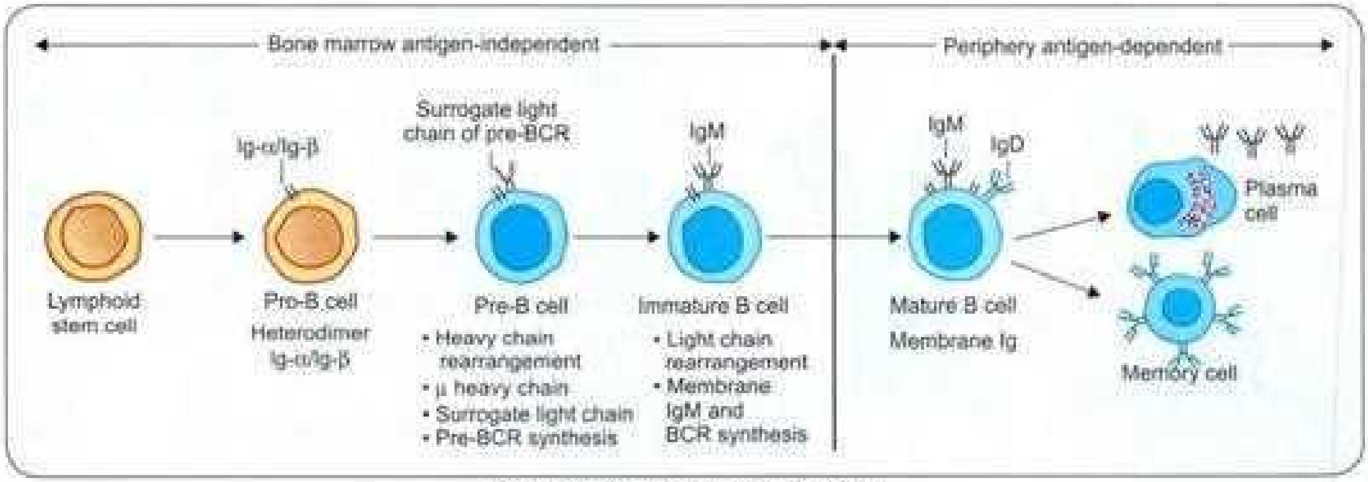


Fig. 14.7: B lymphocyte development

Development of B Cells in Bone Marrow

Initial stages of B cell proliferation occur in bone marrow; independent of exposure to antigen.

Pro-B Cells (Progenitor B Cells)

They are the earliest bone marrow cells of B cell lineage. They do not produce immunoglobulin (Ig) but express a heterodimer Igα/Igβ that forms a part of the B cell receptor (BCR) in future.

Pre-B Cells (Precursor B Cells)

Pro-B cells differentiate into pre-B cells by undergoing Ig heavy chain genes rearrangement. This stage is characterized by translation of heavy chain genes.

- **μ heavy chain** is synthesized first and then it accumulates in cytoplasm
- **Surrogate light chain:** Pre-B cells do not form light chains; but synthesize some similar but smaller peptides, which are not true light chains. They are called surrogate light chain
- **Pre-BCR synthesis:** μ chain and surrogate light chains complex with heterodimer Igα/Igβ to form pre-B cell receptor
- Pre-BCR always allows the expression of only one out of the two alleles coding for an Ig chain by inhibiting the other allele. This phenomenon is called as **allelic exclusion** by which the clonal specificity of B cells is maintained.

Immature B Cells

Pre-B cells proliferate into immature B cells which are characterized by the following properties:

- **Light chain genes rearrangement** takes place and following which the light chains are expressed. By allelic exclusion, only one type of light chain (either kappa or lambda) is allowed to express
- **Membrane IgM:** Heavy chain μ and its light chain join to form complete IgM molecule, which is complexed with heterodimer Igα/Igβ on the B cell surface to form B cell receptor (Fig. 14.8)

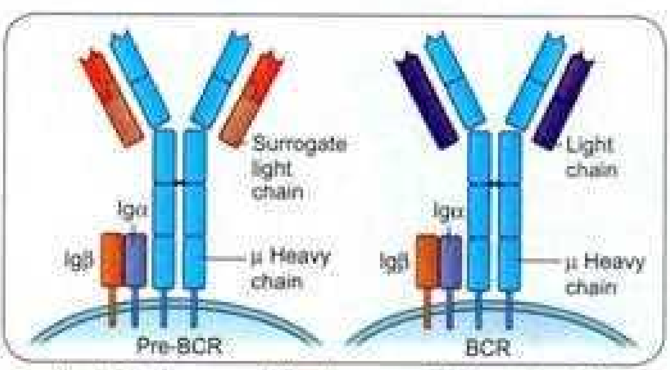


Fig. 14.8: Structure of B cell receptor (BCR) and pre-BCR

- **Tolerance:** Some of the immature B cells are capable of recognizing self-antigens. Tolerance to those B cells are essential for prevention of autoimmunity. Following contact with a self-antigen, the tolerance is developed either by:
  - **Receptor editing:** A process by which the Ig genes coding light chains are rearranged so that a different (edited) B cell receptor is produced which no longer recognizes the self-antigen or
  - **Negative selection:** By apoptosis of self-reacting immature B cells in spleen.

Development of B Cells in Peripheral Lymphoid Organs

Immature B cells migrate from bone marrow to peripheral lymphoid organs (lymph node and spleen) where they transform into mature B cells following contact with appropriate antigen.

Mature or Naive B Cells

Most mature B cells (95%) belong to the follicular B cell type and produce surface receptor IgD in addition to IgM. They play an important role in humoral immune response (described in detail in Chapter 15).

- Following antigenic stimulus, the mature B cells transform into activated B cells (lymphoblasts) which further differentiate into either effector B cells, i.e. plasma cells (majority) or memory B cells
- **Plasma cells** (antibody secreting cells): They are oval, large (15 μm size), with an eccentrically oval nucleus containing large blocks of peripheral chromatin (cartwheel appearance) and the cytoplasm containing abundant organelles. They have a short life span of two or three days.

However, there are few rare mature B cell types such as B-1 cell and marginal zone B cells which have limited diversity and are components of innate immunity.

- **B-1 cells:** They are found mostly in the peritoneal cavity, coated by surface markers IgM (natural antibodies) and CD5 molecules; but lack IgD
- **Marginal-zone B cells:** They are present at the edges of lymphoid follicles of spleen and are produced in response to the polysaccharide antigens.

B cells are the main components of humoral immunity; produce five classes of antibodies, which in turn have various biological functions (described in Chapters 11 and 15).

Differences between T cell and B cell are given in Table 14.4.

Table 14.4: Differences between T cell and B cell

Property	T cell	B cell
Origin	Bone marrow	Bone marrow
Maturation	Thymus	Bone marrow
Peripheral blood	70–80% of total lymphocytes	10–15% of total lymphocytes
Antigen recognition receptors	T cell receptors complexed with CD3	B cell receptor-surface IgM or IgD complexed with Igα/Igβ
CD markers	CD 3, 4, 8	CD19, 21, 24
Thymus specific Ag	Present	Absent
Microvilli on the surface	Absent	Present

NATURAL KILLER CELLS

Natural killer (NK) cells are large granular lymphocytes that constitute 10-15% of peripheral blood lymphocytes. They are derived from a separate lymphoid lineage. Similar to cytotoxic T cells, NK cells also are involved in destruction of virus infected cells and tumor cells (described in Chapter 15).

OTHER CELLS OF IMMUNE SYSTEM

Macrophage

Macrophages were first described by Russian scientist Metchnikoff (1883) who suggested that the monocyte-macrophage system plays a vital role in host defense by performing two important functions— (1) phagocytosis and (2) antigen presentation.

Monocytes/macrophages originate from bone marrow, from a separate lineage, i.e. from the granulocyte-monocyte progenitor cells.

Monocytes: They are present in blood; they are the largest blood cells measuring 12-20 µm size. They do not divide and have an average transit time of 8 hours in blood; then they migrate to tissues.

Macrophages: When monocytes migrate to tissues, they transform into macrophages (Fig. 14.9). Macrophages differ from monocytes in the following:

- 5-10 folds larger than monocytes
- Contain more lysozymes and cell organelles
- Produce more lytic enzymes and cytokines
- Possess greater phagocytic activity
- Have a longer life in tissues (months to years).

Most macrophages are motile, travel by amoeboid movement throughout the tissues and are called as free or wandering macrophages. While, some reside in particular tissue, become non-motile and are called fixed macrophages. Macrophages in various tissues are designated by different names (Table 14.5).

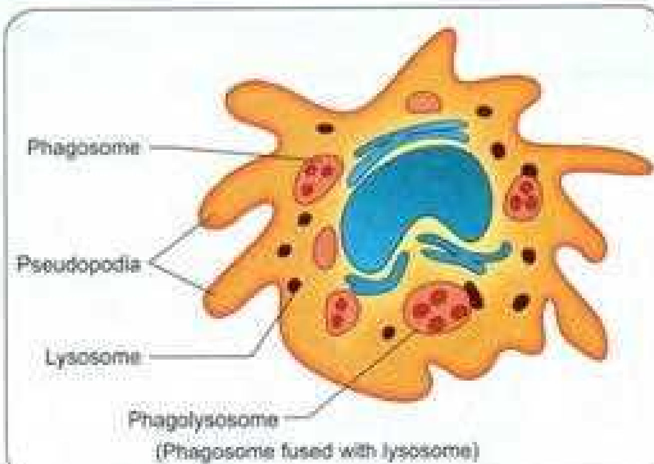


Fig. 14.9: Macrophage

Secretory Products of Macrophages

Activated macrophages in turn produce a number of secretory products which mediate various functions (Table 14.6).

Functions of Macrophage

- **Phagocytosis:** Macrophages are the principle cells involved in phagocytosis. Macrophages from various tissues are together called as the mononuclear phagocyte system (or previously known as the reticuloendothelial system). They also remove old dying cells from the body. The steps involved in phagocytosis are as follows (Fig. 14.10):
  - **Recognition:** Attachment of the microbe to the receptors present on the surface of macrophage, such as toll like receptors or immunoglobulin G (IgG)
  - **Engulfment:** Microbe is ingested with subsequent formation of a phagocytic vacuole (phagosome)

Table 14.5: Types of macrophages

Body sites	Macrophage designation
Peripheral blood	Monocytes
Tissues	Macrophages
Liver	Kupffer cells
Brain	Microglial cells
Kidney	Mesangial cells
Lungs	Alveolar macrophages
Bone	Osteoclasts
Inflammation site	Epithelioid cells, multinucleated cell (Langhans giant cells)
Connective tissues	Histiocytes
Placenta	Hofbauer cell
Lymphoid follicle	Tingible body macrophage

Table 14.6: Secretory products of activated macrophages

Secretory Products	Examples
Enzymes	Lysozyme, acid hydrolases, elastases, Phosphatases, lipases, collagenases
Free radicals	**Reactive oxygen intermediates** • Superoxide anion (O_2^-) • Hydroxyl radicals (OH) • Hydrogen peroxide (H_2O_2) • Hypochlorite anion(ClO^-) **Reactive nitrogen intermediates** • Nitric oxide (NO) • Nitrogen dioxide (NO_2) • Nitrous acid (HNO_2)
Cytokines	• Interferon α, β • Interleukins (IL-1, IL-6, IL-8, IL-12) • Tumor necrosis factor- α (TNF- α)
Growth factors	• Colony-stimulating factors (CSF) • Platelet-derived growth factor (PDGF) • Platelet-activating factor (PAF) • Transforming growth factor β (TGF-β)
Coagulation factors	• Factor V, VII, IX, X • Prothrombin
Complement factors	C5, C8 properdin, factor B, D, I

- **Fusion** of lysosome with phagosome to form phagolysosome
- **Killing or degradation** of the ingested microbes which is accomplished largely by both:
  - Oxygen independent killing—degradation by lysosomal enzymes
  - Oxygen dependent killing by generating free radicals (Table 14.6).

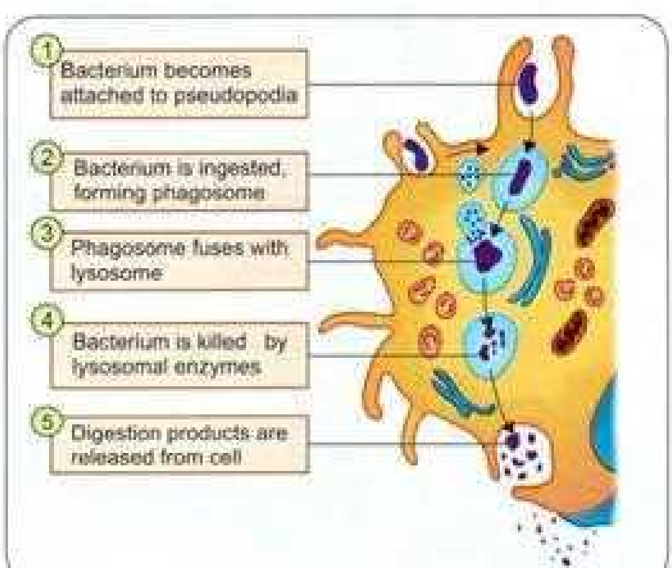


Fig. 14.10: Mechanism of phagocytosis

- **Antigen presentation:** Macrophages also promote adaptive immunity, by acting as antigen presenting cells (APCs). Macrophages capture the antigen, process into smaller antigenic peptides and present the antigenic peptides along with the MHC class II molecules to the helper T cells; thus facilitating helper T cell activation
- **Activated macrophages:** On exposure to certain cytokines such as interferon-γ, macrophages become activated. The activated macrophages have greater phagocytic ability and produce many cytokines that act against intracellular bacteria, virus infected cells and tumor cells. They also express higher level of MHC class II, hence can act as efficient APCs
- **Secretory products** of macrophages have various biological functions (Table 14.6):
  - Interleukin 1 (IL-1): It promotes inflammatory responses, fever, and activate helper T cells
  - IL-6 and TNF-α: They promote innate immunity, (inflammation and fever) and eliminate the pathogens
  - Interferon α and β: They have antiviral activity
  - TNF-α: It lyses the tumor cells (antitumor activity)
  - Growth factors, such as CSF (colony-stimulating factor)—promote hematopoiesis
  - Following tissue injury, various mediators are secreted from macrophage; which help in tissue repair and scar formation.

Dendritic Cells

Dendritic cells are specialized antigen presenting cells of immune system (Fig. 14.11).

- **Naming:** They possess long membranous cytoplasmic extensions resembling dendrites of neurons; hence, they are named as dendritic cells
- **Origin:** Dendritic cells originate from bone marrow, but the pathway is uncertain. They either develop as a separate lineage from stem cells or may originate from the macrophage lineage
- **Types:** Dendritic cells are widely distributed; present in various tissues (Table 14.7)
- **Function:** Dendritic cells are nonphagocytic in nature. They are the most efficient APCs; their main function is to capture, process and present the antigenic peptides on their cell surface to the helper T cells
  - They carry high level of MHC class II and co-stimulatory B7 molecules
  - They act as messengers between the innate and the adaptive immune systems
- **Follicular dendritic cells:** They are present in lymphoid follicles. They differ from other dendritic cells by the fact

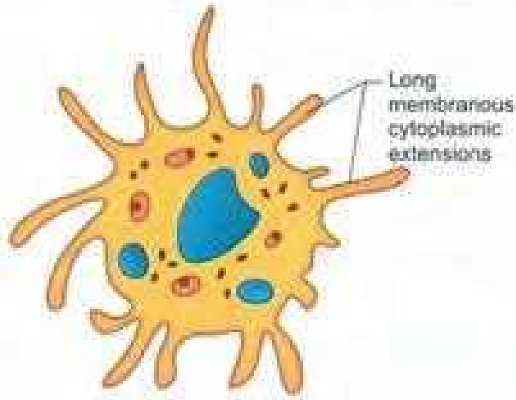


Fig. 14.11: Dendritic cell

that they recognize antigen-antibody complex rather than antigen alone. They do not act as APCs and do not express MHC class II or B7 molecules (for detail refer Chapter 15).

Granulocytic Cells

Granulocytes (e.g. neutrophils, eosinophils and basophils) are a category of white blood cells, characterized by the presence of granules in their cytoplasm. They differ from each other by cell morphology and cytoplasmic staining and function.

Neutrophils

They have a multilobed nucleus and a granulated cytoplasm that stains with both acid and basic dyes. They are often called as polymorphonuclear leukocyte (PMN) because of their multilobed nucleus.

- Their cytoplasm is heavily granular; contains several granules such as myeloperoxidase, lysozyme, defensins, elastase, gelatinase, etc.
- Neutrophils constitute 50–70% of the circulating white blood cells (WBCs). However, the level is greatly increased in presence of infection under the influence of certain cytokines, such as IL-8
- They are the principal phagocytes of innate immunity; the mechanism of microbial killing is similar to that of macrophages, i.e. both by oxygen dependent and independent mechanisms.

Eosinophils

They have a bilobed nucleus and a granular cytoplasm that stains red with the acid dye eosin.

- They are also phagocytic, constitute only 1–3% of total leukocytes, but the number is greatly increased in certain allergic conditions and helminthic infections
- Interleukin-5 is believed to be the eosinophil chemotactic factor.

Table 14.7: Distribution and functions of dendritic cells

Types of dendritic cells	Site	Function
Langerhans cells*	Skin and mucosa	Antigen presentation, express high MHC-II and B7 molecules
Interstitial dendritic cells	Organs (lungs, liver, spleen, etc.)	
Interdigitating dendritic cells	Thymus	
Circulating dendritic cells	Blood and lymph	
Follicular dendritic cells	Lymph nodes	• B cells maturation, and differentiation • MHC-II and B7 molecules absent • Coated with Ag-Ab complex

*Langerhans cell is a dendritic cell; whereas Langhans giant cell is a macrophage

Basophils

They are nonphagocytic granulocytes that contain several secreting granules. They have a lobed nucleus and heavily granulated cytoplasm that stains with the basic dye, methylene blue. They resemble mast cells in their function. Granules are rich in histamine and other mediators that play a major role in certain allergic responses.

Mast Cells

Mast cells are present in various body sites, such as skin, connective tissues of various organs, and mucosa (respiratory and intestinal). Like circulating basophils, mast cells also contain cytoplasmic granules rich in histamine and other active substances and play an important role in the development of certain allergic (type I hypersensitivity) reactions.

MAJOR HISTOCOMPATIBILITY COMPLEX

The major histocompatibility complex (MHC) is a group of genes coding for a set of host cell surface molecules that bind to peptide fragments derived from pathogens and display them on the host cell surface for recognition by the appropriate T-cells.

- These are present in almost all the human cells, but first discovered on the surface of leukocytes; hence in humans, the MHC coded proteins are also called as human leukocyte antigens (HLA)
- MHC molecules serve as a unique identification marker for every individual as the genetic sequence of MHC genes is different for every individual
- Following transplantation of a graft, the recipient mounts an immune response against the graft's MHC molecule and vice versa. Greater the difference of the MHC gene

sequence between the graft and the recipient, greater is the immune response and greater is the rejection of the graft
- The acceptance or rejection of the graft is directly dependent on the MHC molecules of the graft and the recipient. As the MHC molecules determine the compatibility between the graft and host tissues, they are named as **histocompatibility antigens**.

MHC GENES (HLA COMPLEX) AND THEIR PRODUCTS

In humans, HLA complex coding for MHC proteins are located in **short arm of chromosome-6**. The HLA complex extends over 4000 kbp length covering >100 genes. The genes are clustered in three regions named as MHC region-I, II and III (Fig. 14.12).

MHC Region-I

It is about 2000 kbp in length, comprises of three class I genes called HLA-A, HLA-B and HLA-C genes which code for HLA-A, HLA-B and HLA-C proteins respectively, each one is capable of forming the α-chain of MHC class I molecules.
- MHC-I proteins are located on the surface of all nucleated cells (except sperm cells) and platelets. They are absent in RBCs
- They present the peptide antigen to CD8 T cells.

MHC Region-II

It spans over 1000 kbp length; comprises of three regions—(1) DP, (2) DQ and (3) DR genes encoding DP, DQ and DR proteins respectively, each one is capable of forming the α and β-chain of MHC class II molecules. In addition, MHC II region also contains certain other non-classical genes, such as DO, DM, LMP and TAP (transporter associated with antigen processing) that help in antigen processing and presentation.
- MHC-II proteins are located on the surface of antigen presenting cells
- They present the peptide antigen to CD4 T cells.

MHC Region-III

It is also 1000 kbp in length. It is not involved in antigen presentation, instead it carries genes that code for complement factors (C2, C4, C3 convertase, factor B and properdin), heat shock protein (HSP70) and tumor necrosis factor (TNF-α and β) and steroid 21-hydroxylases.

STRUCTURE OF MHC MOLECULE (FIG. 14.13)

MHC Class I Molecule

It is composed of α chain (glycoprotein, 45kDa) coded by HLA class I genes and β2 *microglobulin* (non-glycosylated 12 kDa protein, encoded by a non MHC gene from chromosome 15).
- The α chain is folded further and organized into three extracellular globular domains—α1, α2 and α3 (each containing 90 amino acids) and a cytoplasmic tail
- The association of β2 microglobulin with α chain is necessary for the expression of MHC I molecules on to the cell surface. In **Daudi cells** (a type of human B cell tumor cell which are not able to produce β2 microglobulin), it is observed that they synthesize MHC-I but do not express them on cell surface.

Role of MHC Class I Molecules

- The antigen peptide binding groove of class I MHC molecule (i.e. the site, where the antigen peptide binds) is formed by the cleft between α1 and α2 domains
- The α3 domain binds to CD8 molecule present on cytotoxic T cells during antigen presentation.

MHC Class II Molecule

It comprises of one α chain (33 kDa) and one β chain (28 kDa). The α and β chains in turn consist of two domains

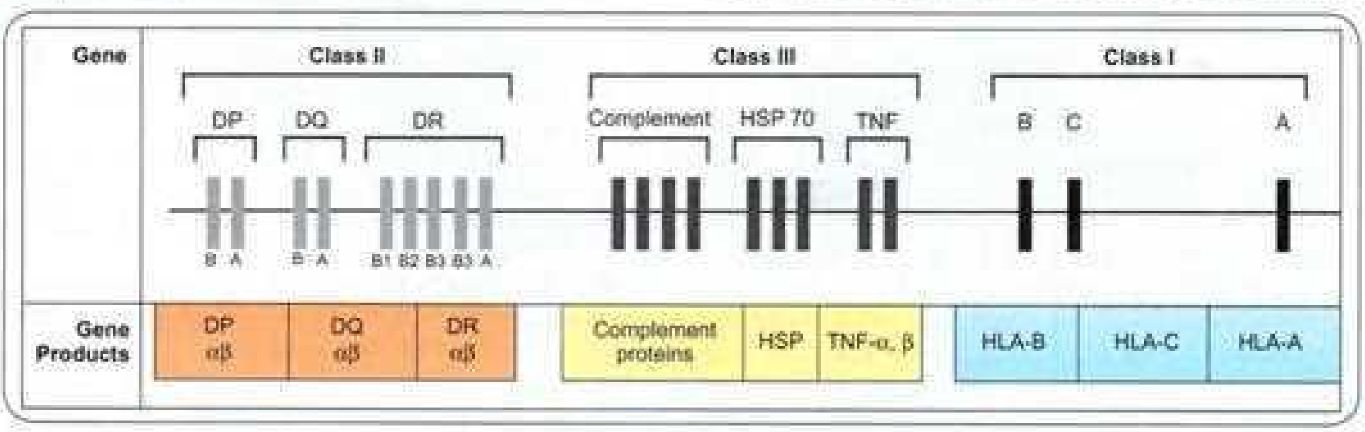


Fig. 14.12: Structure of human MHC complex

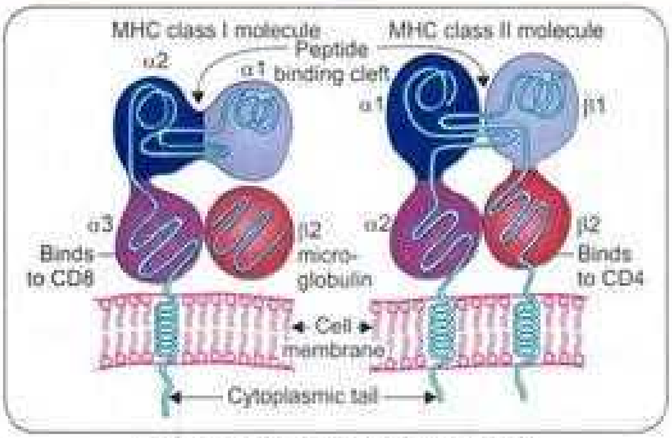


Fig. 14.13: Structure of MHC molecules

each—(1) α1 and α2 and (2) β1 and β2, respectively and cytoplasmic tails.

- The antigen peptide binding groove is formed by the cleft between α1 and β1 domains
- The β2 domain interacts with CD4 molecule of helper T cells during antigen presentation.

Differences between MHC class I and II molecules are described in Table 14.8.

MHC Polymorphism

MHC molecules are involved in antigen processing and presentation to the T cells. Each MHC molecule specifically presents a certain antigenic peptide to the T cells. Since there is a wide spectrum of different antigenic peptides (with specific sequences) derived from various antigens; there is a likewise need of different MHC molecules capable of recognizing these peptides. This is made possible by the polymorphic feature of MHC genome; by which it is capable of producing a wide array of MHC molecules with vast antigenic specificities. There are three mechanisms by which MHC molecules show such a high level of polymorphism.

1. **Multiple gene loci:** Both MHC class I and II molecules are coded by multiple genes. Gene of each locus codes for a similar but not identical chain. For example:
   - MHC I molecules (α chain) are coded by any of the three loci of class I region, i.e. HLA-A or B or C loci
   - MHC II molecules (α and β chains) are coded by any of the three loci of class II region, i.e. DP or DQ or DR loci.
2. **Multiple alleles for each locus:** In a given species, extraordinarily large numbers of different alleles are known to exist for each locus. MHC genes are one of the most polymorphic genes known. For example:
   - For class I MHC region in humans, there are 240 alleles for HLA-A, 470 alleles for HLA-B and 110 alleles for HLA-C. The MHC class I region of any individual would have one of the allele from each HLA-A, B and C allele bank
   - So, there are total 240 × 470 × 110 number of theoretical combinations possible for class I MHC region. These

Contd...

Table 14.8: Differences between MHC class I and MHC class II molecules

	MHC class I	MHC class II
Present on	All nucleated cells (except sperms) and platelets	Antigen presenting cells (APCs)
Peptide antigen is	Presented to CD8 T cells	Presented to CD4 T cells
Nature of peptide antigen	Endogenous or intracellular (viral/ tumor antigen)	Exogenous
Peptide antigen (size)	8–10 amino acid long	13–18 amino acid long
Antigen presentation pathways	Cytosolic pathway	Endocytic pathway
Peptide-binding site	α1/α2 groove	α1/β1 groove
CD4 or CD8 binding site	α3 binds to CD8 molecules on T_c cells	β2 binds to CD4 on T_H cells

Contd...

alleles encode for products that vary from one another by 5–10% of their DNA sequence
   - Similar polymorphism also exists for alleles of class II DP, DQ and DR loci.
3. **Codominant expression:** MHC genes are expressed in *codominant fashion*, i.e. the alleles inherited from parents (one from father and one from mother) are simultaneously and equally expressed.

Regulation of MHC Expression

There are several regulatory mechanisms that control the expression of MHC genes in different cell types.

- **Transcription factors:** MHC genes have promoter sequences at their 5' end which are regulated by certain transcription factors such as **CIITA**, and **RFX** (both bind to MHC II promoter genes and increase their transcription). Defects in CIITA, and RFX cause one of the form of **Bare lymphocyte syndrome**
- **Cytokines** also influence MHC expression
  - IFN-γ activates both MHC-I and II promoter genes
  - IL-4 increases expression of class II MHC molecules on resting B cells.
- **Corticosteroid and prostaglandins** decrease the expression of MHC II molecules
- **In many viral infections,** the viral antigens inhibit various components of MHC-I (e.g. adenovirus proteins inhibit TAP, cytomegalovirus proteins inhibit β2 microglobulin). As a result, MHC-I expression is suppressed.

MHC and Disease Susceptibility

Many HLA alleles have been associated with increased susceptibility to certain diseases (Table 14.9). The relative

Table 14.9: Diseases associated with certain HLA alleles

HLA allele	Associated disease
HLA B27	Ankylosing spondylitis, Reactive arthritis (*Yersinia*, *Salmonella*, gonococcus) and Reiter's syndrome
DR-2	Multiple sclerosis, Goodpasture's syndrome
DR-3	Myasthenia gravis, systemic lupus erythematosus
DR-3/DR-4	Insulin-dependent diabetes mellitus
DR-4	Rheumatoid arthritis
A3/B14	Hereditary hemochromatosis

risk of occurrence of the disease in presence of the identified allele varies. For example, HLA B27 is strongly associated with ankylosing spondylitis (90 times higher risk than those not expressing HLA B 27).

SOLUBLE PRODUCTS OF LYMPHOID CELLS

CYTOKINES

Definition

Cytokines are chemical substances which serve as messengers, mediating interaction and communication between the various cells of immune system.

Major Classes of Cytokines

Present nomenclature of cytokines includes all the compounds that were known earlier by various names, such as:
- Lymphokines—produced by lymphocytes
- Monokines—produced by monocytes and macrophages
- Interleukins—produced by WBCs and acting on the same or different WBCs
- Chemokines—involved in chemotaxis and other leukocyte behavior.

Properties of Cytokines

Cytokines are comparable to growth factors and hormones in many ways such as all of them act at very low concentration (picomoles) and through specific receptors. However, there are some differences also.
- Growth factors are produced constitutively while cytokines are inducible, i.e. produced only after the activation of their cells of origin
- Hormones have mostly endocrine effects; whereas cytokines have broad range of effects, which include (Fig. 14.14):
  - Autocrine effect—that acts on the same cell
  - Paracrine effect—that acts on the adjacent cell
  - Endocrine effect—that acts on a cell present at a distant site.

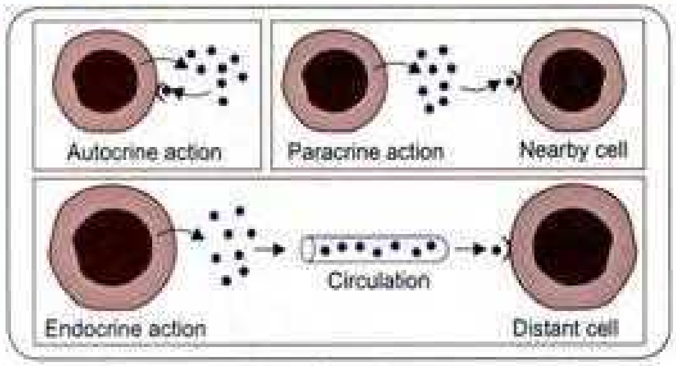


Fig. 14.14: Action of cytokines on target cell

- Unlike hormones and growth factors which work mostly independently, cytokines can work together and there are various types of interactions occurring between cytokines:
  - **Pleiotropy and redundancy effect:** Pleiotropy refers to same cytokine having different actions on different target cells, whereas redundancy implies to different cytokines producing the same effect on the same target cell (Fig. 14.15)
  - **Synergy and antagonism effect:** Two cytokines may augment each other's action producing a larger effect (synergism) or may oppose each other's action (antagonism)
  - **Cascade effect:** It refers to a series of effects mediated by different cytokines. One cytokine acts on a target

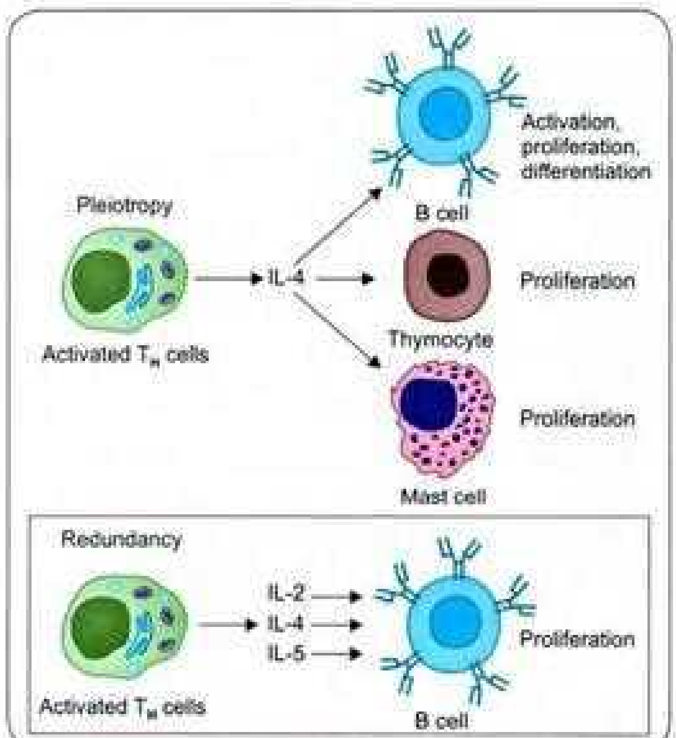


Fig. 14.15: Pleiotropy and redundancy effect of cytokines

cell to produce another cytokine which in turn acts on another target cell and so on.

Structure of Cytokines

Cytokines are glycoproteins with molecular weight less than 30 kDa. Most cytokines display high degree of α-helix structure but no β-structure. Cytokines characterized so far belong to one of the four groups—(1) the hematopoietin family, (2) the interferon family, (3) the chemokine family, or (4) the tumor necrosis factor family; each interacting with a separate class of cytokine receptors present on target cell surface.

Functions of Important Cytokines

Though cytokines are secreted by a wide variety of cells, the major producers are T_H cells and macrophages. Cytokines produce a range of overlapping functions on the target cells/tissues, which can be broadly categorized into two groups:

1. Promote development of cellular and humoral responses of adaptive immunity:
   - Interferon-γ
   - Cytokines such as IL-2, IL-4, IL-5
2. Cytokines promote various responses of innate immunity:
   - Induction of inflammatory responses—by IL-1, IL-8, TNF-α
   - Regulation of hematopoiesis—by colony-stimulating factors, IL1, IL-3, IL-7, IL-9, IL-11, etc.
   - Antiviral activity—By interferon- α and β
   - Antitumor activity—By TNF- α and β
   - Pyrogenic activity—By TNF- α, IL-1 and IL-6.

The functions of individual cytokines are summarized in Table 14.10. Cytokines of cellular and humoral immune responses are also discussed in Chapter 15.

Table 14.10: Sources and functions of cytokines

Cytokine	Cytokine secreting cells	Target cells and functions
Interleukins (IL)		
IL-1	Produced by all nucleated cells, but principal sources are APCs, such as macrophages, monocytes, dendritic cell, B cells and endothelial cell	• T_H cells—IL-1 produced by APCs stimulates T_H cells activation and proliferation ➢ Promotes IL2 secretion by T_H cells ➢ Induces IL-2 receptor expression on T_H cells ➢ Induces ↑MHC-II expression on APCs • B cell—promotes B cell development and maturation • Liver—induces synthesis of acute phase reactant proteins • Hypothalamus—induction of fever • Macrophage and neutrophil activation—↑expression of ICAM
IL-2	T_H 1 cells	Induces proliferation activated T_H cells, T_C cells and some NK cells (Previously called as T cell growth factor)
IL-3	T_H cell, NK cell, mast cell	• Stimulates hematopoiesis (acts as multi-CSF) • Mast cell degranulation—↑ histamine secretion
IL-4	T_H 2 cells	• T_H cells—promotes T_H 2 cell activity and inhibits T_H 1 cell • B cell—promotes B cells activation and proliferation and induces B cell class switch over to produce IgE, IgG4, IgG1; previously called as B cell growth factor • Macrophage and APCs—induce ↑MHC-II expression
IL-5	T_H 2 cells	Promotes eosinophil growth and differentiation
IL-6	T_H 2 cells, macrophages	IL-1 and TNF like effects (synergistic effect) Promotes B cell proliferation and antibody production
IL-7	Bone marrow/thymic stromal cells	Serves as a growth factor for T cell and B cell precursors
IL-8	Macrophages, endothelial cells	Attracts neutrophils, NK cells, eosinophils and basophils
IL-9	T_H cells	Hematopoietic and thymopoietic effects
IL-10	T_H 2 cells	Reduces cytokine production by T_H1 cell
IL-11	Bone marrow stromal cells	• Hematopoietic effect (B cell and platelet development) • Liver—induces synthesis of acute phase reactant protein
IL-12	Macrophages	Promote T_H 1 cell induction and inhibit T_H 2 activity; promotes CMI responses NK cell stimulatory factor
IL-13	T_H 2 cells	Mimic IL-4 function
IL-17	$CD4^+$ activated memory T_H cell	Initiates and maintains inflammation

Contd...

Contd...

Cytokine	Cytokine secreting cells	Target cells and Functions
Interferons (IFN)*		
IFN-α	Leukocytes	Antiviral activity
IFN-β	Fibroblasts	Antiviral activity
IFN-γ	T_H and T_C cells, NK cells	• Macrophage—activates the resting macrophages into activated macrophage • B cells—activate B cells to produce IgG • Promotes inflammation of delayed type of hypersensitivity (along with TNF-β) • T_H2 cell—inhibits T_H 2 cell proliferation
Tumor necrosis factors (TNF)		
TNF-α	Macrophage	• IL-1 like effect • Tumor cells—promote vascular thrombosis and tumor necrosis • Inflammatory cells—induce cytokine secretion • Induces lipolysis, causes extensive weight loss associated with chronic inflammation
TNF- β	T_H 1 cell and T_C cell	Tumor cells—similar effect like TNF-α Macrophage—enhance phagocytic activity
Colony-stimulating factor (CSF)		
GM-CSF	Fibroblasts, endothelium, T cells, macrophages	Macrophage and granulocyte growth stimulation
G-CSF	Bone marrow stromal cells, macrophages	Granulocyte growth stimulation
M-CSF	Fibroblasts, endothelium	Macrophage growth stimulation
Others		
TGF- β	Macrophages, mast cells T and B cells, platelet	• Inhibit T and B cell proliferation and hematopoiesis • Promotes wound healing • Promotes class switching of B cells to the IgA class

Abbreviations: ICAM-1, intercellular adhesion molecule 1; TGF-β, transforming growth factor- β; APC, antigen presenting cell

*Interferons are discussed in detail in Chapter 41

Cytokines and Diseases

Pathogenesis of many diseases is characterized by increased expression of cytokines or their receptors. Common examples include the following:

- **Septic shock** due to gram-negative bacteria, such as *Escherichia coli* or *Neisseria meningitidis* is mediated by the endotoxins released by bacteria that stimulate macrophages to produce IL-1 and TNF-α
- **Toxic shock syndrome** is caused by the toxin released from *Staphylococcus aureus*, which activates T cells nonspecifically leading to massive cytokine release and that in turn activates macrophages to release large quantities of IL-1 and TNF-α
- **Cancers:** Several malignancies have been associated with ↑IL-6, e.g. cervical cancer, bladder cancer, etc.
- **Chaga's disease** is caused by a parasite named *Trypanosoma cruzi* which is shown to inhibit the IL-2α receptor, thus blocking the IL-2 action → leading to inhibition of T_H1 activity → immunosuppression.
- Cytokine storm (see highlighted box).

Cytokine Storm

It is a condition, where the cytokines are produced in excess leading to **hypercytokinemia** which can cause significant damage to body tissues and organs.

- Normally, the production of cytokines is kept in check by the body. However, in some instances, the reaction becomes uncontrolled, and too many immune cells are activated in a single place
- The precise reason for this is not entirely understood but may occur in a number of infectious and noninfectious diseases, including graft versus host disease (GVHD), acute respiratory distress syndrome (ARDS), sepsis, Ebola, avian influenza, smallpox, and systemic inflammatory response syndrome (SIRS).

Cytokines used in Therapy

Cytokines offer great promise for the treatment of a number of diseases. Many strategies have been followed to create a cytokine or anticytokine state in the body depending on the need. The strategies are:

❖ **Use of cytokines (e.g. interferons) as drug:**
- Interferon-α is used for the treatment of hepatitis B, hepatitis C, hairy cell leukemia, multiple myeloma and chronic myeloid leukemia (CML)
- Interferon-β is used for the treatment of multiple sclerosis
- Interferon-γ is used for the treatment of chronic granulomatous disease.

❖ **Cytokine-toxin conjugates** are used to destroy the target cells; here the cytokines help in binding to the target cells so that the toxin can act on them.

EXPECTED QUESTIONS

I. Essay:

1. Describe in detail about the structure and function of various lymphoid organs and cells of immune system.

II. Write short notes on:

1. Major histocompatibility antigen.
2. Cytokines.
3. Dendritic cells.
4. Development of T cells.
5. Development of B cells.
6. MALT.

III. Multiple Choice Questions (MCQs):

1. **T cell area of lymph node is:**
   a. Cortex b. Medulla
   c. Paracortical area d. All of the above
2. **Which one of the following cytokine induces fever?**
   a. IL-2 b. IL-1
   c. IL-4 d. IL-5
3. **All of these are antigen presenting cells (APCs), *except*:**
   a. T cells b. B cells
   c. Dendritic cells d. Macrophage
4. **Cell type which lacks HLA antigen is:**
   a. Monocyte b. Thrombocyte
   c. Neutrophil d. Red blood cell
5. **Interferon gamma is secreted by:**
   a. Macrophage b. Fibroblasts
   c. Activated T-cell d. Neutrophils
6. **All of the following are example of peripheral or secondary lymphoid organs, *except*:**
   a. Bone marrow b. Lymph node
   c. Spleen d. MALT
7. **All the following about thymus are true, *except*:**
   a. Developed from third/fourth brachial pouch
   b. Nurse cells are found in cortex
   c. Hassall's corpuscles are found in medulla
   d. Congenital aplasia of thymus is seen in DiGeorge syndrome
8. **Which of the following represents the T cell area of lymph node ?**
   a. Cortex b. Medulla
   c. Paracortical area
   d. Primary and secondary follicles
9. **Which of the following represents the T cell area of spleen?**
   a. Periarteriolar lymphoid sheath (PALS)
   b. Marginal zone
   c. Red pulp d. All of the above
10. **Defect in spleen predisposes to all of the following infection, *except*:**
    a. *Staphylococcus aureus*
    b. *Streptococcus pneumoniae*
    c. *Neisseria meningitides*
    d. *Haemophilus influenzae*
11. **M cells act as the portal of entry of following microbes, *except*:**
    a. *Salmonella* b. *Shigella*
    c. *E. histolytica* d. Poliovirus
12. **T cell receptors (TCR) require association with which of the following CD molecule to interact with an antigen?**
    a. CD1 b. CD3
    c. CD4 d. CD8
13. **γδ T cells—all are true, *except*:**
    a. Constitute 50% of total T cells
    b. They do not require antigen presentation
    c. They are part of innate immunity
    d. Example include intraepithelial lymphocytes (IELs) in gut mucosa
14. **Which of the following is the most common outcome of double positive (DP) T cells during T cell development?**
    a. Positive selection b. Death by neglect
    c. Negative selection d. All of the above
15. **The phenomenon of allelic exclusion occurs during:**
    a. B cell development b. T cell development
    c. NK cell development
    d. Macrophage development
16. **IPEX syndrome occurs due to defect in which cells?**
    a. γδ T cells b. Treg cells
    c. T17 cells d. NK/T cells
17. **Which is not an example of macrophage?**
    a. Monocytes b. Kupffer cells
    c. Hassall's corpuscles d. Epithelioid cells
18. **Which type of dendritic cells help in B cell development?**
    a. Interstitial b. Interdigitating
    c. Circulating d. Follicular

Answers

1. c **2.** b **3.** a **4.** d **5.** c **6.** a **7.** a **8.** c **9.** a **10.** a **11.** c **12.** b **13.** a **14.** b **15.** a **16.** b **17.** c **18.** d

Immune Responses: Cell-mediated and Antibody-mediated

15
CHAPTER

Chapter Preview

- Antigen presentation
- Helper T cells (activation and differentiation)
- Cell-mediated immune response
- Humoral/antibody-mediated immune response

INTRODUCTION

Immune response refers to the highly coordinated reaction of the cells of immune system and their products. It has two arms (Fig. 15.1).

Humoral or Antibody-mediated Immune Response (AMI)

It provides protection to the host by secreting *antibodies*; that can bind and neutralize microbial antigens circulating free or present on the surface of the host cells and in the extracellular spaces, but have no role against intracellular antigens. If antibodies were the only agents of immune response, pathogens that manage to evade them by being in the intracellular environment would have escaped the immune response. Nevertheless, this is not the case.

Cell-mediated Immune Response (CMI)

It plays a crucial role in providing protection against intracellular microbes as well as tumor cells. Although CMI is mainly T cell mediated (especially cytotoxic T cells); however, various other effector cells such as natural killer (NK) cells, macrophages, granulocytes are also components of CMI.

CMI and AMI are Interdependent

CMI and AMI cannot work individually, but they are highly dependent on each other (Fig. 15.1). Cytokines released from T cells stimulate B cells to produce antibodies. Similarly, many effector cells of CMI such as macrophages and NK cells use antibodies as receptors to recognize the target cells for killing.

CMI also regulates the humoral immunity by releasing cytokines from activated T cells that stimulate the B cells to transform into antibody secreting plasma cells.

There are certain initial events that must take place before the induction of either CMI or AMI. These events are common to both CMI and AMI, and they occur irrespective of the type of immune response that will follow. These events include:

- Antigen presentation to helper T cells
- Activation and differentiation of helper T cells into either T_H1 or T_H2 subsets.

Helper T (T_H) cells are the central key that regulate the type of immune response that is going to occur. Activated helper T cells differentiate into either T_H1 or T_H2 subsets. Induction of T_H1 cells secrete cytokines that stimulate cell mediated response, whereas if T_H2 cells are differentiated, they secrete certain cytokines that in turn induce the B cells to produce antibodies.

ANTIGEN PRESENTATION

For induction of immune responses, recognition of antigens by T cells is essential. T cells cannot recognize the native and free antigens, but they do so only after the antigen is processed into smaller antigenic peptides containing specific epitopes which are subsequently combined with MHC molecules (class I or II) and presented on the host cell surface.

Antigen-presenting Cells (APCs)

Although antigen presentation refers to presentation of antigenic peptide to both T_H (helper T cells) and T_C (cytotoxic T cells) by complexing with MHC-II and I respectively; however, antigen-presenting cells (APCs) in strict sense implies only to those cells (e.g. dendritic cell, macrophage, etc.) that present the antigenic peptide along with MHC class II to T_H cells (Table 15.1).

Cells presenting antigenic peptides along with MHC class I molecules to T_C cells are not included under APCs.

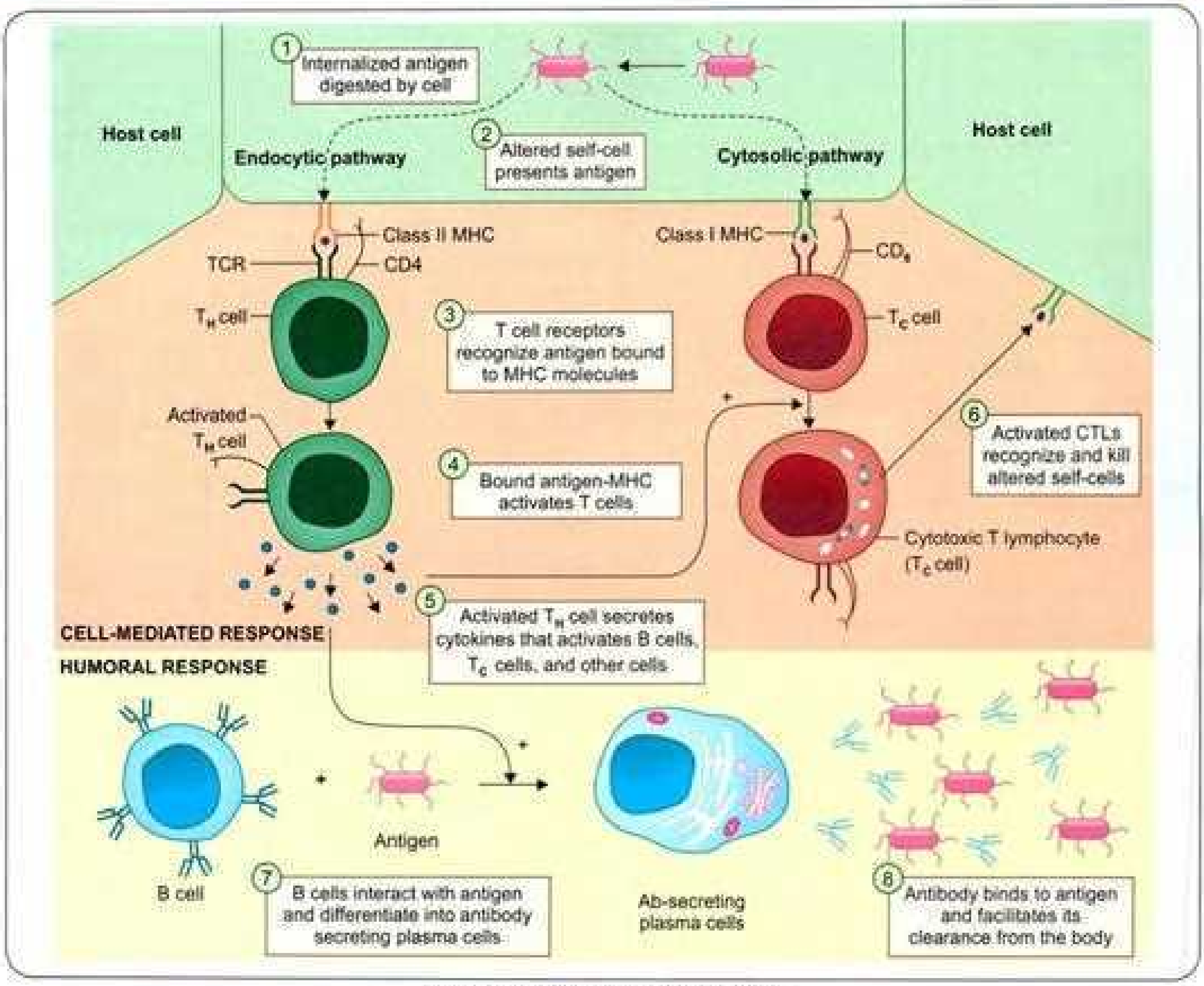


Fig. 15.1: Overview of immune response

Abbreviations: MHC, major histocompatibility complex; CTL, cytotoxic T lymphocyte; Ab, antibody.

Table 15.1: Antigen-presenting cells (APCs)

Professional APCs	Nonprofessional APCs
Dendritic cells	Fibroblasts (skin)
Macrophages	Thymic epithelial cells
B cells	Pancreatic beta cells
	Vascular endothelial cells
	Glial cells (brain)
	Thyroid epithelial cells

These cells are usually virus infected cells or tumor cells. They are often referred to as **target cells** as the activated T_C cells cause lysis of these cells.

Dendritic cells, macrophages and B cells are the major APCs and are called **professional APCs**. There are some other non-professional cells that can occasionally present antigens to helper T cells (Table 15.1).

Antigen Processing Pathways

For induction of immune response (both CMI and AMI), antigens must be presented to T_H cells. In addition, for CMI induction, antigen presentation to T_C cells is essential. Two well defined pathways are used by the immune system for this purpose. They differ from each other in their mechanism and target antigen, as described below (Table 15.2):

1. **Cytosolic pathway:** Here, the endogenous (intracellular) antigens such as viral antigens and tumor antigens are processed and presented along with MHC class I molecules to CD8 T cells.

Table 15.2: Differences between cytosolic and endocytic pathways of antigen presentation

Property	Cytosolic pathway	Endocytic pathway
Antigen processed	Endogenous	Exogenous
Antigen is complexed with	MHC I molecules	MHC II molecules
Antigen is presented to	T_C cells	T_H cells

2. **Endocytic pathway:** In this pathway, the exogenous antigens (extracellular microbes and their products, e.g. toxins) are processed and complexed with MHC class II molecules and presented to T_H cells. The cells involved in endocytic pathway include the APCs such as macrophages, dendritic cells and B cells.

HELPER T CELLS (ACTIVATION AND DIFFERENTIATION)

Helper T cell (T_H) activation and differentiation is the central event that regulates both the components of immune response; CMI and AMI.

Activation of Helper T Cells

Signal Generation

Activation of T_H cells requires generation of three specific signals (Fig. 15.2).

1. **Antigen-specific signal:** It involves binding of antigenic peptide present in the groove of MHC-II on APCs to TCR (T cell receptor) present on surface of T_H cells. CD4 molecules of T_H cells also interact with β2 domain of MHC-II.
2. **Costimulatory signal:** It involves binding of CD28 molecule on T_H cells to B7 molecules on APCs.

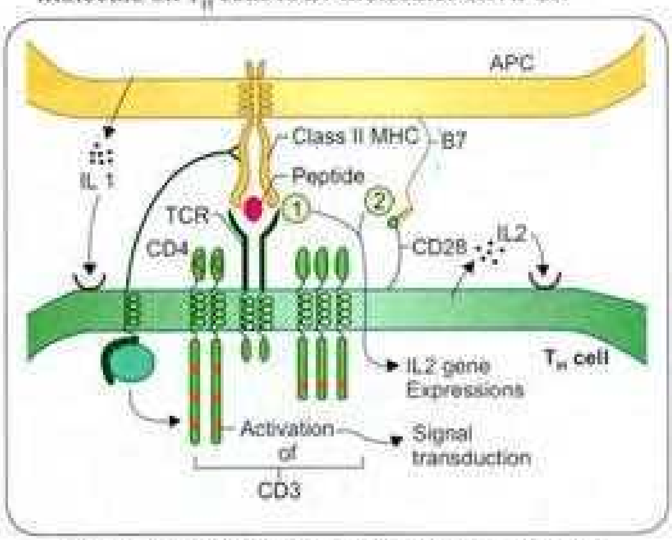


Fig. 15.2: Activation of T_H cell by interacting with APC

Abbreviations: APC, antigen-presenting cells; MHC, major histocompatibility complex; TCR, T cell receptor.

3. **Cytokine signal:** APCs (macrophages) secrete interleukin-1 (IL-1) which acts on T_H cells.

Signal Transduction

Following induction of signal, its transmission is essential for T_H cell activation. Signal transduction is initiated at CD4 molecule which interacts with CD3 complex, which in turn transmit the signal leading to activation of T_H cells.

Differentiation of Helper T cells

Activated T_H cells secrete increased amount of IL-2 as well as IL-2 receptor (IL2R or CD25). IL-2 binds to its receptors on the same T_H cell and also on other T_H cells and induces the naive T_H cells to proliferate and differentiate. T_H cells get activated and become lymphoblast cells which subsequently differentiate into memory and effector T_H cells.

Effector T_H cells

They are derived either from the naive T_H cells or pre-existing memory T_H cells following antigenic stimulus. They are short lived (few days to weeks). They further **differentiate into either T_H1 or T_H2** subsets. This differentiation is very crucial as they secrete distinct cytokines that further mediate specific functions.

Cytokines secreted by T_H1 cell stimulate cytotoxic T cells and induce cell mediated immune response; while cytokines secreted by T_H2 cell stimulate B cells producing different classes of antibodies (humoral immune responses). **IL12** secreted by macrophage plays an important role in the differentiation of T_H cells. It promotes T_H1 subset proliferation.

- **T_H1 cells** produce IL-2, interferon-γ (IFN-γ) and tumor necrosis factor-β (TNF-β); each has specific function (Table 15.3)
- **T_H2 cells** secrete IL-4, IL-5, IL-6, IL-10 and IL-13. They activate the B cells to transform into plasma cells which in turn secrete antibodies (Table 15.3).

Memory T Cells

They are derived from activated T_H cell. They have longer life span (months to years). They are in resting stage, but following subsequent antigenic stimulus, they become activated and differentiated into effector T_H cells. They express CD45RO isoform of common leukocyte antigen CD45, as compared to naive T cells which express CD45RA.

CELL-MEDIATED IMMUNE RESPONSE

The term cell-mediated immune response (CMI) refers to destruction of cells carrying intracellular microbes and other abnormal cells, such as tumor cells by various

Table 15.3: Role of cytokines secreted by T_H1 and T_H2 cells

T_H1 cytokines and their functions	
IL-2	• Promotes activation of T_H and T_C cells • Activates NK cells to become LAK cells
IFN-γ	• Activates the resting macrophages into activated macrophages • Activates B cells to produce IgG • Promotes inflammation of delayed type of hypersensitivity (along with TNF-β) • Inhibits T_H2 cell proliferation
TNF-β	Enhances phagocytic activity of macrophage
T_H2 cytokines and their functions	
IL-4	• Inhibits T_H1 cell differentiation • Stimulates B cells to produce IgE and also IgG4 and IgG1
IL-5	• Enhances proliferation of eosinophils • Both IL-4 and IL-5 together provide protection against helminthic infections and also mediate allergic reaction
IL-6	Promotes B cell proliferation and antibody production
IL-10	Inhibits T_H1 cell differentiation

Abbreviations: LAK cell, lymphokine-activated killer cells; IFN, interferons; IgG, immunoglobin G; TNF, tumor necrosis factor; NK, natural killer.

specific and nonspecific cells of immune system, of which the most important is cytotoxic T (T_C) cells.

Role of CMI

CMI mediates the following immunological functions:

❖ Provides immunity against microbes residing in intracellular milieu:
  - For obligate intracellular organisms, CMI remains the only effective immune response. Examples include all viruses, some bacteria (*Mycobacterium, Chlamydia* and *Rickettsia*), some parasites (*Plasmodium, Leishmania, Trypanosoma* and *Cryptosporidium*) and some fungi (*Pneumocystis*)
  - For facultative intracellular organisms, humoral immunity is active as long as the organism is extracellular. Once they come to intracellular milieu, CMI takes the leading role. Examples include Bacteria like *Listeria, Salmonella* and *Yersinia* and fungi such as *Histoplasma* and *Cryptococcus.*

❖ Provides immunity against tumor cells and other damaged and altered cells

❖ Mediates delayed hypersensitivity (type IV hypersensitivity)

❖ Plays key role in transplantation immunity and graft-versus-host (GVH) reaction.

Effector Cells of CMI

CMI can be mediated by both antigen specific and nonspecific effector cells (Table 15.4). They perform their

Table 15.4: Effector cells of CMI

Effector cells of CMI	Antigen specificity
Cytotoxic T cells	Specific
NK cells	Nonspecific
Cells performing ADCC (NK cells, macrophages, neutrophil and eosinophils)	Nonspecific

Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; NK, natural killer.

function by direct killing of the target cells (e.g. virus infected cells or tumor cells).

❖ The most important mediator of CMI is cytotoxic T cell which is antigen specific

❖ However, many other nonspecific effector cells such as macrophages, NK cells, neutrophils, and eosinophils also contribute to CMI

❖ Although CMI has many features distinct from humoral immune response but it is not completely independent. The nonspecific effector cells use antibodies as receptors to recognize the target cells for killing.

Cytotoxic T Lymphocytes

CD8 cytotoxic T lymphocytes (CTL or T_C) are the principal effector cells of CMI, involved in the destruction of target cells such as virus infected host cells and tumor cells. Naive T_C cells (or CTL precursors) respond to viral or tumor peptide antigens which are processed by the target host cells (by cytosolic pathway) and presented along with MHC class I molecules. Activated T_C in turn secretes cytotoxic enzymes that lyse the target cells.

Activation of CTL

Generation of activated CTL from naive T_C cells requires induction of at least three signals (Fig. 15.3):

1. **Antigen-specific signal:** It is induced by binding of TCR-CD3 complex of naive T_C cells to MHC I-peptide complex of target cells. CD8 of T_C cells also interacts with α3 domain of MHC-I.

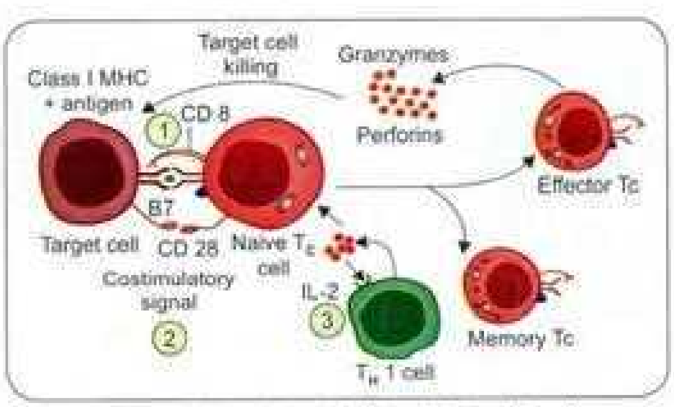


Fig. 15.3: Activation and differentiation of T_C cells

2. **Costimulatory signal:** CD28 of naive T_C cells interacts with B7 molecule on target cells.
3. **Third signal:** IL-2 (secreted by T_H1 cell) acts on high-affinity IL-2 receptor on T_C cells.

Following induction, the transmission of signal occurs in a way similar to that described for T_H cells.

Functions of CTL (Target Cell Lysis)

The activated T_C cells produce two types of lethal enzymes; called perforins and granzymes.

- **Perforins** produce pores in the target cell membrane; through which granzymes are released inside
- **Granzymes** are serine proteases; they induce cell death by apoptosis through caspase pathway.

Natural Killer Cells

Natural killer cells are large granular lymphocytes that constitute 10–15% of peripheral blood lymphocytes.

- They are derived from a separate lymphoid lineage. NK cells are cytotoxic, but antigen nonspecific
- They are part of innate immunity, act as first line of defense and do not require prior contact with the antigen.

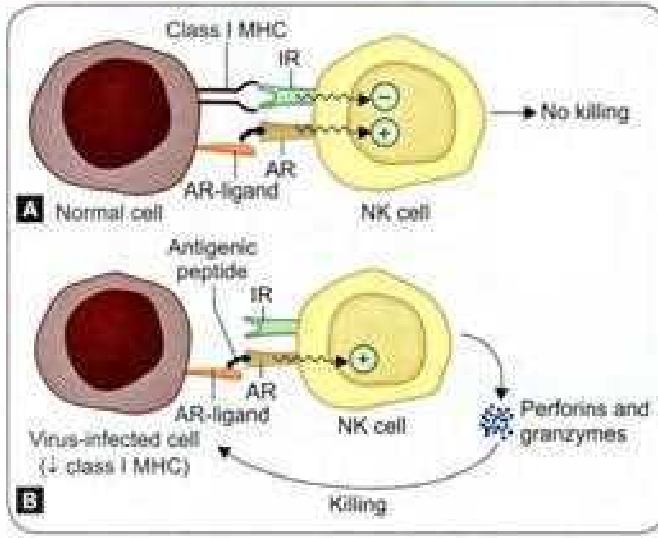
NK cells act against virus infected cells and tumor cells till the T_C cells are activated and take over the function. However, they differ from T_C cells in many other aspects (Table 15.5) such as:

- **Natural killer cell markers:** NK cells lack the T cell markers such as CD3, CD4 or CD8 molecules (hence are also called null cells), instead possess specific surface markers such as CD16 and CD56
- **No MHC restriction:** NK cells can recognize the ligands (antigens) without MHC presentation
- **Innate immunity:** NK cells are part of innate immunity; they do not require the prior exposure to microbial antigen

Table 15.5: Comparison between NK cells and T_C cells

Property	NK cells	T_C cells
Surface markers	CD16 and CD56	CD3, CD8
MHC restriction	No	MHC-I restricted
Memory	No	Yes
Immunity	Part of innate immunity	Part of acquired immunity
Target cell	Virus infected cells Tumor cells	Same as NK cells
Mechanism of destruction	Perforins and granzymes (constitutive)	Same as NK cells (inducible)
Immune response	CMI	CMI

Abbreviations: MHC, major histocompatibility complex; CMI, cell-mediated immunity.



Figs 15.4A and B: NK cell-mediated cytotoxicity. **A.** In normal cell; **B.** In virus infected cell

Abbreviations: AR, activation receptor; IR, inhibition receptor.

- **No memory:** NK cells do not differentiate into memory cells.

Mechanism of NK Cell-mediated Cytotoxicity

Receptor Interaction

Natural killer cells are not MHC restricted. They directly recognize certain ligands (e.g. glycoproteins) present on the surface of altered host cells like virus-infected cells or tumor cells. However, such ligands are also present on normal cells. Still, NK cells are capable of distinguishing normal host cells from the altered cells (Figs 15.4A and B). This is mediated by two types of receptors present on NK cell surface **(theory of opposing-signals model).**

- **Activation receptors** (e.g. NKR-P1, CD16): When these receptors are engaged with ligands present on the target cells; NK cells become activated
- **Inhibitory receptors** (such as C-type lectin inhibitory receptors): They recognize a part of MHC I molecule (HLA-E) which is present on the surface of all normal nucleated cells
  - Binding of inhibitory receptors to MHC-I molecules generates an inhibitory signal that suppresses the NK cells even if they are bound to the activation receptors. This is because the inhibitory signal is the dominant signal and hence it overrides the activation signal
  - However, in virus infected cells and tumor cells, the MHC-I expression is remarkably reduced. In such cases, there would not be any inhibitory signal. Hence, binding of activation receptor to its ligand leads to activation of NK cells.

Target Cell Destruction

Mechanism of target cell lysis by NK cells is similar to that of T_C cells, i.e. via secreting perforins and granzymes. Perforins form pores on target cells, through which granzymes enter and lyse the target cells (Fig. 15.4B). The only difference is that, the enzymes are constitutively expressed in NK cell cytoplasm (i.e. they are cytotoxic all the time, even without exposure to the antigen).

Alternate Mechanisms of NK Cell Activity

- NK cells respond to IL-12 produced by macrophages and secrete IFN-γ, which in turn activates the macrophages. Then, the activated macrophages phagocytose and kill the microbes
- NK cells also mediate their function via ADCC (described below).

Antibody-dependent Cell-mediated Cytotoxicity (ADCC)

A number of nonspecific cytotoxic cells express receptors (FcR) on their surface that can bind to the Fc region of any immunoglobulin.

- Following contact with a target cell coated with an antibody, these FcR bearing cells can bind to Fc portion of the antibody coated on the target cells, and subsequently cause lysis of the target cell
- Although these cytotoxic cells are nonspecific for the antigen, the specificity of the antibody directs them towards the specific target cells. This type of cytotoxicity is referred to as **antibody-dependent cell-mediated cytotoxicity (ADCC)**.

ADCC is exhibited by various cells such as NK cells, macrophages, monocytes, neutrophils, and eosinophils. They release various cytotoxic factors into the target cells like perforins, granzymes, lytic enzymes, free radicals, TNF, etc. (Fig. 15.5). However, there is no complement dependent cytolytic activity.

- NK cells secrete perforins, and granzymes. Neutrophils release lytic enzymes
- Eosinophils can release lytic enzymes and perforins; play an important role in providing immunity against helminths
- Macrophages produce lytic enzymes and TNF.

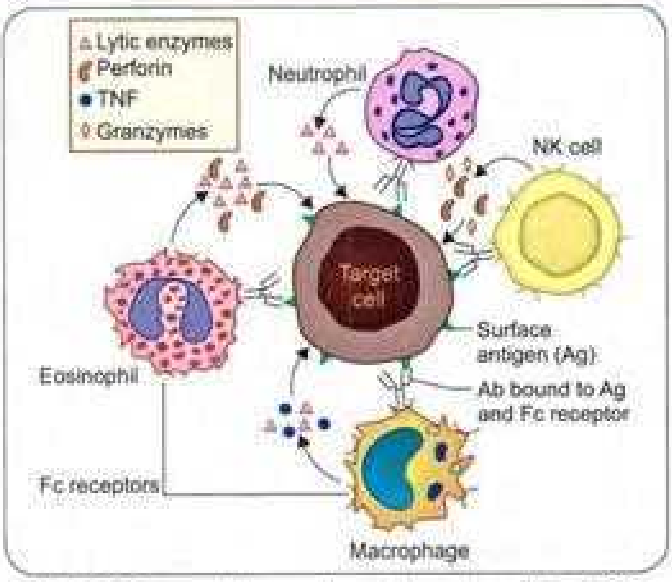


Fig. 15.5: Cytotoxic factors released by various cells in ADCC

Assessment/detection of CMI

There are several methods for detection of CMI.

- The mixed-lymphocyte reaction (MLR) is an in vitro system for assaying T-cell proliferation in a cell-mediated response
- Cell-mediated lympholysis (CML) is another in vitro assay for testing the cytotoxic function of effector cells of CMI
- The graft-versus-host (GVH) reaction in experimental animals provides an in vivo system for studying cell-mediated cytotoxicity.

HUMORAL/ANTIBODY-MEDIATED IMMUNE RESPONSE

Antibody-mediated immune response (AMI) provides protection to the host by **secreting antibodies;** that prevent invasion of microbes present on the surface of the host cells and in the extracellular environment, but has no role against intracellular microbes. AMI occurs through the following three sequential steps:

1. Activation of B cells following contact with the microbial antigen (B cells act as APCs).
2. Proliferation and differentiation of B cells into effector cells (antibody producing plasma cells) and memory cells.
3. Effector function: Production of antibodies by plasma cells which in turn counter act with the microbes in many ways, such as neutralization, opsonization, complement activation, etc.

Activation of B Cells

Antigens that activate B cells fall into two categories.

1. Most antigens are **thymus-dependent (TD);** they activate B cells indirectly via activation of T cells. TD antigens are processed by APCs, presented to T_H cells following which the activated T_H cells secrete cytokines that in turn activate the B cells.
2. The thymus independent (TI) antigens (e.g. bacterial capsule) are not processed by APC. They can directly

activate B cells without the help of T cell induced cytokines (for details refer Chapter 10).

TD antigens induced activation of B cell is described below.

Antigen Presentation of B Cells to Activated T_H Cells

The first and foremost step that occurs is recognition of microbial antigen (TD antigen) by B cell membrane immunoglobulin receptors (mIg) followed by receptor-mediated endocytosis of antigen. Then the antigen is processed into smaller antigenic peptides that are presented in complex with MHC-II to activated T_H cells (by endocytic pathway). This leads to induction of three signals.

Signal Induction

The naive B cells are in the resting stage. Activation requires induction of three signals (Fig. 15.6).

1. **Signal 1:** It is induced by the cross linking of IgM on B cell membrane with the microbial antigen.
2. **Signal 2:** It is an additional signal provided by binding of CD40 on B cell with CD40L (ligand) on activated T_H cells.
3. **Signal 3:** It is usually a cytokine stimulus. Cytokines produced by the activated T_H cells bind to specific cytokine receptor on B cells.

Signal Transduction

Following induction of signal, its transmission is essential for B cell activation.

- Signal transduction is initiated by the B-cell receptor (BCR). The BCR comprises of two parts (Fig. 15.7)
  1. Antigen-binding membrane Ig
  2. Ig-α/Ig-β heterodimer
- Following antigen cross linkage to membrane Ig, the Ig-α/Ig-β heterodimer is activated and in turn transmits the signal, ultimately leading to activation of B cells.

Proliferation and Differentiation of B Cells

As described in Chapter 14, the naive B cells, released from bone marrow go and house in the B cell areas of peripheral lymphoid organs (e.g. cortex of lymph node and marginal zone of spleen). There, the naive B cells are organized to form primary lymphoid follicles.

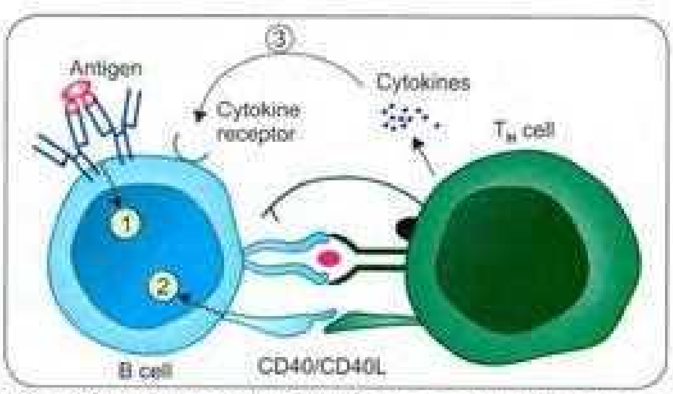


Fig. 15.6: Antigen presentation of B cells to activated T_H cells and signal induction

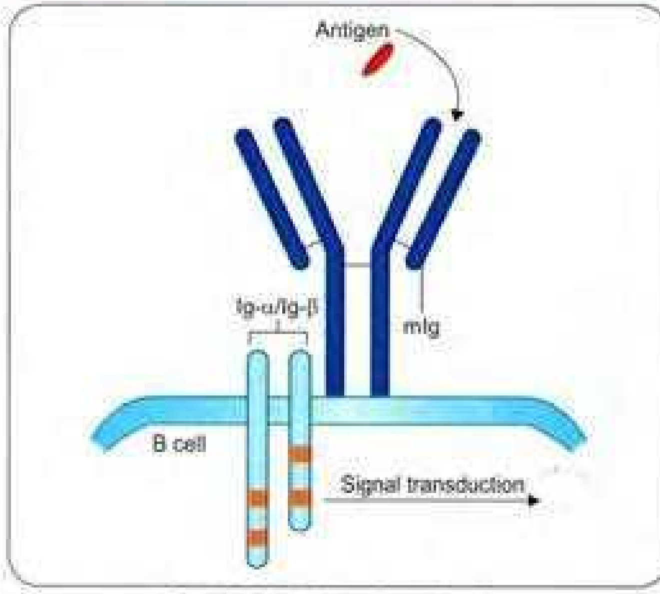


Fig. 15.7: B cell signal transduction

- Following the antigenic exposure, the naive B cells are activated and then they proliferate
- Eventually, the primary lymphoid follicles transform into secondary lymphoid follicles
- Secondary lymphoid follicles bear a germinal center which in turn has two areas; dark zone and light zone. Events occurring in the secondary lymphoid follicles are as follows.

Events in the Dark Zone of Germinal Center (Fig. 15.8)

The activated B cells differentiate into larger dividing cells called **centroblasts**, which further transform into smaller non dividing cells called **centrocytes** by expressing membrane Ig.

- Centroblasts express the membrane Ig by undergoing a type of mutation called **somatic hypermutations**. These are point mutations arising due to insertion or deletion in the variable region of Ig gene
- This results in alteration of the membrane Ig affinity by which it binds with the corresponding antigen. Thus, the resultant centrocytes would bear membrane Ig with altered affinity
- Because somatic hypermutations occur randomly; they generate membrane Ig with both high and low affinity
  - The centrocytes with low affinity membrane Ig undergo apoptosis and then are phagocytosed by special type of macrophages found in lymphoid follicles called **tingible body macrophages**
  - The centrocytes with high affinity membrane Ig are allowed to survive, following which they migrate to

the light zone. The process of enhancement of affinity of membrane Ig for antigen binding is called **affinity maturation**.

Events in the Light Zone of Germinal Center (Fig. 15.8)

- **Binding of centrocytes to follicular dendritic cells:** The centrocytes with high affinity membrane Ig undergo maturation by binding to a special type of dendritic cell called **follicular dendritic cell** (see box below). Then the mature centrocytes undergo class switch over

Follicular Dendritic Cells

The follicular dendritic cells (FDC) are special type of dendritic cells which differ from the other types of dendritic cells in various ways.

- They do not act as APCs and do not express MHC class II. Instead, they bear Fc receptors that recognize Ag-Ab complex
- Consequently, the antigen is unable to move and is retained in the lymphoid follicle for prolonged periods so that the centrocytes can come and bind to the antigens present in Ag-Ab complex
- This allows the FDCs to interact with the centrocytes which results in the selection of the centrocytes with high affinity membrane Ig.

- **Class switch over:** Early in the immune response, IgM is the predominant immunoglobulin secreted by the B cells. But as the maturation progresses, the same B cells undergo a phenomenon called **class switch over** to produce Ig of other classes (Fig. 15.8)
  - Class switch over occus in the light zone of lymphoid follicles, where the positively selected centrocytes interact with activated T_H cells and receive a cytokine signal for class switching
  - Binding of cytokines produced by T_H cells to cytokine receptors present on centrocytes surface induces class switch over
  - Different cytokines induce production of different classes of Ig by switching mechanism (Table 15.6).
- **Differentiation of centrocytes into plasma cells and memory cells:**
  - After undergoing class switch over, the selected centrocytes further undergo differentiation into effector cells (plasma cells) and memory cells in the light zone of germinal center
  - Plasma cells are large antibody-secreting cells; produce secretory Ig enormously, but do not synthesize membrane Ig. They do not have MHC-II molecules and do not undergo further class switch over
  - Memory cells bear high affinity membrane Ig molecules of all classes as compared to naive B cell that bear only low affinity IgM or IgD membrane Ig. They are long lived cells which respond to the secondary antigenic stimulus.

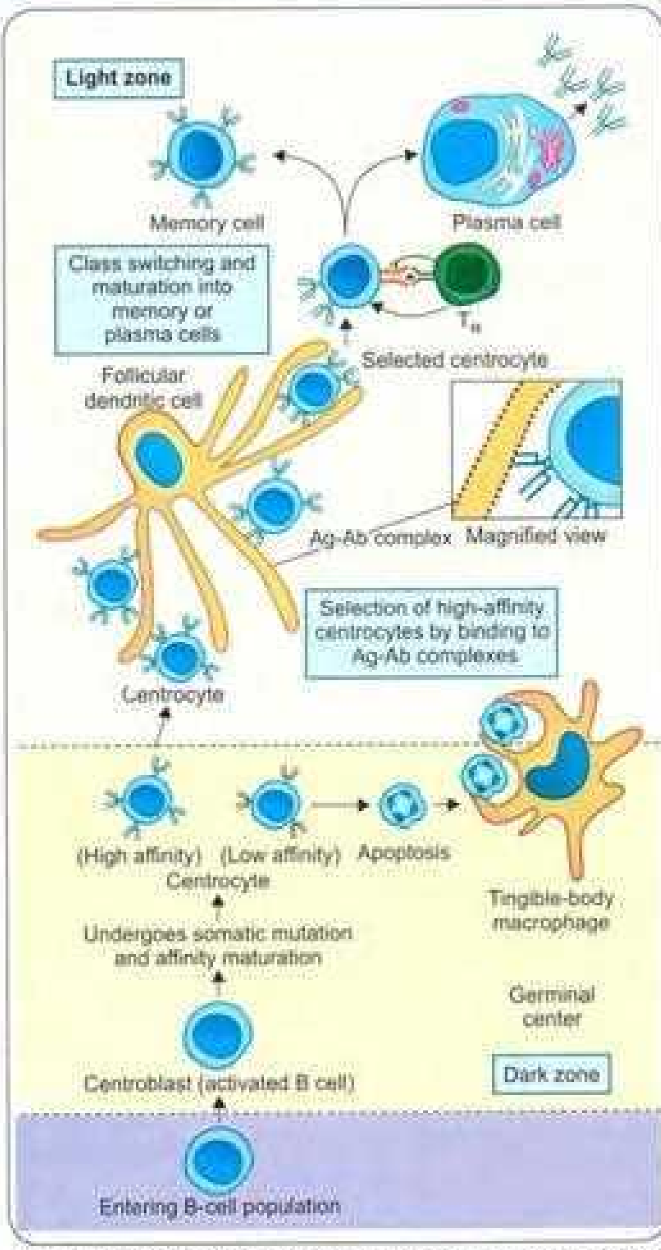


Fig. 15.8: Differentiation of B cells in secondary lymphoid follicles

Table 15.6: Cytokines secreted by T_H cells and the respective Ig class/subclass they induce

Cytokine(s)	Ig class produced
IFN-γ	IgG2a or IgG3
IL-5 + TGF-β	IgA or IgG2b
IL-4	IgE or IgG1 or IgG4
IL-2, 4, 5	IgM
IL-4, 5, 6 + IFN-γ	IgG

Effector Functions of AMI

Antibodies secreted from plasma cells mediate a number of biological functions through their Fc portions that bind to Fc receptors (FcRs) expressed by many cell types.

- **Promotes opsonization:** FcRs present on phagocyte surface recognize antibody coated microbes, bind to them and that leads to enhanced phagocytosis (Fig. 15.9)
- **Transcytosis:** Poly-Ig receptors are expressed on the inner (basolateral) surface of epithelial cells (facing the blood). They bind to dimers of IgA and multimers of IgM antibodies and transfer them through the cell to their apical (outer) surface and into the lumen of an organ (e.g. the intestine). This is a process referred to as **transcytosis** and is responsible for the accumulation of antibodies in the lumen of the organ (Fig. 15.10)
- **Mediates mucosal immunity:** Transcytosis of IgA to gut lumen provides mucosal immunity by neutralizing the microbes at local mucosal sites
- **Activates complement-mediated inflammation and cytolysis:** Antigen antibody complex activates the classical complement pathway (Fig. 15.11). The final complement factors (C5-C9), also called **membrane attack complex** which has lethal activity by forming pores on the target cells
- **Promotes ADCC:** Though ADCC is principally cell mediated (described under CMI section); antibodies direct the cells to reach to the target cells. ADCC is important to provide immunity against:
  - Helminths (eosinophil-IgE mediated)
  - Tumor cells and virus infected cells (NK cell-IgG mediated).

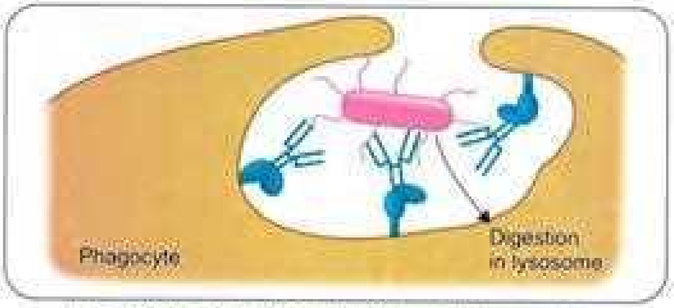


Fig. 15.9: Opsonization of bacteria and phagocytosis

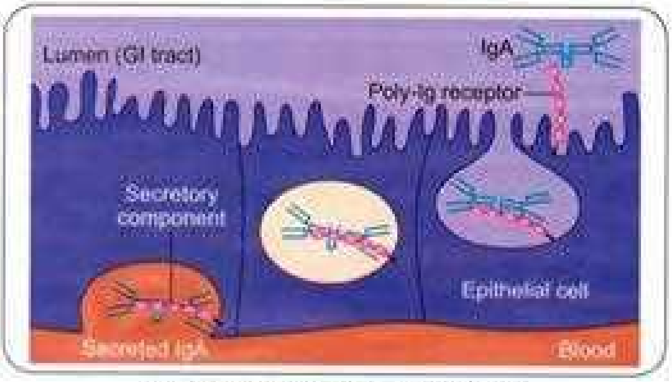


Fig. 15.10: Transcytosis of dimeric IgA

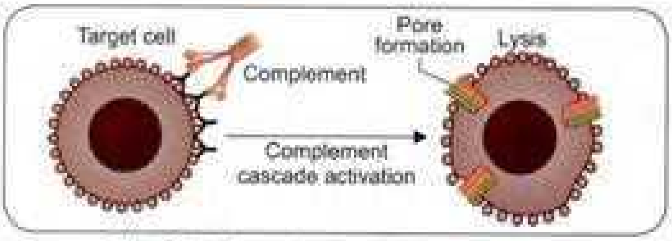


Fig. 15.11: Complement-mediated cytolysis

EXPECTED QUESTIONS

I. Essay:

1. Describe in detail about the mechanism of cell-mediated immune response.
2. Describe in detail about the mechanism of antibody-mediated immune response.

II. Write short notes on:

1. Antigen presentation.
2. ADCC.

III. Multiple Choice Questions (MCQs):

1. Cell-mediated immunity is by virtue of:
a. NK cell b. Eosinophil
c. Cytotoxic T cells d. All above

2. Macrophages are major source of:
a. IL-1 b. IL-5
c. IL-7 d. IFN-γ

3. Perforins are produced by:
a. Plasma cells b. Suppressor T cells
c. Cytotoxic T cells d. Memory helper T cells

4. Professional antigen-presenting cells (APCs) include all, *except:*
a. Dendritic cells b. Macrophages
c. Fibroblasts (skin) d. B cells

5. Cytosolic pathway of antigen presentation—all are true, *except:*
a. Endogenous antigens processed
b. Antigen is complexed with MHC I molecule
c. Antigen is presented to T_C cells
d. Occurs only in antigen presenting cells

6. T_H2 cells secrete all the following cytokines, *except:*
a. IL-2 b. IL-4
c. IL-5 d. IL-6

Answers

1. d **2.** a **3.** c **4.** c **5.** d **6.** a

Hypersensitivity

16
CHAPTER

Chapter Preview

- Definition and classification
- Type I hypersensitivity reaction
- Type II hypersensitivity reaction
- Type III hypersensitivity reaction
- Type IV hypersensitivity reaction

The purpose of immune response is to eliminate the foreign antigens that have entered into the host. In most instances, immune response leads to only a subclinical or localized inflammatory response which just eliminates the antigen without causing significant damage to the host. However, at times, this response becomes abnormal; leads to exaggerated inflammatory response which causes extensive tissue damage or sometimes even death.

HYPERSENSITIVITY REACTIONS

Definition

The term hypersensitivity or allergy refers to the injurious consequences in the sensitized host, following subsequent contact with specific antigens.

Gell and Coombs Classification

Following an antigen contact, hypersensitivity may occur immediately or after a few days. It may result from abnormality of either humoral or cell-mediated immune response. Based on the above two features, Gell and R Coombs classified hypersensitivity reactions into four types (Table 16.1).

Immediate Hypersensitivity Reactions

These reactions occur immediately, within few minutes to few hours of antigen contact, as a result of abnormal exaggerated humoral response (antibody mediated). This can be further classified into three types based on the type of effector mechanisms:

1. **Type I hypersensitivity reaction:** It is IgE-mediated, which causes mast cell degranulation following a contact with soluble antigen.
2. **Type II hypersensitivity reaction:** It is IgG (or rarely IgM) mediated, which causes complement activation or antibody-dependent cellular cytotoxicity (ADCC) in response to cell surface bound antigens.
3. **Type III hypersensitivity reaction:** It is immune complex-mediated; which are formed due to interaction between soluble antigen and antibody (usually IgG), resulting in an abnormal inflammatory response.

Delayed Hypersensitivity Reaction

Delayed hypersensitivity reaction occurs after few days of antigen contact, as a result of abnormal cell-mediated immune response. This is also called type IV hypersensitivity reaction. It is mediated by a specific subset of T_H cells called delayed hypersensitivity T cells or T_{DTH} cell.

TYPE I HYPERSENSITIVITY REACTION

The hallmark of type I hypersensitivity reaction is production of IgE by sensitized B cells following a contact with an allergen which inturn induces mast cell degranulation. The pharmacologically active mediators released from these granules cause vasodilation, vascular and smooth muscle contraction and increased vascular permeability. These changes ultimately lead to localized response (called **atopy**) and systemic response (called **anaphylaxis**).

Allergens

Allergens are foreign antigens that induce allergy. List of allergens is given in Table 16.2.

Experiments to Demonstrate Type I Reaction

Several experiments were conducted in the past to demonstrate type I hypersensitivity reactions.

Table 16.1: Features of various types of hypersensitivity reactions

	Type I	Type II	Type III	Type IV
Immune response altered	Humoral	Humoral	Humoral	Cell mediated
Immediate or delayed	Immediate	Immediate	Immediate	Delayed
Duration between appearance of symptoms and antigen contact	2–30 minutes	5–8 hour	2–8 hours	24–72 hours
Antigen	Soluble	Cell surface bound	Soluble	Soluble or bound
Mediator	IgE	IgG	Ag-Ab complex	T_{DTH} cell
Effector mechanism	Mast cell degranulation	• ADCC • Complement-mediated cytolysis	Complement activation and inflammatory response	Macrophage activation leads to phagocytosis or cell cytotoxicity
Desensitization to the allergen	Easy, but short-lasting	Easy, but short-lasting	Easy, but short-lasting	Difficult, but sustained
Typical manifestations	• Anaphylaxis • Asthma • Atopic dermatitis	• Transfusion reactions • Rh incompatibility • Hemolytic anemia	• Arthus reaction • Serum sickness • Glomerulonephritis • Rheumatoid arthritis	• Tuberculin test • Granuloma formation in tuberculosis, leprosy, etc. • Contact dermatitis

Abbreviation: ADCC, antibody- dependent cellular cytotoxicity

Table 16.2: Common allergens associated with type I hypersensitivity reaction

Allergen types	Examples
Food	Nuts, egg, peas, sea food, beans, milk
Plants and pollens	Rye grass, rag weed
Proteins	Foreign serum, vaccines
Drugs	Penicillin, sulfonamides, local anesthetics and salicylates
Insect bite products	Venom of bee, wasp, ant, cockroach calyx and dust mites
Others	Mold spores, animal hair and dander

P-K Reaction

K Prausnitz and H Kustner (1921) injected serum from an allergic person into a nonallergic individual intradermally. Later when the appropriate antigen was injected at the same site, a wheal and flare reaction (analogous to hives) developed at the site. Thus, they were the first to demonstrate that antibodies in the serum are responsible for the allergy and it is transferable from one person to another.

❖ The wheal and flare response occurs in three stages as follows:
  1. Begins with the appearance of an erythematous area at the site of injury, followed by
  2. Development of a flare (erythema) surrounding the site.
  3. Finally, a wheal (swelling and congestion) forms at the site as fluid leaks under the skin from the surrounding capillaries.

❖ They named the response as the **P-K reaction** and such serum factors that reacted with the allergen were called **P-K antibodies**, or **reaginic antibodies**. After the discovery of IgE (K. Ishizaka, 1960), it became clear that these serum factors are nothing, but IgE antibodies.

Schultz Dale Phenomenon

This was done to demonstrate anaphylaxis in vitro; by exposing isolated tissues such as intestine or muscle segments of sensitized guinea pigs to the allergens.

Theobald Smith Phenomenon

This was done to demonstrate anaphylaxis in vivo by injecting the allergen into guinea pigs.

Mechanism of Type I Hypersensitivity

Type I hypersensitivity reaction occurs through two phases; the sensitization and effector phases, both occurring with an interval of 2–3 weeks (Fig. 16.1).

Sensitization Phase

This occurs when an individual is exposed for the first time to the sensitizing or priming dose of an allergen.

❖ Sensitization is most effective when the allergen is introduced parenterally, but may occur by any route, including ingestion or inhalation
❖ In susceptible individuals, very minute doses can be sufficient to sensitize the host
❖ The allergen is processed by the antigen presenting cells and the antigenic peptides are presented to the CD4 helper T cells

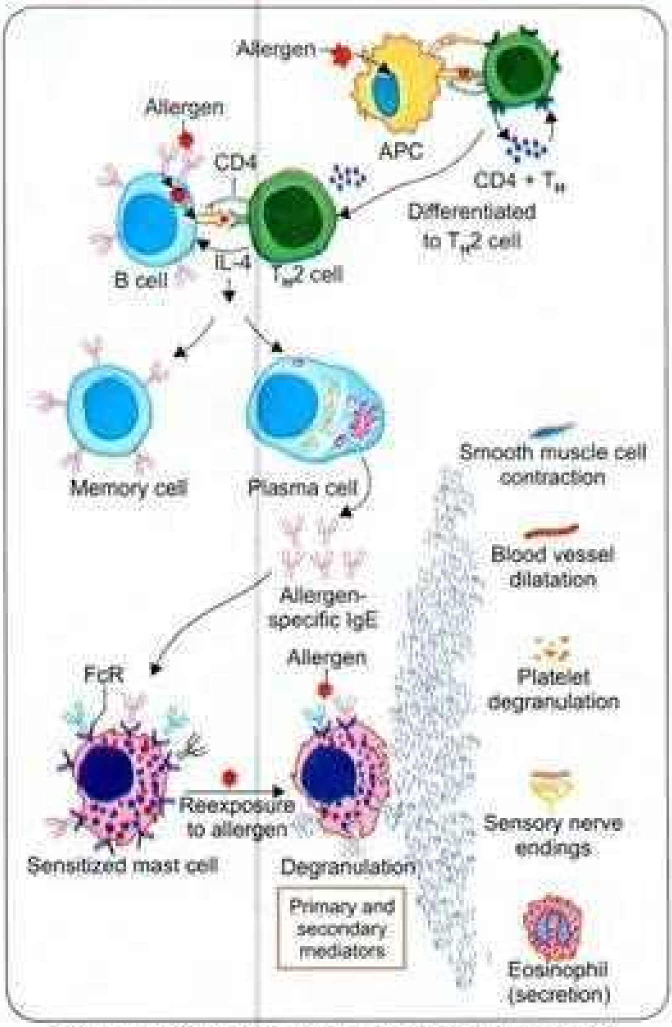


Fig. 16.1: Mechanism of type I hypersensitivity reaction

- Activated T_H cells are differentiated into T_H2 cells which in turn secrete interleukin 4 (IL-4)
- IL-4 induces the B cells to differentiate into IgE producing plasma cells and memory cells. Many molecules of IgE with specificities against various epitopes of the allergen may be produced
- Secreted IgE migrate to the target sites, and coat on the surface of mast cells and basophils. Fc region (the C_H3 and C_H4 domains) of IgE binds to high affinity Fc receptors (e.g. FcεR1) present on mast cell surface
- Such sensitized mast cells (coated with IgE) will be waiting for interaction with the subsequent antigenic challenge.

Effector Phase

When the same allergen is introduced subsequently (shocking dose), it directly encounters with the Fab region of IgE coated on mast cells.

- IgE cross linkage initiates degranulation: Allergen bound to IgE triggers the mast cells (and basophils) activation and degranulation. Granules in turn release a number of pharmacologically active chemical mediators that lead to the various manifestations of type-1 reaction
- The memory B cells further differentiate into plasma cells that produce IgE
- **Degranulation in two phases:** Mast cells and basophils undergo degranulation in two phases
  1. Primary mediators: The preformed chemical mediators which are already synthesized by mast cells, are immediately released, e.g. histamine and serotonin (Table 16.3).
  2. Secondary mediators: The mast cells synthesize them following stimulation by allergen and release, e.g. prostaglandins and leukotrienes (Table 16.3).
- **Pharmacological actions:** The chemical mediators perform several pharmacological actions, such as bronchial and other smooth muscle contraction, increased vascular permeability and vasodilation (Table 16.3)
- **Symptoms:** These actions in combinations, produce symptoms such as breathlessness, hypotension and shock leading to death at times.

Table 16.3: Mediators of type I hypersensitivity

Primary mediators	Action
Histamine, heparin and serotonin	↑Vascular permeability ↑Smooth-muscle contraction
Eosinophil chemotactic factor (ECF-A)	Eosinophil chemotaxis
Neutrophil chemotactic factor (NCF-A)	Neutrophil chemotaxis
Proteases	Bronchial mucous secretion Degradation of blood-vessel and basement membrane
Secondary mediators	**Action**
Platelet-activating factor	Platelet aggregation and degranulation; Contraction of pulmonary smooth muscles
Leukotrienes (slow reactive substance of anaphylaxis, SRS-A)	↑ Vascular permeability; contraction of pulmonary smooth muscles
Prostaglandins	↑Vasodilation; Contraction of pulmonary smooth muscles Platelet aggregation
Bradykinin	↑Vascular permeability; smooth-muscle contraction
Cytokines (IL-1 and TNF-α)	Systemic anaphylaxis; ↑ Expression of cell adhesion molecules (CAMs) on venular endothelial cells

Manifestations of Type I Reaction

Manifestations are grouped into immediate and late.

Immediate Manifestations

Systemic Anaphylaxis

It is an acute medical emergency condition, characterized by severe dyspnea, hypotension, and vascular collapse leading to death at times.

- It occurs within minutes of exposure to allergen and unless treated promptly, may lead to fatality
- Allergens: Wide range of allergens have been shown to trigger anaphylaxis in susceptible humans, including the venom (from bee, wasp, and ant stings); drugs (such as penicillin, insulin), antitoxins, seafood and nuts
- Epinephrine (adrenalin) is the drug of choice for systemic anaphylactic reactions.

Localized Anaphylaxis (Atopy)

Here, the reaction is limited to a specific target tissue or organ, mostly the epithelial surfaces at the entry sites of allergen. These allergies afflict more than 20% of people. They almost always run in families (i.e. inherited) and are collectively called **atopy**. Examples include:

- **Allergic rhinitis (or hay fever):** It is the most common atopic disorder, affecting 10% of the population. This results from exposure to airborne allergens with the conjunctiva and nasal mucosa leading to appearance of various symptoms such as ↑watery secretions of the conjunctiva, nasal mucosa, and upper respiratory tract, as well as sneezing and coughing
- **Asthma:** It is the second most common atopic manifestation. It differs from hay fever in involvement of lower respiratory mucosa, resulting in contraction of the bronchial smooth muscles and airway edema, ↑mucus secretion; all together leading to bronchoconstriction and dyspnea. The stimulus may or may not be an allergen. Accordingly, asthma can be classified as:
  - Allergic asthma: It is induced by air-borne or blood-borne allergens, such as pollens, dust, fumes, insect products, or viral antigens
  - Intrinsic asthma: It is independent of allergen stimulation; induced by exercise or cold.
- **Food allergy:** Various foods also can induce localized anaphylaxis in atopic individuals. The food allergens (e.g. nuts, egg, seafood, etc.) can either stimulate the mast cells lining gut mucosa to cause GI symptoms such as diarrhea and vomiting or may be carried in the blood stream to distant sites (e.g. when the allergen is deposited on skin, causes local wheal and flare like reaction called **atopic urticaria** (or hives)
- **Atopic dermatitis (allergic eczema):** It is an inflammatory disease of skin that is frequently associated with young children with family history of atopy. It often develops during infancy, manifested as erythematous skin eruptions which are filled with pus. The skin lesions have an increased response of T_H2 cells and eosinophils
- **Drug allergy:** Various drugs (such as penicillin, sulfonamides, etc.) may produce type I hyper-sensitivity responses which may be either local reactions or even sometimes produce systemic anaphylaxis.

Late Manifestations

The immediate phase of type 1 reaction is followed, 4-6 hours later, by an inflammatory response. This phase lasts for 1-2 days and leads to tissue damage.

- **Mediators:** They are released in acute phase along with cytokines (IL-3, IL-5, IL-8), ECF and NCF; induce recruitment of various inflammatory cells, such as neutrophils, eosinophils, macrophages, and lymphocytes, etc. Among the infiltrates, eosinophils and neutrophils predominate; each accounting for 30% of the total inflammatory cells influx
- **Eosinophil influx:** It is favored by ECF (eosinophil chemotactic factor), IL-5 and GM-CSF. Eosinophils express Fc receptors for IgG and IgE and thus bind directly to antibody-coated allergens. This in turn causes release of toxic granules from eosinophils which contribute to the chronic inflammation of the bronchial mucosa that characterizes persistent asthma
- **Neutrophil infiltration:** It is induced by NCF (neutrophil chemotactic factor), and other cytokines such as IL-8. Activated neutrophils release various mediators which further potentiate inflammatory tissue damage and thickening of basement membrane.

Factors Influencing Type I Hypersensitivity

1. Genetic Makeup

Host genetic factors play an important role in mounting an immune response against an allergen.

- Some individuals mount a normal response where as some mount an exaggerated immune response. Allergen to one individual may not be allergic to other individual
- There are several gene loci identified which encode proteins that are involved in the regulation of immune responses towards the allergens
- It is also observed that if both the parents are allergic there is 50% chance that the child will be allergic and when only one parent is allergic, the chance of the child being allergic drops down to 30%.

2. Allergen Dose

The dose of the allergen has a definite impact on the type of immune response produced. It is observed that repeated small doses of allergen induce a persistent IgE response in mice; while higher dosage leads to transient IgE response with a shift towards IgG response.

3. T_H1 vs T_H2 Response

The balance between T_H1 and T_H2 response determines the response of an individual towards an allergen.

- T_H1 response produces cytokine interferon-γ, which is inhibitory to type I hypersensitivity; whereas T_H2 response induced cytokine IL-3, IL-4 and IL-5 promotes IgE-mediated allergic response
- Hence, accordingly atopic and non-atopic individuals would demonstrate a predominant T_H2 and T_H1 response to an allergen respectively.

Detection of Type I Hypersensitivity

Skin Prick Test

Small amounts of suspected potential allergens are introduced at different skin sites either by intradermal injection or by superficial scratching.

- If a person is already sensitized to the allergen, a local wheal and flare response develops within 30 minutes at the inoculation sites (Fig. 16.2)
- Advantage: Skin test is relatively inexpensive and allows screening of a large number of allergens at one go
- Disadvantage: It may occasionally sensitize the individual to new allergens and in some rare cases may induce late-phase reaction or even systemic anaphylactic shock.

Radioimmunosorbent Test (RIST)

It quantitatively detects the **total serum IgE** antibody up to nanogram levels.

- It is a highly sensitive technique, based on the radioimmunoassay
- The patient's serum (containing IgE) is made to react with agarose beads or paper disks coated with anti-IgE. After the beads or disks are washed, radiolabeled anti-IgE is added
- The radioactivity of the beads or disks, measured with a gamma counter, is proportional to the level of IgE in the patient's serum (Fig. 16.3A).

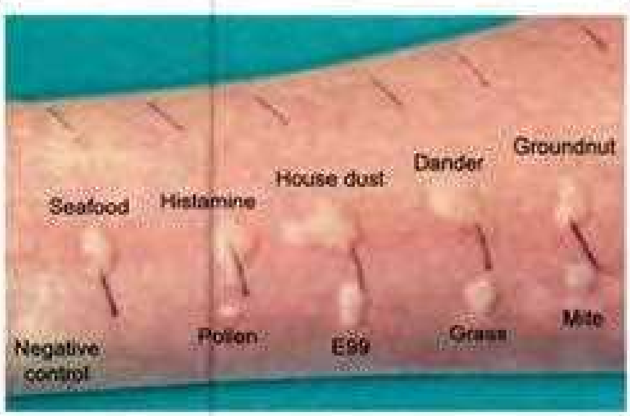


Fig. 16.2: Skin testing by intradermal testing of allergens into forearm

Radioallergosorbent Test (RAST)

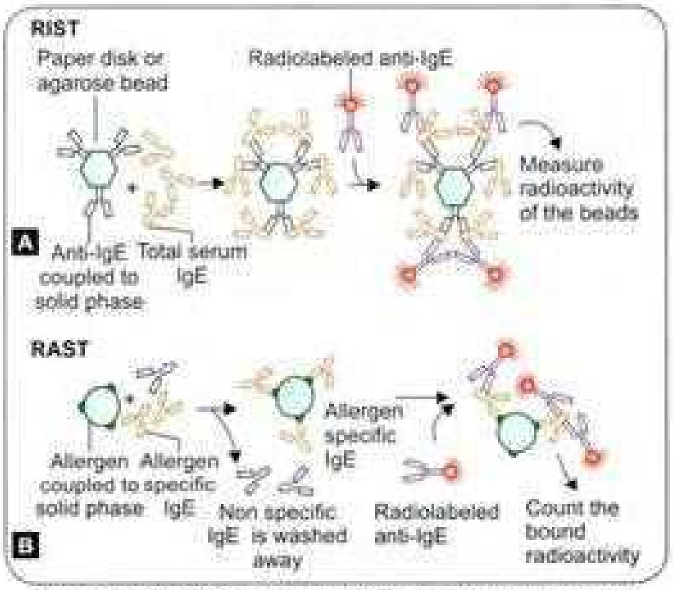
It is an another method similar to RIST, but it quantifies the serum level of **allergen specific IgE**.

- Here, instead of anti-IgE, the allergen itself is coated to beads or disks, so that when the patient's serum is added, only the allergen specific IgE would bind to the beads or disks
- After washing the unbound nonspecific IgE, the amount of allergen specific IgE is then measured by adding radiolabeled anti-IgE, followed by counting the bound radioactivity by a gamma counter (Fig. 16.3B).

TREATMENT — Type I hypersensitivity reaction

- **Avoidance of contact with known allergens:** The first and foremost step is identification and avoidance of contact with known allergens such as dusts, house pets, allergic food, etc. However, it is not practically possible to avoid all allergens especially air born allergens, such as pollens
- **Hyposensitization:** Repeated exposure to increased subcutaneous doses of allergens can reduce or eliminate the allergic response to the same allergen
  - This occurs probably due to either (1) a shift of IgE response towards IgG or (2) a shift of T_H2 response towards T_H1 response, which secrete IFN-γ that in turn can suppress the IgE response

Contd...



Figs 16.3A and B: Principles of. **A.** Radioimmunosorbent test (RIST); **B.** Radioallergosorbent test (RAST)

Contd...

TREATMENT **Type I hypersensitivity reaction**

> Here, the IgG acts as blocking antibody because it competes with IgE for binding to the allergen. The IgG-allergen immunocomplex can be removed later by phagocytosis.

- **Monoclonal anti-IgE:** Humanized monoclonal anti-IgE can bind and block the IgE; but useful only if the IgE is not already bound to high affinity Fc receptors.
- **Drugs:** Several drugs are useful in suppressing type 1 response through various mechanisms (Table 16.4).

TYPE II HYPERSENSITIVITY REACTION

In type II reactions, the host injury is mediated by **antibodies** (IgG or rarely IgM) which interact with various types of antigens, such as:

- Host cell surface antigens (e.g. RBC membrane antigens like blood group and Rh antigens)

Table 16.4: Drugs used in type I hypersensitivity

Drugs	Mechanism of action
Antihistamines	Block H1 receptors on target cells; hence antagonize the effects of histamine released
Epinephrine (adrenaline)	Stimulates cAMP production in mast cells; thereby prevents mast cell degranulation
Cortisone	Blocks conversion of histidine to histamine and stimulates cAMP levels in mast cells
Theophylline	Prolongs high cAMP levels in mast cells
Cromolyn sodium	Blocks Ca^{2+} influx into mast cells

Abbreviation: cAMP, cyclic adenosine monophosphate.

- Extracellular matrix antigens or
- Exogenous antigens absorbed on host cells (e.g. a drug coating on RBC membrane).

After Ag-Ab binding occurs, the Fc region of antibody initiates the type II reactions by the following three broad mechanisms (Figs 16.4A and B).

Complement-dependent Reactions

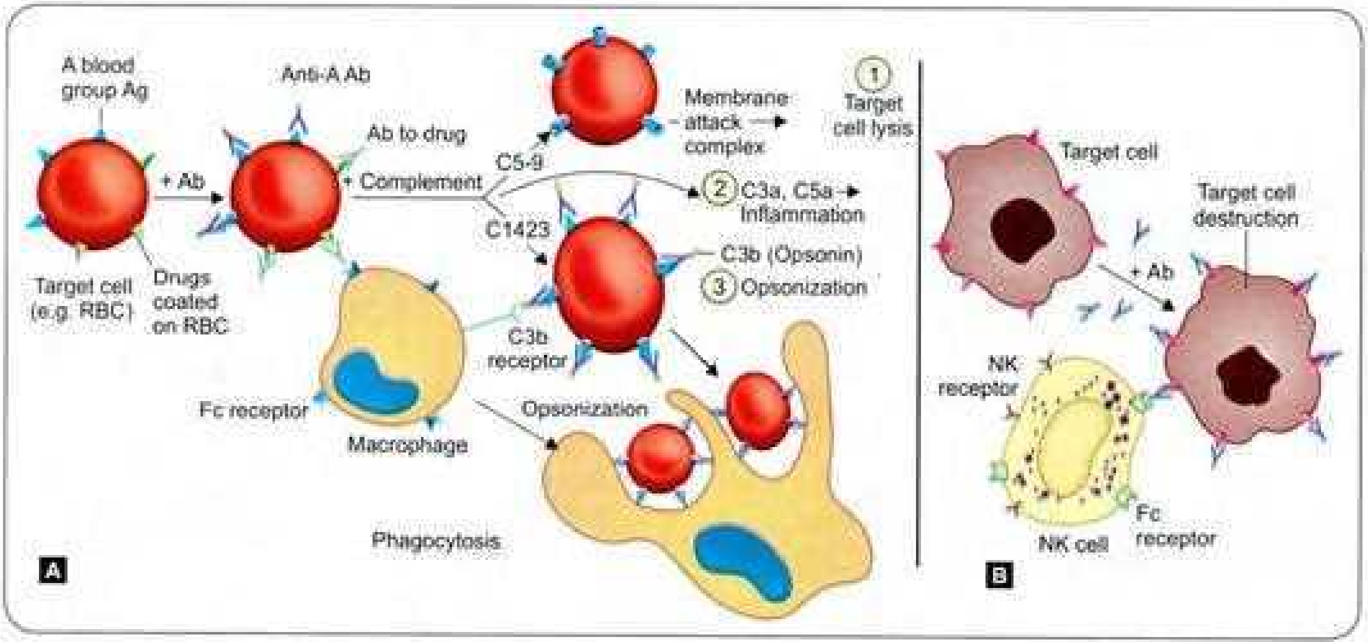
The Fc region of antibody (bound with antigen) can activate the classical pathway of complement system. Activation of classical pathway leads to host cell injury which is mediated by the following three mechanisms (Fig. 16.4A).

1. **Complement-dependent cytolysis:** The membrane attack complex (C5-C9) formed by the activation of classical pathway can produce pores which lead to lysis of the target cells.
2. **Complement-dependent inflammation:** The by-product of complement pathways such as C3a and C5a are chemoattractants; hence can induce inflammatory response leading to tissue injury.
3. **Opsonization:** By-products of complement pathway, such as C3b and C4b act as opsonins. They deposit on the target cells. Phagocytes, such as macrophage and neutrophil can engulf such C3b and C4b coated target cells via complement receptors.

Complement Mediated Type II Reactions

Antibody-dependent complement mediated type II hypersensitivity is observed in various clinical conditions such as:

Contd...



Figs 16.4A and B: Different mechanisms of antibody mediated type II hypersensitivity reactions. **A.** Complement-dependent reactions; **B.** Antibody-dependent cellular cytotoxicity (ADCC)

Abbreviations: RBC, red blood cell; NK, natural killer.

Contd...

- **Transfusion reaction (ABO incompatibility):** RBCs from an incompatible donor are destroyed after being coated with recipient antibodies directed against the donor's blood group antigens (Fig. 16.4A)
- **Erythroblastosis fetalis (Rh incompatibility):** Rh negative mother having anti-Rh antibodies due to prior exposure to Rh positive blood (due to previous pregnancy or blood transfusion), can cross the placenta and cause destruction of Rh-positive fetal RBCs
- **Autoimmune hemolytic anemia, agranulocytosis, or thrombocytopenia:** All these result due to production of autoantibodies to individual's own membrane antigens of RBCs/granulocytes/platelets respectively
- **Drug-induced hemolytic anemia:** Drug or its metabolic products may get adsorbed onto RBC membrane. If antibodies are formed against the drug, these antibodies will bind with the adsorbed drug on RBC surface and lead to complement activation and lysis of RBCs. For example, following quinine therapy used for malaria (resulting in black water fever) and penicillin therapy (Fig. 16.4A)
- **Pemphigus vulgaris** (autoantibodies against desmosomal proteins that lead to disruption of epidermal intercellular junctions).

Antibody-Dependent Cellular Cytotoxicity (ADCC)

IgG antibodies can coat on the target cells by interacting with the surface antigens through Fab region. The Fc portion of IgG in turn binds to Fc receptors on various effector cells such as NK cells which result in destruction of the target cells (Fig. 16.4B).

- ADCC is involved in destruction of the targets that are too large to be phagocytozed, e.g. parasites, tumors or graft rejection
- Although ADCC is typically mediated by IgG antibodies, in certain instances (e.g. eosinophil-mediated killing of parasites) IgE antibodies are used.

Autoantibody Mediated (Antibody-dependent Cellular Dysfunction or ADCD)

In this condition, the host produces certain autoantibodies which bind and disturb the normal function of human self-antigens.

- **Anti-receptor Ab:** Antibodies may be directed against human receptors, resulting in either inhibition or excessive activation of the receptors leading to host injury
  - **Activation of receptor, e.g. Graves' disease:** Here, the autoantibodies produced are called LATS (long-acting thyroid stimulators), which stimulate the thyroid cells to upregulate the production of thyroid hormones
  - **Inhibition of receptor, e.g. myasthenia gravis:** In this condition, anti-acetylcholine (ACh) receptor antibodies are produced; which block the ACh receptors, leading to profound muscular weakness.
- **Other examples of ADCD:**
  - Goodpasture syndrome (antibody produced against type IV collagen)
  - Pernicious anemia (antibody directed against intrinsic factor)
  - Rheumatic fever (antibody against streptococcal antigens cross reacting with heart)
  - Myocarditis in Chagas disease.

TYPE III HYPERSENSITIVITY REACTION

Type III hypersensitivity reactions are as a result of excess formation of immune complexes (Ag-Ab complexes) which initiate an inflammatory response through activation of complement system leading to tissue injury (Fig. 16.5).

- **Antigen involved:** Immune complexes can involve exogenous antigens such as bacteria and viruses or endogenous antigens such as DNA
- **Removal of immune complexes:** Mere formation of immune complexes does not result in type III hypersensitivity reaction
  - Under normal circumstances, the immune complexes are rapidly cleared by activation of complement system

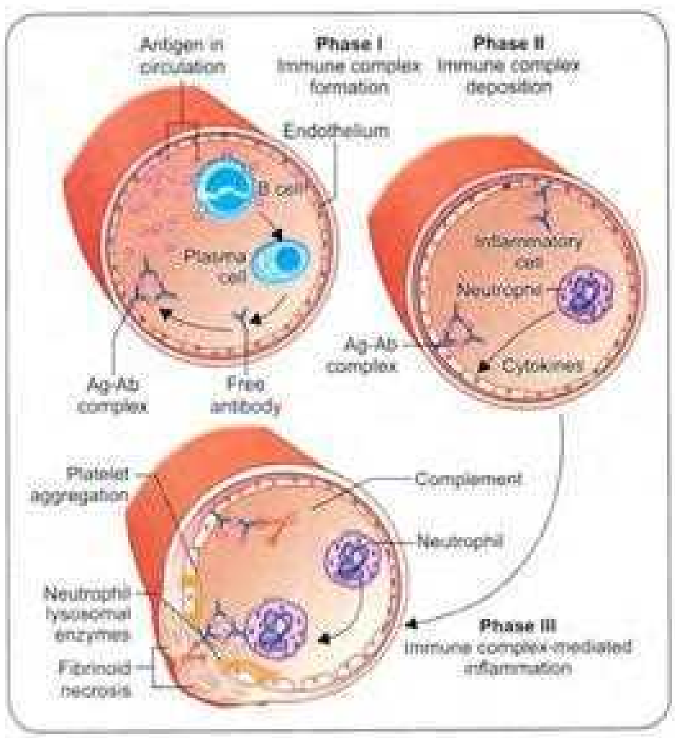


Fig. 16.5: Mechanism of systemic type III hypersensitivity reaction

- Immune complexes coated with complements are either directly phagocytosed by macrophages/monocytes or are bound to RBCs and carried to liver and spleen where they are phagocytosed.

- However, in some situations, the immune system may be exposed to excess dose of antigen over long period of time such as in chronic infection, autoimmune diseases, and repeated exposure to environmental pollutants. This leads to formation of excessive immune complexes.

Soluble vs Insoluble Immune Complexes

Balance between level of antigen and antibody decides the nature of immune complex that is going to be formed.

- In case of antibody excess or antigen-antibody equivalence, immune complexes formed are large and insoluble; which tend to localize near the site of antigen administration to produce a localized type III reaction
- However, in situations when the antigen is in excess (particularly monovalent antigens), small soluble complexes are formed which tend to travel through blood and get deposited in various sites producing a generalized type III reaction.

Mechanism of Tissue Injury

Classical Complement Activation

The Ag-Ab-immune complexes stimulate the classical pathway of complement; the products of which mediate the tissue injury in type III reaction.

- **Anaphylatoxin:** Complement by-products C3a and C5a being anaphylatoxins, induce localized mast cell degranulation with consequent increase in vascular permeability
- **Chemoattractant:** C3a and C5a also act as chemoattractants, causing recruitment of neutrophils to the site of immune complex deposition
- **Role of neutrophils:** Neutrophils attempt to phagocytose the large immune complexes, but fail in doing so. Instead, they release large number of lytic enzymes from the secretory granules (through **frustrated phagocytosis**) which causes extensive tissue damage.

Platelet Activation

Immune complexes bind to the Fc receptors on platelets leading to their activation. Platelet aggregation (leads to microthrombi formation) and vasoactive amines released from activated platelets, both together cause tissue ischemia leading to further tissue damage.

Activation of Hageman Factor

Activation of Hageman factor leads to activation of kinin, which in turn causes vasodilatation and edema.

Types of Type III Hypersensitivity Reaction

Type III reactions are either localized or generalized.

Localized or Arthus Reaction

Arthus reaction is defined as localized area of tissue necrosis due to vasculitis resulting from acute immune complex deposition at the site of inoculation of antigen.

The reaction is produced experimentally (NM Arthus, 1903) by injecting an antigen into the skin of a previously immunized animal, e.g. rabbit (i.e. excess of preformed antibodies against the injected antigen are already present in the circulation). The circulating antibodies bind with the antigen in the dermis and form immune complexes. These immune complexes fix the complement, resulting in localized immune complex mediated inflammatory response called **Arthus reaction**.

In humans, localized Arthus reaction is seen in some situations, such as:

- **In skin:** (1) following insect bites or (2) during allergic desensitization treatment wherein repeated injections of the same antigen is given for long periods
- **In lungs**, following inhalation of bacteria, fungi, spores or proteins may produce intrapulmonary lesions. Examples include conditions causing extrinsic allergic alveolitis, such as:
  - Farmer's lung: It develops following inhalation of actinomycetes (*Saccharopolyspora* species) from mouldy hay
  - Bird-Fancier's disease: This develops following inhalation of serum proteins in dust derived from dried pigeon's feces.

Generalized or Systemic Type III Reactions

The pathogenesis of systemic immune complex disease can be divided into two phases:

1. **Formation of small sized soluble Ag-Ab complexes** in the circulation, which occurs following the entry of a large dose of antigen into the body.
2. **Induces inflammatory reaction:** Deposition of the immune complexes in various tissues, thus initiating an inflammatory reaction in various sites throughout the body such as: blood vessels (vasculitis), glomerular basement membrane (glomerulonephritis), and synovial membrane (arthritis). This has been linked to the pathogenesis of various diseases (Table 16.5).

Serum Sickness

This is another historical example of type III reaction. This condition is not seen nowadays, it was seen in the past,

Table 16.5: Diseases associated with generalized type III hypersensitivity reactions

Connective tissue disorders: Result due to autoantibodies forming immune complexes with self-antigens
- SLE (systemic lupus erythematosus): Anti-DNA Ab
- Rheumatoid arthritis: Ab against human immunoglobulin
- PAN (polyarteritis nodosa)

Parasitic diseases: Resulting from immune complex deposition
- Nephrotic syndrome in *Plasmodium malariae*
- Katayama fever in schistosomiasis
- African trypanosomiasis

Bacterial diseases: Resulting from immune complex deposition
- *Streptococcus pyogenes*: Post-streptococcal glomerulonephritis
- *Mycobacterium leprae* (Lepra reaction type 2)

Viral diseases: With immune complex deposition
- Hepatitis B (arthritis)
- Hepatitis C (arthritis)
- Infectious mononucleosis (Epstein-Barr virus)
- Dengue (arthritis)

Others:
- Hyperacute graft rejection
- Subacute bacterial endocarditis
- Serum sickness

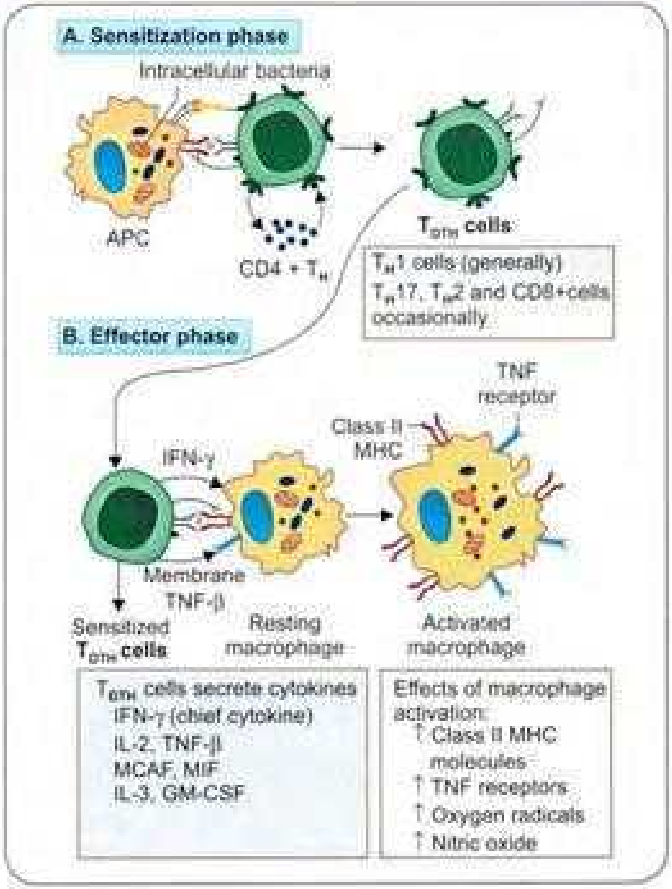
following serum therapy, i.e. administration of foreign serum, e.g. horse anti-tetanus serum, to treat tetanus cases.
- ❖ The horse serum proteins being foreign can induce antibody formation in the host, leading to generation of large number of immune complexes
- ❖ Typically, after 7-8 days, the individuals begin to show various manifestations which are collectively called **serum sickness**. The symptoms include fever, weakness, vasculitis, edema, erythema and rarely lymphadenopathy and glomerulonephritis
- ❖ It subsides gradually once the immune complexes are cleared and free antibodies accumulate.

TYPE IV HYPERSENSITIVITY REACTION

Type IV hypersensitivity reactions differ from other types in various ways:
- ❖ It is delayed type (occurs after 48-72 hours of antigen exposure)
- ❖ It is cell-mediated; characteristic cells called **T_{DTH} cells** (delayed type of hypersensitivity T cells) are the principal mediators of type IV reactions
- ❖ Tissue injury occurs predominantly due to activated macrophages.

Mechanism of Type IV Reactions

Type IV hypersensitivity reactions occur through two phases—(1) sensitization and (2) effector phases (Figs 16.6A and B).



Figs 16.6A and B: Mechanism of delayed type hypersensitivity: **A.** Sensitization phase; **B.** Effector phase

Abbreviations: APCs, antigen presenting cells; MHC, major histocompatibility complex; TNF, tumor necrosis factor; IFN, interferons; DTH, delayed type hypersensitivity; MCAF, monocyte chemotactic and activating factor; MIF, migration inhibitory factor; GM-CSF, granulocyte monocyte colony stimulating factor.

Sensitization Phase

This is the initial phase of 1-2 weeks occurring following antigenic exposure (Fig. 16.6A).
- ❖ During this period, the antigen presenting cells (APCs) process and present the antigenic peptides along with MHC-II to the helper T cells. T_H cells are differentiated to form T_{DTH} cells
- ❖ Most T_{DTH} cells are derived from T_H1 cells; but occasionally other T cells, such as $CD8^+$ T cells and $CD4^+$ T_H17 can also act as T_{DTH} cells.

Effector Phase

The T_{DTH} cells, on subsequent contact with the antigen, secrete variety of cytokines which attract and recruit various inflammatory cells (e.g. macrophages) at the site of DTH reaction (Fig. 16.6B).

Cytokines Secreted from T_{DTH} Cells

- **Interferon-γ-:** It is the key cytokine of type IV reaction. It activates the resting macrophages into activated macrophages which are highly competent for microbial killing; mediated through several mechanisms such as:
  - ↑ Expression of MHC-II molecules so that they can act as efficient APCs
  - ↑ TNF receptors
  - ↑ Levels of oxygen radicals and nitric oxide.
- **Interleukin-2 (IL-2):** It acts in autocrine manner; stimulates the proliferation of T_{DTH} cells
- **MCAF** (Monocyte chemotactic and activating factor) and **TNF β**–Help in migration of monocytes from blood to the site of DTH and transforming them into tissue macrophages
- **MIF** (migration inhibitory factor): It further inhibits migration of macrophages from the site of DTH
- **IL-3** and **GM-CSF** (granulocyte-monocyte colony stimulating factor)-help in local synthesis of monocytes.

Role of DTH: Protective vs Tissue Damage Response

Through type IV hypersensitivity reactions, host attempts to provide defense against many intracellular microorganisms such as *M. tuberculosis* as well as several chemicals and nickel salts (Table 16.6). Always, the attempts do not result in protection.

Table 16.6: Examples of delayed-type hypersensitivity (DTH)

Intracellular pathogens inducing DTH	
Intracellular bacteria • *Mycobacterium leprae* • *M. tuberculosis* • *Listeria monocytogenes* • *Brucella abortus*	**Intracellular fungi** • *Pneumocystis jirovecii* • *Candida albicans* • *Histoplasma capsulatum* • *Cryptococcus neoformans*
Intracellular viruses • Herpes simplex virus • Variola (smallpox) • Measles virus	**Skin test to demonstrate DTH** • Tuberculin test (Mantoux test) • Lepromin test • Montenegro test (leishmaniasis) • Frei test—done in LGV
Contact dermatitis	
Following exposure to contact antigens: Nickel, poison ivy, poison oak, picryl chloride	
Other examples of DTH	
Noninfectious conditions • Diabetes mellitus type 1 • Multiple sclerosis • Peripheral neuropathies • Hashimoto's thyroiditis	• Crohn's disease • Chronic transplant rejection • Graft-versus-host disease
Granuloma formation seen in Tuberculosis, sarcoidosis, schistosomiasis and other trematode infections	**Other example** Lepra reaction type I

Abbreviation: LGV, lymphogranuloma venereum.

Protective Response

Under normal circumstances, the pathogens are usually cleared with little tissue damage; mediated by the enhanced microbicidal potency of activated macrophages.

Tissue Damage Response

However, in conditions, when the intracellular microbes escape the macrophage killing mechanisms; the enhanced phagocytic activity and release of various lytic enzymes by the activated macrophages in an attempt to kill the pathogen leads to nonspecific tissue destruction.

Pathology of DTH Reaction (Granuloma Formation)

Continuous DTH reaction for killing the intracellular microbes (especially persistent and/or nondegradable antigens) leads to formation of granuloma (e.g. tubercles in leprosy and tuberculosis).

- The initial T_H cell infiltrate is progressively replaced by macrophages in 2–3 weeks. Macrophages transform into two type of cells:
  1. They become large, flat, and eosinophilic; denoted as **epithelioid cells**.
  2. The epithelioid cells occasionally fuse (induced by IFN-γ) to form **multinucleated giant cells**.

Granuloma consists of an inner zone of epithelioid cells, typically surrounded by a collar of lymphocytes and a peripheral rim of fibroblasts and connective tissue (Fig. 16.7).

Tuberculin Test

Tuberculin test is the prototype of delayed hypersensitivity. In sensitized individuals, (i.e. who possess sensitized T_{DTH}

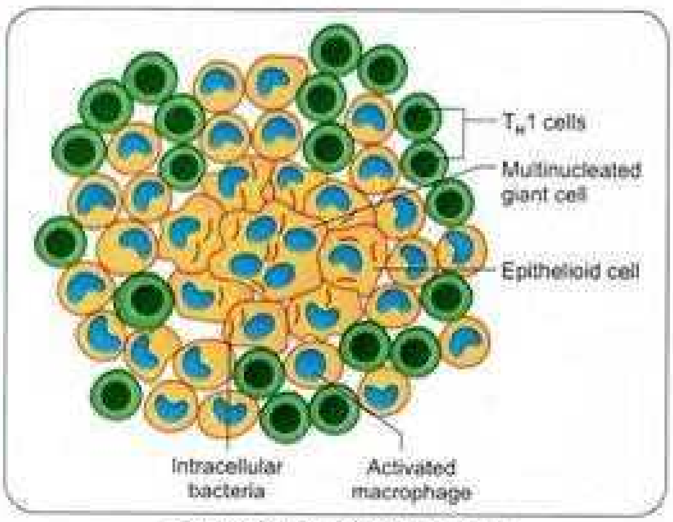


Fig. 16.7: Structure of granuloma

cells due to prior contact with *M. tuberculosis*); when a preparation of tuberculin antigen (glycerol extract of the tubercle bacillus) is injected intradermally, a local reaction develops after 48–72 hours consisting of induration surrounded by erythema.

Contact Dermatitis

Many antigens such as nickel, poison oak, etc. (Table 16.6) act by producing DTH response:

- Most of these substances are haptens; they complex with skin proteins, which act as carrier to make the haptens immunogenic
- This hapten-skin protein complex is internalized by skin APCs (e.g. Langerhans cells), then presented to T_H cells to induce a TDH reaction
- Activated macrophages release lytic enzymes which result in skin lesions (e.g. redness and pustule seen following contact with poison oak).

EXPECTED QUESTIONS

I. Essay:

1. Define and classify hypersensitivity reactions. Write in detail about type IV hypersensitivity reaction.
2. Neha, a 17-year student who has recently joined MBBS, has come back to the hostel after the first vacation. After entering to her hostel room, she suddenly developed an episode of severe sneezing, and dyspnea. She had to be admitted to the casualty and when asked, she told that she has faced similar episodes since her childhood.
   a. What type of immune reaction is this?
   b. Describe the pathogenesis of this condition and management.

II. Write short notes on:

1. Type II hypersensitivity reaction.
2. Immune complex mediated hypersensitivity reaction.

III. Multiple Choice Questions (MCQs):

1. **Type I hypersensitivity is mediated by which of the following immunoglobulins?**
   a. IgA b. IgG
   c. IgM d. IgE
2. **Wheal and flare is whichtype of hypersensitivity reaction:**
   a. Type I b. Type II
   c. Type IV d. Type V
   e. Mediated through allergen specific IgE
3. **Type I hypersensitivity includes all of the following except:**
   a. Hemolytic anemia b. Anaphylaxis
   c. Extrinsic asthma d. Hay fever
4. **The type of hypersensitivity reaction in myasthenia gravis is:**
   a. Type I b. Type II
   c. Type III d. Type IV
5. **A positive tuberculin test is an example of:**
   a. Type I hypersensitivity
   b. Type II hypersensitivity
   c. Type III hypersensitivity
   d. Type IV hypersensitivity
6. **Major cytokine involved in type I hypersensitivity:**
   a. IL-1 b. IL-2
   c. IL-4 d. IL-6
7. **About radioimmunosorbent test (RIST) and radioallergosorbent test (RAST), Which is false?**
   a. RIST- Quantitatively detects the total serum IgE
   b. RAST- Quantifies the serum level of allergen specific IgE
   c. RAST- IgE is coated to beads or disks
   d. RIST- Anti-IgE is coated to beads or disks
8. **Hyposensitization as a treatment of type I hypersensitivity, Which of the following statement is worng?**
   a. Repeated exposure to small doses of allergen is given
   b. Shift of IgE response towards IgG occurs
   c. Shift of T_H2 response towards T_H1 may occur
   d. IgG acts as blocking antibody because it competes with IgE for binding to the allergen
9. **Example of antibody-dependent cellular dysfunction or ADCD:**
   a. Graves' disease b. Hemolytic anemia
   c. Pemphigus vulgaris d. Transfusion reaction
10. **Type II hypersensitivity reaction, where antibody binds to exogenous antigens absorbed on host cells:**
    a. Transfusion reaction (ABO incompatibility)
    b. Erythroblastosis fetalis (Rh incompatibility)
    c. Autoimmune hemolytic anemia
    d. Drug-induced hemolytic anemia
11. **Which of the following statement is wrong about type III hypersensitivity reaction?**
    a. Antibody excess—produce a localized type III reaction
    b. Antigen excess—produce a systemic type III reaction
    c. Antigen-antibody equivalence—produce a localized type III reaction
    d. Soluble immunocomplexes—produce a localized type III reaction
12. **DTH T cells can be derived from all, *except*:**
    a. T_H1 cells b. T_H2 cells
    c. CD8 T cells d. CD4+ TH17

Answers

1. d **2.** a **3.** a **4.** b **5.** d **6.** c **7.** c **8.** a **9.** a **10.** d **11.** d **12.** b

Autoimmunity

17
CHAPTER

Chapter Preview

- Immunological tolerance
- Mechanisms of autoimmunity
- Autoimmune diseases
- Single organ or cell type autoimmune diseases
- Systemic autoimmune diseases
- Laboratory diagnosis of autoimmune diseases

Autoimmunity is a condition in which the body's own immunologically competent cells or antibodies act against its self-antigens resulting in structural or functional damage. Paul Ehrlich had first introduced the concept of autoimmunity; he termed this condition as "horror autotoxicus."

- Normally immune system does not react to its own antigens due to a protective mechanism called tolerance. Any breach in tolerance mechanisms predisposes to several autoimmune diseases
- Therefore, before going into the details of mechanisms of autoimmunity; it is essential to know about the various tolerance mechanisms that the human immune system possesses.

IMMUNOLOGICAL TOLERANCE

Immunological tolerance is a state in which an individual is incapable of developing an immune response against his own tissue antigens. It is mediated by two broad mechanisms—central tolerance and peripheral tolerance.

Central Tolerance

This refers to the deletion of self-reactive T and B lymphocytes during their maturation in central lymphoid organs (i.e. in the thymus for T cells and in the bone marrow for B cells).

- **In thymus:** During the T cell development in thymus, if any self-antigens are encountered, they are processed and presented by thymic antigen presenting cells (APCs) in association with self-MHC. Any developing T cell that expresses a receptor for such self-antigen is negatively selected (i.e. deleted by apoptosis). Therefore, the resulting peripheral T-cell pool is devoid of self-reactive cells
- **In bone marrow:** When developing immature B cells in the bone marrow encounter a self-antigen during their development, the tolerance is developed by:
  - **Receptor editing:** It is a process by which many of the B cells reactivate the machinery of antigen receptor gene rearrangement (mainly genes coding for light chains), so that a different (edited) B cell receptor will be produced which no longer recognizes the self-antigen
  - **Negative selection:** After receptor editing, if the B cells again recognize a self-antigen, then they are destroyed by subjecting them to apoptosis.

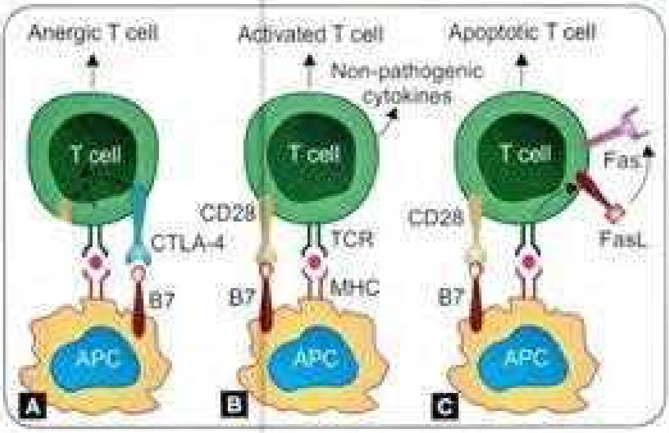
However, the process of central tolerance is not completely perfect. Many self-reactive T and B cells bearing receptors for self-antigens escape into the periphery. Hence, for counteracting those lymphocytes, peripheral tolerance takes a lead role.

Peripheral Tolerance

This refers to several back-up mechanisms that occur in the peripheral tissues to counteract the self-reactive T cells that escape central tolerance. It is provided by several mechanisms (Fig. 17.1).

- **Ignorance:** The self-reactive T cells might never encounter the self-antigen which they recognize and therefore remain in a state of ignorance
- **Anergy:** It can be defined as unresponsiveness to antigenic stimulus. The self-reactive T cells interact with the APCs presenting the self-antigen, but the co-stimulatory signal is blocked. The B7 molecules on APC bind to CTLA-4 molecules on T cells instead of CD28 molecules (Fig. 17.1A).

  Note: Normally, T cell activation requires two signals- Main signal (provided by antigen MHC complex of APC



Figs 17.1A to C: Mechanisms of peripheral tolerance. **A.** Anergy; **B.** Phenotypic skewing; **C.** Apoptosis

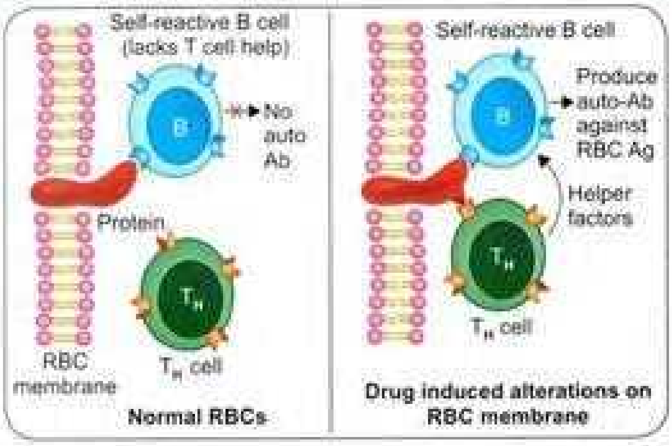


Fig. 17.2: T cells help to stimulate self-reacting B cells

interacts with TCR on T cell) and a co-stimulatory signal (B7 molecules on APCs bind to CD28 on T cells). If self-antigens are processed and presented by APCs, that do not bear the co-stimulators, a negative signal is delivered, and the cell becomes anergic.

- **Phenotypic skewing:** Self-reactive T cells interacting with APCs presented with self-antigens, undergo full activation, but might secrete nonpathogenic cytokines and chemokine receptors profile, hence although they are activated, fail to induce autoimmune response (Fig. 17.1B)
- **Apoptosis by AICD:** Self-reactive T cells are activated after interacting with APCs presented with self-antigens. But the activation of T cells induces upregulation of Fas ligand which subsequently interacts with the death receptor Fas leading to apoptosis. This mechanism is called as activation-induced cell death (AICD) (Fig. 17.1C)
- **Regulatory T cells (T_{reg} cells):** T_{reg} cells can down regulate the self-reactive T cells through secreting certain cytokines (e.g. IL-10 and transforming growth factor β [TGF-β]) or killing by direct cell-to-cell contact
- **Dendritic cells (DCs):** When certain dendritic cells such as immature DCs and tolerogenic DCs capture the self-antigen for processing, they down regulate the expression of molecules of costimulatory ligands such as CD40 and B7 molecules or act indirectly by induction of regulatory T cells
- **Sequestration of self-antigen:** Certain self-antigens can evade immune recognition by sequestration in immunologically privileged sites, e.g. corneal proteins, testicular antigens and antigens from brain.

B cells can also exhibit peripheral tolerance. The self-reacting B cells that have escaped (10%) the central tolerance at bone marrow are further destroyed at spleen by several mechanisms such as downregulation of a B cell growth factor called B cell activating factor (BAFF).

MECHANISMS OF AUTOIMMUNITY

Autoimmunity results due to breakdown of one or more of the mechanisms of immunological tolerance.

Breakdown of T Cell Anergy

Normal cells that do not usually express costimulatory molecules (B7) can be induced to do so. Such induction may occur in presence of tissue necrosis and local inflammation. This mechanism has been postulated for—

- Multiple sclerosis
- Rheumatoid arthritis
- Psoriasis.

Failure of AICD

Failure of the autoreactive activated T cells to undergo activation-induced cell death (AICD), i.e. apoptosis via Fas-Fas ligand can lead to autoimmunity. It is observed in patients suffering from systemic lupus erythematosus (SLE).

Loss of T_{reg} Cells

Autoimmunity can result following the loss of regulatory T cell-mediated suppression of self-reactive lymphocytes.

Providing T Cell help to stimulate self-reacting B Cells

Antibody response to self-antigens occurs only when potentially self-reactive B cells receive help from T cells. For example, in autoimmune hemolytic anemia, administration of certain drugs may result in drug-induced alterations in the red cell surface that create antigens which can be recognized by helper T cells (Fig. 17.2).

Release of Sequestered Antigens

The sequestered antigens are usually viewed as foreign to the immune system as they are never been exposed to the tolerance mechanisms during development of immune system. Injury to the organs leads to release of such sequestered antigens which are very well capable of mounting an immune response. Spermatozoa and ocular antigens released after trauma or surgery can cause post-vasectomy orchitis and post-traumatic uveitis.

Infectious agents may participate in the pathogenesis of autoimmunity by the following mechanisms:

Molecular Mimicry

Some microorganisms share antigenic determinants (epitopes) with self-antigens, and an immune response against such microbes would produce antibodies that can crossreact with self-antigen.

- For example, acute rheumatic fever results due to antibodies formed against streptococcal antigens (M protein), cross react with cardiac antigens (glycoproteins), due to antigenic cross reactivity
- Molecular mimicry involving T-cell epitopes—Examples include multiple sclerosis, where T cell clones reacting to myelin basic protein probably would have been induced by reacting against peptides derived from many microbes including viruses.

Polyclonal Lymphocyte Activation

Several microorganisms and their products are capable of causing polyclonal (i.e. antigen-nonspecific) activation of T cells or B cells.

- **Polyclonal T cell activation:** Superantigens released from microbes (e.g. *Staphylococcus aureus*), polyclonally activate the T cells directly by binding to antigen non-specific Vβ region of T cell receptors
- **Polyclonal B cell activation:** It can be induced by products of various microbes such as Epstein-Barr virus, HIV, etc.

Exposure of Cryptic Self-epitopes

Research has proved that "molecular sequestration" of antigens is much more common than anatomic sequestration.

- During development of immune system, not all epitopes of an antigen are effectively processed and presented to T cells. There are some nondominant cryptic epitopes which remain sequestrated. Hence, T cell clones reacting against such epitopes are not deleted
- Such cryptic self-epitopes can be released secondary to inflammation at a site of tissue injury, which can induce increased protease production and differential processing of released self-epitopes by APCs.

Epitope Spreading

The self-peptides released due to persistent inflammation induce tissue damage (as occurs in chronic microbial infection) and are processed and presented by APCs along with microbial peptides. It is possible that, there may occur a shift or spread of T cell recognition to self-epitopes presented on APCs rather than recognizing microbial epitope.

Bystander Activation

It is the nonspecific activation of bystander self-reactive T_H1 cells. Activation of microorganism-specific T_H1 cells leads to cytokine influx which causes an increased infiltration of various nonspecific T cells at the site of infection.

AUTOIMMUNE DISEASES

The immunological attack of self-reacting T lymphocytes or autoantibodies on tissues leads to the development of various autoimmune diseases. There are broad ranges of autoimmune diseases which can either be localized into single organ/cell type or may involve many organs and cause systemic manifestations (Table 17.1).

Laboratory Diagnosis of Autoimmune Diseases

Autoimmune diseases are diagnosed by detection of various autoantibodies in serum of the patients;

Table 17.1: Autoimmune diseases and immune response produced with their clinical manifestations

Single organ or cell type autoimmune diseases		
Disease	**Self-antigen present on**	**Type of immune response and important features**
Autoimmune anemias		
Autoimmune hemolytic anemia	RBC membrane proteins	Autoantibodies to RBC antigens trigger complement mediated lysis or antibody-mediated opsonization of the RBCs
Drug-induced hemolytic anemia	Drugs alter the red cell membrane antigens	Drugs such as penicillin or methyldopa interact with RBCs so that the cells become antigenic
Pernicious anemia	Intrinsic factor (a membrane-bound protein on gastric parietal cells)	Autoantibodies to intrinsic factor block the uptake of vitamin B12; leads to megaloblastic anemia
Idiopathic thrombocytopenic purpura	Platelet membrane proteins (glycoproteins IIb-IIIa or Ib-IX)	Auto-antibodies against platelet membrane antigens leads to ↓platelet count
Goodpasture syndrome	Renal and lung basement membranes	Auto-antibodies bind to basement-membrane antigens on kidney glomeruli and the alveoli of the lungs followed by complement mediated injury leading to progressive kidney damage and pulmonary hemorrhage

Contd...

Contd...

Myasthenia gravis	Acetylcholine receptors	Blocking type of autoantibody directed against Ach receptors present on motor nerve endings, leads to progressive weakening of the skeletal muscles
Graves' disease	Thyroid-stimulating hormone (TSH) receptor	Anti-TSH-autoantibody (stimulates thyroid follicles, leads to hyperthyroid state)
Hashimoto's thyroiditis	Thyroid proteins and cells	Autoantibodies and T_{DTH} cells targeted against thyroid antigen leads to suppression of thyroid gland • Seen in middle-aged females • Hypothyroid state is produced (↓ production of thyroid hormones)
Post-streptococcal glomerulonephritis	Kidney	Streptococcal antigen–antibody complexes are deposited in glomerular basement membrane
Systemic autoimmune diseases		
Disease	**Self-antigen present on**	**Type of immune response and important features**
Systemic lupus erythematosus (SLE)	Autoantibodies are produced against various tissue antigens such as DNA, nuclear protein, RBC and platelet membranes	• **Age and sex:** Women (20-40 years of age) are commonly affected; female to male ratio is 10:1 • **Immune complexes** (self Ag-autoAb) are formed; which are deposited in various organs • Major symptoms: Fever, **butterfly rash** over the cheeks, arthritis, pleurisy, and kidney dysfunction
Rheumatoid arthritis	Here, a group of auto-antibodies against the host IgG antibodies are produced called **RA factor**. It is an IgM antibody directed against the Fc region of IgG Anticitrullinated peptide antibodies (**ACPA**) are also produced	• Age and sex: Women (40–60 years of age) affected • Autoantibodies bind to circulating IgG, forming IgM-IgG complexes that are deposited in the joints and can activate the complement cascade **Major symptoms:** • Main feature: Arthritis (chronic inflammation of the joints, begins at synovium; most common joints involved are small joints of the hands, feet and cervical spine) • Other features: Hematologic, cardiovascular, and respiratory systems are also frequently affected
Sjögren syndrome	Ribonucleoprotein (RNP) antigens SS-A (Ro) and SS-B (La) present on salivary gland, lacrimal gland, liver, kidney, thyroid	Auto-antibodies to the RNP antigens SS-A (Ro) and SS-B (La), lead to immune-mediated destruction of the lacrimal and salivary glands resulting in dry eyes (*keratoconjunctivitis sicca*) and dry mouth (xerostomia)
Scleroderma (Systemic sclerosis)	Nuclear antigens such as DNA topoisomerase and centromere present in heart, lungs, GIT, kidney, etc.	Helper T cell (mainly) and auto-antibody mediated Excessive fibrosis of the skin, throughout the body **Two types** • Diffuse scleroderma: Autoantibodies against DNA topoisomerase I (anti-Scl 70) is elevated • Limited scleroderma: ↑Anticentromere antibody, characterized by CREST syndrome—calcinosis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia
Seronegative spondyloarthropathies	Sacroiliac joints and other vertebrae Several types: • Ankylosing spondylitis • Reiter syndrome • Psoriatic arthritis • Spondylitis with inflammatory Bowel disease • Reactive arthritis	**Common characteristics:** They present as rheumatoid arthritis like features, but differ from it by: • Association with HLA-B27 • Pathologic changes begin in the ligamentous attachments to the bone rather than in the synovium • Involvement of the sacroiliac joints, and/or arthritis in other peripheral joints • Absence of RA (hence the name "seronegative") • Auto-Ab and immune complex mediated
Multiple sclerosis	Brain (white matter)	Self-reactive T cells produce characteristic inflammatory lesions in brain that **destroy the myelin** sheath of nerve fibers; lead to numerous neurologic dysfunctions

- **Autoimmune hemolytic anemia:** Diagnosed by Coombs test, in which the red cells are incubated with an anti-human IgG antiserum. If IgG autoantibodies are present on the red cells, the cells are agglutinated by the antiserum
- **Goodpasture syndrome:** Biopsies from patients are stained with fluorescent-labeled anti-IgG and anti-C3b reveal linear deposits of IgG and C3b along the basement membranes

- **SLE** is diagnosed by:
  - Detection of autoantibodies against various nuclear antigens by indirect immunofluorescence assay (most widely used) and ELISA-based techniques
    - **Antinuclear antibody (ANA):** Positive in >90% of cases, used as screening method (Fig. 17.3)
    - Anti-double stranded DNA (dsDNA): Highly specific, used for confirmation of cases
    - Anti-Sm antibodies.
  - **Lupus band test:** It is a direct immunofluorescence test, can detect deposits of immunoglobulins and complement proteins in the patient's skin
  - **LE cell test:** The lupus erythematosus (LE) cell test was commonly used for diagnosis, but it is no longer used because the LE cells are only found in 50–75% of SLE cases.
- **Scleroderma:** Anti-Scl 70 antibody is raised, detected by indirect immunofluorescence assay
- **Sjögren's syndrome:** Is diagnosed by detection of SS-A (or anti-Ro) and SS-B (or anti-La) antibodies by indirect immunofluorescence assay
- **Rheumatoid arthritis:** RA is diagnosed by detection of two important autoantibodies-RA factor and ACPA
  - ***RA factor*** (by latex agglutination test): RA factor is an IgM autoantibody directed against Fc portion of IgG
    - RA factor detection has good sensitivity (negative in only 15% of cases)
    - False positive detection of RA factor is seen in other autoimmune diseases.
  - ***Anticitrullinated peptide antibodies* (ACPA):** It is an autoantibody to citrullin protein. It is positive only in 67% of cases; but is *highly specific*
  - ***Rose-Waaler test*** to detect RA factor is of historical importance, no longer used now.

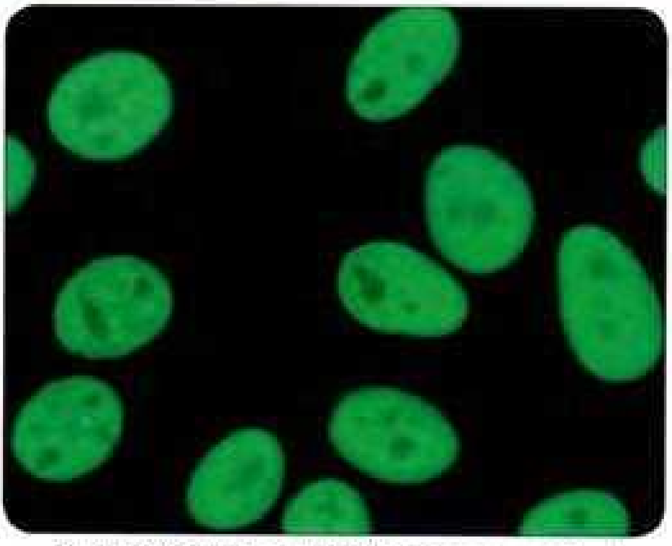


Fig. 17.3: Antinuclear antibody (homogeneous pattern) by indirect immunofluorescence staining

Source: Biological Reference Reagents, Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*).

EXPECTED QUESTIONS

I. Essay:

1. Define autoimmunity. Classify various autoimmune diseases and briefly explain various mechanisms involved in the development of autoimmunity with suitable examples.

II. Multiple Choice Questions (MCQs):

1. **Lens antigens of the eye are a type of:**
   a. Sequestered antigens
   b. Neoantigens
   c. Cross reacting antigens
   d. None of the above
2. **Autoimmunity can be caused due to all of the following, *except*:**
   a. The pressure of forbidden clones
   b. Expression of cryptic antigens
   c. Negative selection of T- cells in the thymus
   d. Release of sequestered antigens
3. **All of the following are systemic autoimmune diseases, *except*:**
   a. Hashimoto's thyroiditis
   b. Systemic lupus erythematosus
   c. Rheumatoid arthritis
   d. Scleroderma
4. **Autoantibodies bind to basement-membrane antigens on kidney glomeruli and the alveoli. This is the hallmark of:**
   a. Goodpasture syndrome
   b. Myasthenia gravis
   c. Graves' disease
   d. Hashimoto's thyroiditis
5. **Autoantibodies to ribonucleoprotein (RNP) antigens SS-A (Ro) and SS-B (La) are produced in which disease?**
   a. Sjögren syndrome
   b. Systemic sclerosis
   c. Multiple sclerosis
   d. Systemic lupus erythematosus (SLE)
6. **Anti-citrullinated peptide antibodies (ACPA) are diagnostic for:**
   a. Systemic lupus erythematosus (SLE)
   b. Rheumatoid arthritis
   c. Sjögren syndrome
   d. Scleroderma

Answers

1. a **2.** c **3.** a **4.** a **5.** a **6.** b

Immunodeficiency Disorders

18
CHAPTER

Chapter Preview

- Definition and classification
- Primary immunodeficiency diseases
  - Humoral immunodeficiency (B cell defects)
  - Cellular immunodeficiency (T cell defects)
  - Combined immunodeficiencies
  - Disorders of phagocytosis
- Secondary immunodeficiencies

DEFINITION AND CLASSIFICATION

Immunodeficiency is a state where the defence mechanisms of the body are impaired, leading to enhanced susceptibility to microbial infections as well as to certain forms of cancer.

Immunodeficiency diseases are broadly classified as primary or secondary.

- Primary immunodeficiency diseases result from inherited defects affecting immune system development
- Secondary immunodeficiency diseases are secondary to some other disease process that interferes with the proper functioning of the immune system (e.g. infection, malnutrition, aging, immunosuppression, autoimmunity, or chemotherapy).

PRIMARY IMMUNODEFICIENCY DISEASES

Most primary immunodeficiency diseases are genetically determined and can be further classified into diseases resulting from deficiency of either specific immunity (i.e. humoral or cellular or both) or nonspecific host defense mechanisms (mediated by complement proteins and cells such as phagocytes or NK cells) (Table 18.1).

However, the distinction of diseases affecting specific immunity (humoral or cellular) components is not clearcut. In particular, T-cell defects almost always lead to impaired antibody synthesis, and hence isolated deficiencies of T cells are usually indistinguishable from combined deficiencies of T and B cells.

Table 18.1: Classification of primary immunodeficiency diseases

Humoral immunodeficiency (B cell defects)
• Bruton disease (X-linked agammaglobulinemia)
• Common variable immunodeficiency
• Isolated IgA deficiency
• Hyper-IgM syndrome
• Transient hypogammaglobulinemia of infancy
Cellular immunodeficiencies (T cell defects)
• DiGeorge syndrome (thymic hypoplasia)
• Chronic mucocutaneous candidiasis
• Purine nucleoside phosphorylase (PNP) deficiency
Combined immunodeficiencies (B and T cell defects)
• Severe combined immunodeficiencies
➢ Cytokine receptor mutation
➢ Adenosine deaminase (ADA) deficiency
• Wiskott–Aldrich syndrome
• Ataxia telangiectasia
• Nezelof syndrome
Disorders of phagocytosis
• Chronic granulomatous disease
• Myeloperoxidase deficiency
• Chediak–Higashi syndrome
• Leukocyte adhesion deficiency
• Lazy leukocyte syndrome
• Job's syndrome or Hyper-IgE syndrome
• Tuftsin deficiency
• Shwachman's disease
Disorders of complement*
• Complement component deficiencies
• Complement regulatory protein deficiencies

*Described in detail in Chapter 13

Table 18.2: Examples of infections in immunodeficiencies

Pathogen type	T-cell defect	B-cell defect	Granulocyte defect	Complement defect
Bacteria	Bacterial sepsis	*Streptococcus pneumoniae* *Staphylococcus aureus* *Haemophilus influenzae*	Staphylococci *Pseudomonas, Nocardia*	*Neisseria* Other pyogenic infections
Viruses	Cytomegalovirus Epstein–Barr virus Severe varicella Chronic infections with respiratory and intestinal viruses	Enterovirus encephalitis	—	
Fungi	*Candida, Pneumocystis jirovecii*	—	*Candida, Aspergillus*	
Parasites	—	Giardiasis	—	
Special features	Aggressive disease with opportunistic pathogens, failure to clear infections	Recurrent sinopulmonary infections, sepsis, chronic meningitis	—	

The type of infections in a given patient depends largely on the component of the immune system that is affected (Table 18.2).

- Patients with defects in humoral immunity, complement, or phagocytosis typically suffer from recurrent infections with pyogenic bacteria
- On the other hand, those with defects in cell-mediated immunity are prone to infections caused by viruses, fungi, and intracellular bacteria.

Most primary immunodeficiencies come to attention early in life (between 6 months and 2 years of life); usually because of the susceptibility of infants to recurrent infections.

HUMORAL IMMUNODEFICIENCY (B CELL DEFECTS)

Bruton Disease (X-linked Agammaglobulinemia)

Bruton disease is one of the more common forms of primary immunodeficiency. It is characterized by:

- Failure of pre-B cells to differentiate into immature B cells in the bone marrow—due to absence of an enzyme called **Bruton's tyrosine kinase** which is involved in transformation of pre-B cell into immature B cell
- As a result, there occurs total absence of B cells and plasma cells in the circulation, with depressed serum levels of all classes of immunoglobulins. However, Pre-B cells are found in normal numbers in bone marrow and the T-cell-mediated responses are also normal
- The B-cell maturation stops at pre-B cell stage; after the synthesis of heavy-chain without forming the light chains. Hence the cytoplasm of pre-B cell may have incomplete immunoglobulins
- Bruton's tyrosine kinase is X-linked; hence, this disease is seen primarily in males; nevertheless, sporadic cases have been described in females
- Secondary infections are seen after 6 months of age, (when maternal antibodies are depleted), such as:
  - Recurrent bacterial infections caused by pathogens that are usually cleared by antibody opsonization (e.g. *Haemophilus influenzae, Streptococcus pneumoniae,* or *Staphylococcus aureus*) leading to acute and chronic pharyngitis, sinusitis, otitis media, bronchitis, and pneumonia
  - Viruses that are cleared by neutralizing antibodies, e.g. enteroviruses
  - Parasites which are usually resisted by secretory IgA, e.g. *Giardia lamblia*.
- Autoimmune diseases (such as SLE and dermatomyositis) also occur in up to 20% of cases.

Common Variable Immunodeficiency

This is a heterogeneous group of both sporadic and inherited forms of the disease characterized by hypogammaglobulinemia, increased susceptibility to infection, autoimmune disorders (hemolytic anemia, pernicious anemia), as well as lymphoid tumors. The clinical manifestations are superficially similar to those of Bruton diseases; but differ in the following aspects:

- Both sexes are affected equally
- Onset of symptoms is much later, in the second or third decade of life
- It is also B cell development defect; B cells may be present in circulation in normal numbers, but they appear defective in their ability to differentiate into plasma cells and secrete immunoglobulins
- The diagnosis is usually one of exclusion (after other causes of immunodeficiency are ruled out); the basis of the immunoglobulin deficiency is variable (hence the name)
- The defect in the antibody production has been variably attributed to intrinsic B-cell defects, deficient T-cell helper, or excessive T-cell suppressor activity.

Isolated IgA Deficiency

IgA deficiency is the most common of all the primary immunodeficiency diseases, affects about 1 in 700 white individuals.

- In healthy normal individuals, IgA is predominant in mucosal secretions and involves in providing immunity at mucosal sites of intestine and respiratory tract
- Therefore, the weakened mucosal defences due to IgA deficiency predispose patients to recurrent sinopulmonary infections and diarrhea. There is also a significant (but unexplained) association with autoimmune diseases
- **Pathogenesis:** IgA deficiency occurs due to a block in the terminal differentiation of IgA-secreting B cells to plasma cells, which in turn is due to altered T-cell production of cytokines that drive IgA responses (e.g. TGF-β and IL-5) or due to intrinsic B-cell defect. The levels of other immunoglobulins are usually normal or even excess.

Hyper-IgM Syndrome

Hyper-IgM syndrome is an X-linked disorder; results due to a defect in **isotype class switchover** of B cells.

- Class switchover is a phenomenon by which the same B cell, instead of producing IgM, starts producing other classes of antibodies. Class switch over depends upon two signals generated by helper T cells which influence the B cells—
  - T_H cell induced cytokine
  - Signal generated due to direct contact through the interaction of CD40 molecules on B cells with CD40 ligand (CD40L) on T_H cells.
- **Genetic defect:** In hyper-IgM syndrome, there occur mutations in either CD40L or CD40 genes; prevent the T- and B-cell interaction; thus blocking the class switchover
- A block in class switchover results in lack of synthesis of other classes of antibodies such as IgG, IgA, and IgE with a normal or supernormal levels of IgM
  - Deficiency of IgG leads to defect in opsonization and complement activation (predisposes to recurrent pyogenic infections) and IgA deficiency leads to increased recurrent sinopulmonary infections and diarrhea
  - Excess IgM antibodies can react with blood cells, resulting in autoimmune hemolytic anemia, thrombocytopenia, or neutropenia.
- Because CD40L signals are involved in macrophage activation and thus producing delayed hypersensitivity response, hence patients with defect in CD40L are more susceptible to *Pneumocystis jirovecii* infection
- Hyper-IgM syndrome is X-linked in 70% of the cases affecting males; in the remaining patients, the precise mutations have not been fully characterized.

Transient Hypogammaglobulinemia of Infancy

This occurs due to an abnormal delay in the initiation of synthesis of IgG (or some time IgA or IgM).

- IgG synthesis usually starts by 2 months of age. But in some infants, it is delayed leading to defect in opsonization or complement activation resulting in recurrent otitis media and respiratory infections
- However, spontaneous recovery occurs usually by 18-24 months of age
- Interestingly, these infants show a normal antibody response against vaccines.

CELLULAR IMMUNODEFICIENCY (T CELL DEFECTS)

DiGeorge Syndrome (Thymic Aplasia)

DiGeorge syndrome results from a congenital defect in thymic development leading to defect in T-cell maturation.

- Infants are extremely vulnerable to viral, fungal, intracellular bacterial and protozoan infections
- **Genetic defect:** In 90% of cases, there occurs a deletion affecting chromosome 22q11 which leads to developmental malformation affecting the third and fourth pharyngeal pouches in embryonic life
- As a result, all the structures that develop from third and fourth pharyngeal pouches such as thymus, parathyroid glands, and portions of the face and aortic arch become defective
- Thus, in addition to the thymic defects, there may be associated:
  - Parathyroid gland hypoplasia resulting in neonatal tetany and hypocalcemia
  - Anomalies of the heart and the great vessels (Fallot's tetralogy)
  - Characteristic facial appearance.
- B cells and serum immunoglobulin levels are generally unaffected
- Treatment: Thymus transplantation has been found to be successful in restoration of immune function. In others (with partial defects), immunity may improve spontaneously with age.

Chronic Mucocutaneous Candidiasis

It represents an impaired cell-mediated immunity against *Candida albicans* leading to superficial infections of the skin, mucous membranes, and nails.

- They do not show increased susceptibility to other infections but often associated with endocrinopathies and autoimmune disorders
- Transfer factor therapy, along with amphotericin B has been reported to be effective.

Purine Nucleoside Phosphorylase Deficiency

It is a rare autosomal recessive disorder (chromosome 14), characterized by deficiency of an enzyme of purine metabolism called purine nucleoside phosphorylase (PNP).

- PNP is a key enzyme required for purine degradation; catalyzes the conversion of guanosine to hypoxanthine
- Its deficiency leads to elevated Deoxyguanosine triphosphate levels resulting in T-cell toxicity. However, B cells are not affected
- T cell depletion predisposes to increased susceptibility to infection and autoimmune disorders.

COMBINED IMMUNODEFICIENCIES (B AND T CELL DEFECTS)

Severe Combined Immunodeficiencies (SCID)

SCID represents groups of genetically distinct syndromes; all having in common, defects in both humoral and cell-mediated immune responses.

Types of Genetic Defect in SCID

- **Mutation in cytokine receptor:** Approximately 50-60% of the cases of SCID are X-linked (seen in males), resulting from mutations in the gene encoding the common γ chain shared by the cytokine receptors for IL-7 and others (IL-2, IL-4, IL-9, and IL-15)
  - IL-7 being lymphopoietic growth factor, defective IL-7 receptor signalling leads to defect in survival and expansion of immature B- and T-cell precursors in the bone marrow
  - Defect in IL-15 receptor signaling leads to deficiency of NK cell.
- **The remaining cases of SCID** are inherited as autosomal recessive manner include:
  - **Adenosine deaminase (ADA) deficiency:** It is the most common type of autosomal recessive SCID. ADA is an enzyme required for purine degradation; its deficiency leads to accumulation of deoxyadenosine which is toxic to rapidly dividing immature T lymphocytes. B cell deficiency is not profound
  - **RAG Mutation:** Recombinase-activating genes (RAG) are essential for somatic gene rearrangements of T cell receptor and immunoglobulins. Thus, defect in RAG blocks the development of T and B cells
  - **Jak3 mutation:** Jak3, an intracellular kinase, is essential for signal transduction through the common cytokine receptor γ chain. Hence Jak3 mutation is another way of blocking the cytokine receptor signalling
  - **Class II MHC deficiency:** Mutations that impair the expression of class II MHC molecules prevent the development of CD4+ T cells. This condition is also called the **Bare lymphocyte syndrome**.

Infections

Irrespective of the underlying genetic defect, the affected infants are susceptible to severe recurrent infections by a wide array of pathogens, including *Candida*, *Pneumocystis*, cytomegalovirus and *Pseudomonas* (*see* Table 18.2).

Treatment

Bone marrow transplantation is the mainstay of treatment. Gene therapy replacing the mutated genes has been successful in X-linked cases.

Wiskott-Aldrich Syndrome (WAS)

It is an X-linked recessive disease, characterized by immunodeficiency with thrombocytopenia, eczema, etc. The severity of WAS increases with age.

- It first manifests itself by defective responses to bacterial polysaccharides and by lower IgM levels. IgG levels are usually normal. Paradoxically the levels of IgA and IgE are often elevated
- Other T and B cell responses are normal initially, but with increase of age, there are recurrent bacterial infections and a gradual loss of humoral and cellular responses
- Patients are also prone to develop non-Hodgkin B-cell lymphomas
- Patients may present with bloody diarrhea secondary to thrombocytopenia.

Pathogenesis

The underlying genetic defect is due to a mutation in the gene encoding Wiskott-Aldrich syndrome protein (WASP) present in precursor lymphoid cells of bone marrow. It is a cytoskeletal glycoprotein (sialophorin or CD43), required for actin polymerization.

Ataxia Telangiectasia

The syndrome is characterized by:

- Difficulty in maintaining balance while walking (cerebellar ataxia)
- Appearance of broken capillaries (telangiectasia) in the eyes and choreoathetoid movements (usually noticed in infancy)
- Deficiency of IgA and sometimes IgE
- Profound sinopulmonary infections.

Genetic defect: The primary defect appears to be in a kinase involved in regulation of the cell cycle. The relationship between the immune deficiency and the other defects in ataxia telangiectasia remains obscure.

Nezelof Syndrome

It is an autosomal recessive condition characterized by cellular immunodeficiency resulting from thymus hypoplasia.

- In some patients, B cells are normal, whereas in others a B-cell deficiency may be present, secondary to the T-cell defect
- Affected individuals suffer from chronic diarrhea, viral and fungal infections, and failure to thrive.

DISORDERS OF PHAGOCYTOSIS

Chronic Granulomatous Disease (CGD)

Pathogenesis

Pathogenesis of CGD involves inherited defects in the gene encoding components of oxidase system, e.g. Nicotinamide adenine dinucleotide phosphate (NADP) oxidase of phagocyte which breaks down hydrogen peroxide to generate free oxygen radicals (O_2^-) that are involved in microbial killing. As a result, there occurs decreased oxidative burst which predisposes to recurrent bacterial infections. CGD is a genetic disease that runs in family in two forms:

- In X-linked form (more common, 70%), membrane component of phagocyte oxidase is defective
- In autosomal recessive form, cytoplasmic component of phagocyte oxidase is defective.

Manifestations

- The bacteria involved in the recurrent infections are catalase positive; pyogenic pathogens such as staphylococci, *Pseudomonas* and coliforms. Catalase negative pathogens such as streptococci and pneumococci are handled well
- Patients also undergo excessive inflammatory reactions that result in gingivitis, swollen lymph nodes, and nonmalignant granulomas (lumpy subcutaneous cell masses)
- Nitroblue tetrazolium reduction test (NBT) is used as screening test to detect deficiency of NADPH oxidase activity.

Myeloperoxidase Deficiency

It is a common genetic disorder characterized by deficiency in either quantity or function, of myeloperoxidase, an enzyme produced by neutrophils. Patients present with immune deficiency and recurrent infections, especially with *Candida albicans*.

Chediak–Higashi Syndrome

It is an autosomal recessive disease, characterized by:

- Defective fusion of phagosomes and lysosomes in phagocytes which leads to increased susceptibility to recurrent and severe pyogenic infections
- Abnormalities in melanocytes leading to albinism (lack of skin and eye pigment)
- Abnormalities in cells of the nervous system (associated with nerve defects), and
- Platelets abnormalities, causing bleeding disorders
- Aggressive but non-malignant infiltration of organs by lymphoid cells.

Genetic defect: Pathogenesis of this syndrome is due to a mutation in a protein called LYST which is believed to regulate lysosomal trafficking.

- The mutation impairs the targeting of proteins to secretory lysosomes, which makes them unable to lyse bacteria
- Phagocytes from patients with this immune defect contain *giant granules* but do not have the ability to kill bacteria.

Leukocyte Adhesion Deficiency (LAD)

LAD is rare autosomal recessive disorder, characterized by a defect in the adhesion of leukocytes which results in poor leukocyte chemotaxis particularly of neutrophils. Thus it predisposes to various bacterial and fungal infections. LAD is of two types:

- **Leukocyte adhesion deficiency 1:** Defective leukocyte adhesion because of mutations in β2 integrin subunit (CD18), of the leukocyte cell adhesion molecule, which is found on chromosome 21
- **Leukocyte adhesion deficiency 2:** Defective leukocyte adhesion is because of mutations in fucosyltransferase required for synthesis of sialylated oligosaccharide which is a selectin ligand.

Lazy Leukocyte Syndrome

It is an idiopathic condition due to defect in neutrophil chemotaxis which results in increased pyogenic infections such as gingivitis, abscess formation, pneumonia and neutropenia.

Job's Syndrome (Hyper-IgE Syndrome)

Hyper-IgE syndrome is a rare primary immunodeficiency disease characterized by eczema, recurrent staphylococcal skin abscesses, recurrent lung infections (pneumatocele), eosinophilia and high serum levels of IgE.

Genetic defect: The underlying mechanism is due to a defect in neutrophil chemotaxis. Most cases are sporadic, but some familial cases of Hyper-IgE syndrome have been reported, with either an autosomal dominant (AD) or autosomal recessive (AR) mode of inheritance.

- Autosomal dominant cases are linked to mutations in the STAT3 gene
- Autosomal recessive cases are due to mutations in DOCK8 gene.

Tuftsin Deficiency

Tuftsin is a tetrapeptide (Thr-Lys-Pro-Arg) produced primarily in the spleen, by the cleavage of the Fc-portion of the heavy chain of IgG. It stimulates the bactericidal activity of phagocytes. Tuftsin deficiency, either hereditary or following splenectomy, results in increased susceptibility to certain capsulated organisms such as *H. influenzae*, pneumococci, and meningococci.

Shwachman's Syndrome

It is a rare congenital disorder characterized by neutropenia, exocrine pancreatic insufficiency, bone marrow dysfunction, skeletal abnormalities, and short stature.

SECONDARY IMMUNODEFICIENCIES

Secondary immunodeficiencies, also known as acquired immunodeficiencies, are due to the secondary effects of other diseases, such as:
- Malnutrition (due to inadequate immunoglobulin synthesis)
- Aging (suppression of immune system with age)
- Patients with several infections that supress immune system causing lymphocyte depletion, e.g. HIV (human immunodeficiency virus) infection
- Underlying cancers (particularly those of the bone marrow and blood cells (leukemia, lymphoma, multiple myeloma)
- Underlying proteinuric renal diseases—leads to loss of immunoglobulins
- Sarcoidosis
- Patients on immunosuppressive medications
- Patients receiving chemotherapy or radiation therapy for malignancy.

As a group, the secondary immunodeficiencies are more common than the primary immunodeficiency disorders. Acquired immunodeficiency syndrome (AIDS), the most widespread and important of the secondary immunodeficiency diseases, is discussed in detail in Chapter 48.

EXPECTED QUESTIONS

I. Essay:

1. Define and classify immunodeficiency diseases. Describe in detail about Bruton's disease.

II. Write short notes on:

1. Severe combined immunodeficiencies.
2. DiGeorge syndrome.
3. Chediak-Higashi syndrome.
4. Chronic granulomatous disease.
5. Wiskott-Aldrich syndrome.

III. Multiple Choice Questions (MCQs):

1. **DiGeorge syndrome is due to defect in:**
   a. T cell
   b. B cell
   c. Phagocyte
   d. Complement
2. **All of the following are primary immunodeficiency diseases except:**
   a. DiGeorge syndrome
   b. Nezelof syndrome
   c. Chediak-Higashi syndrome
   d. AIDS
3. **Chronic granulomatous disease is due to deficiency of:**
   a. Tyrosine kinase
   b. NADPH oxidase
   c. Adenosine deaminase
   d. Myeloperoxidase
4. **Disorders of phagocytosis include all, *except:***
   a. Chronic granulomatous disease
   b. Myeloperoxidase deficiency
   c. Chediak-Higashi syndrome
   d. Nezelof syndrome
5. **Combined immunodeficiencies (B and T cell defects) include all, *except:***
   a. Severe combined immunodeficiencies
   b. DiGeorge syndrome
   c. Wiskott-Aldrich syndrome
   d. Ataxia telangiectasia
6. **B-cell defect predisposes to the following infection, *except:***
   a. *Streptococcus pneumoniae*
   b. Enterovirus encephalitis
   c. Giardiasis
   d. Severe varicella
7. **The most common underlying mechanism of severe combined immunodeficiency disease (SCID) is:**
   a. Mutation in cytokine receptor
   b. Adenosine deaminase (ADA) deficiency
   c. Recombinase-activating genes
   d. Jak3 mutation
8. **Wiskott-Aldrich syndrome (WAS): All are true, *except:***
   a. Thrombocytopenia
   b. Low IgA and IgE
   c. Defective response to bacterial polysaccharides
   d. Prone to develop non-Hodgkin B-cell lymphomas
9. **Ataxia telangiectasia all are true, *except:***
   a. Difficulty in maintaining balance while walking
   b. Telangiectasia
   c. Hyper IgA
   d. Profound sinopulmonary infections
10. **Nezelof Syndrome is characterized by:**
    a. Adenoid hypoplasia
    b. Pancreatic deficiency
    c. Thymus hypoplasia
    d. Pituitary deficiency

Answers

1. a **2.** d **3.** b **4.** d **5.** b **6.** d **7.** a **8.** b **9.** c **10.** c

Transplant and Cancer Immunology

19
CHAPTER

Chapter Preview

- Transplant Immunology
  - Classification of transplants
  - Histocompatibility antigens
  - Types of graft rejection
  - Mechanism of graft rejection
  - Prevention of graft rejection
  - Graft-versus-host reaction
- Cancer immunology
  - Tumor antigens
  - Immune surveillance theory
  - Cancer immunotherapy

Organ transplantation and cancer are two situations in which the host immune system plays a deciding role in the survival of such transplants or tumors inside the host.

- In organ transplantation, immune response against the graft is a barrier to successful transplantation, and suppression of the immune system is the key for the transplant survival
- In cancer, the situation is precisely the reverse: suppressed immune system gives opportunity for many tumors to take birth and hence, enhancing the immunity against the tumor cells, is the principle used for treatment of cancers.

TRANSPLANT IMMUNOLOGY

Transplantation refers to transfer of a graft or transplant (cells, tissues, or organs) from one site to another. The individual from whom the transplant is taken is referred to as the **donor**; while the individual to whom it is transplanted, is called **recipient**.

CLASSIFICATION OF TRANSPLANTS

Transplants are classified in various ways.

- **Based on the organ or tissue transplanted:** Examples are kidney, heart and skin grafts, etc.
- **Based on the anatomical site of the graft:**
  - Orthotopic grafts: When the tissue or organ grafts are transplanted to their anatomically 'normal' sites in the recipient, then such grafts are known as orthotopic grafts, e.g. as in skin grafts
  - Heterotopic grafts: They are placed in anatomically 'abnormal' sites, as when thyroid tissue is transplanted in a subcutaneous pocket.
- **Vital and static transplants:**
  - Vital grafts are the live grafts, such as the kidney or heart, are expected to survive and function physiologically in the recipient
  - Static grafts are nonliving structures, like bone or artery which merely provide a scaffolding on which new tissue is laid by the recipient.
- **Based on the genetic relationship** between the donor and the recipient:
  - Autograft: It is a self-tissue transferred from one part of the body site to another in the same individual. Examples include transferring healthy skin to a burned area in burn patients and use of healthy blood vessels of the same person to replace blocked coronary arteries
  - Isograft or syngeneic graft: It is a tissue transferred between genetically identical individuals (e.g. monozygotic twins)
  - Allograft: It is a tissue transferred between genetically non-identical members of the same species (e.g. kidney or heart transplant)
  - Xenograft: It is a tissue transferred between different species (e.g. the graft of a baboon heart into a man).

In humans, allografts are the most commonly used graft in transplant centres; hence our further discussion will be confined to allografts.

HISTOCOMPATIBILITY ANTIGENS

Histocompatibility

Histocompatibility between the graft and recipient would decide whether the graft is going to be accepted or rejected.

- If a graft and recipient tissues are histocompatible to each other (i.e. antigenically similar); then the graft is accepted. Usually, autografts and isografts are histocompatible
- On the contrary, histoincompatible (i.e. antigenically dissimilar) grafts are generally rejected by the recipient. Allografts and xenografts are usually histoincompatible.

Transplantation Antigens

Transplantation antigens are the antigens of allografts against which the recipient would mount an immune response.

- MHC molecules (major histocompatibility antigens) are the most important transplantation antigens
- Apart from that, ABO and Rh blood group systems also play a role in determining the histocompatibility
- **Minor histocompatibility antigens (MHA):** They are the peptides derived from normal cellular proteins of donated organs. Immune response against MHA molecules is weaker; hence they pose problems of rejection less frequently than MHC molecules. One exception is when a graft is transferred from a male donor to a female recipient
    - The graft tissues of a male donor (XY) would have some male-specific minor histocompatibility (H-Y) antigens determined by the Y chromosome which will be absent in the female (XX) recipient
    - Hence, it is observed that the rejection of the grafts when transferred from a male donor to female recipient is more as compared to female to male transplantation
    - This unilateral sex linked histoincompatibility is known as the **Eichwald-Silmser effect**.

TYPES OF GRAFT REJECTION

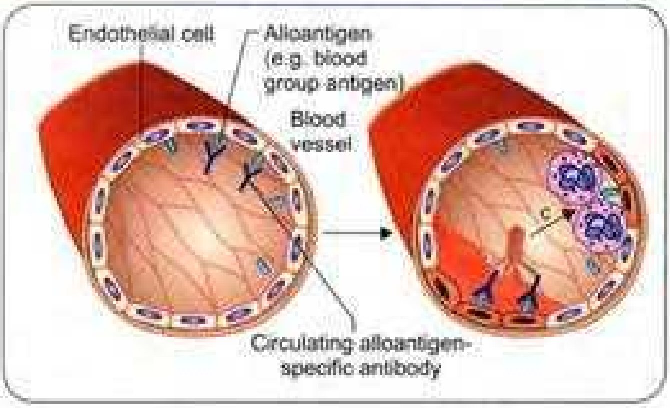
Graft rejection is classified into hyperacute, acute, and chronic, on the basis of time taken for the rejection, types of immune response mounted against the graft and clinical and pathologic features (Table 19.1).

Hyperacute Rejection

This occurs within minutes to hours of transplantation and is characterized by thrombosis of graft vessels and ischemic necrosis of the graft.

Table 19.1: Comparison of various types of graft rejection

Graft rejection	Time taken for rejection	Immune mechanisms involved
Hyperacute	Minutes to hours	Preformed antibodies (anti-ABO and/or anti-HLA)
Acute	Weeks to months	• Cytotoxic T cell mediated • Antibody mediated
Chronic	Months to years	• Chronic DTH mediated • Antibody mediated



Preformed antibodies react with alloantigens on the vascular endothelium of the graft, activate complement (C) and trigger rapid intravascular thrombosis and necrosis of the vessel wall.

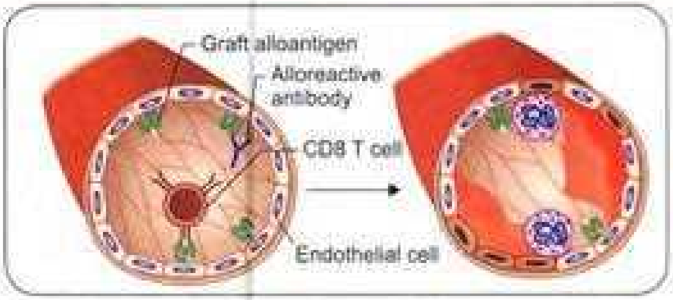
Fig. 19.1: Hyperacute graft rejection

- It is mediated by **circulating antibodies** that are specific for antigens on the graft endothelial cells and that are present before transplantation
- In an individual, exposure to foreign HLA antigens can occur as a consequence of previous blood transfusions, pregnancy, or organ transplantation. Following which, the individual develops antibodies against these antigens. These preformed antibodies may be anti-ABO or anti-HLA specific for allogenic (i.e. graft's) MHC molecules. If an individual with these pre-existing antibodies to a foreign HLA antigen receives a graft (containing same foreign HLA antigen), then the graft will be rejected earlier and more vigorously (Fig. 19.1)
- Hyperacute rejection is not a common problem in clinical transplantation, because it can be avoided by matching the donor and the recipient. Potential recipients are tested for antibodies against the prospective donor's blood group antigens (by cross matching) and HLA antigens (by HLA typing).

Acute Graft Rejection

Acute graft rejection occurs within days or weeks after transplantation. It is due to an active immune response of the host stimulated by alloantigens in the graft.

- Acute graft rejection is mediated by T cells (mainly cytotoxic T cells, occasionally helper T cells) and antibodies specific for alloantigens in the graft
- Cytotoxic T cells directly destroy the graft cells, or cytokines secreted by the helper T cells induce inflammation, which destroys the graft
- Antibodies contribute especially to the vascular component of acute rejection. Antibody-mediated injury to graft vessels is caused mainly by complement activation by the classical pathway (Fig. 19.2)



CD8 T cells react with graft alloantigens and destroy the endothelial and parenchymal cells or antibodies react with alloantigens of the graft's endothelium and causes endothelial cell damage via complement activation.

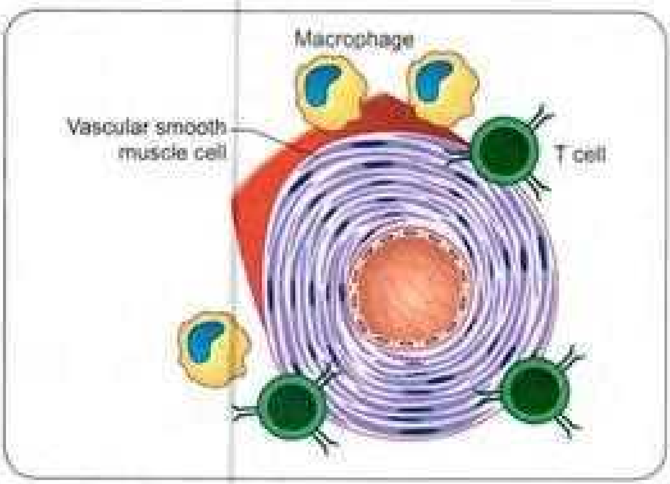
Fig. 19.2: Acute graft rejection

- Current immunosuppressive therapy is designed mainly to prevent and reduce acute rejection by blocking the activation of alloreactive T cells.

Chronic Graft Rejection

Chronic graft rejection is an indolent form of graft damage that occurs over months or years, leading to progressive loss of graft function.

- Chronic rejection may be manifested as fibrosis of the graft and by gradual narrowing of graft blood vessels, called graft arteriosclerosis
- T cells that react against graft alloantigens secrete cytokines, which stimulate the proliferation and activities of fibroblasts and vascular smooth muscle cells in the graft. The smooth cell proliferation in the vascular intima may represent a specialized form of chronic delayed type hypersensitivity (DTH) reaction (Fig. 19.3)
- Alloantibodies also contribute to chronic rejection
- Chronic rejection is refractory to most of the therapeutic options available and is becoming the leading cause of graft failure.



T cells react with graft alloantigens may produce cytokines that induce inflammation and proliferation of intimal smooth muscle cells, leading to luminal occlusion and graft arteriosclerosis

Fig. 19.3: Chronic graft rejection

FACTORS INFLUENCING ALLOGRAFT REJECTION

The rate of allograft rejection varies according to the—

- **Tissue involved**, e.g. skin grafts are rejected faster than other tissues such as kidney or heart
- **Genetic distance** between the donor and recipient—More the genetic distance; faster is the rejection. Autografts and isografts are well accepted
- **Immunological memory:** Rejection is faster when another graft is placed to a recipient from the same donor. This occurs due to the memory cells produced against the first graft would differentiate quickly into effector cells; and that in turn reject the second graft faster.

An example is given below which describes the pathological sequences that take place when a skin graft is placed: (1) as an autograft to the same donor (leads to acceptance), (2) as an allograft to a recipient for the first time (leads to first set rejection), (3) as an allograft to the same recipient for the second time (leads to second set rejection).

Autograft Acceptance

When a skin graft is transplanted to the same individual at a different site, revascularization takes place by day 3–7; followed by healing (within day 7–10) and then resolution and acceptance of the graft (by day 12–14) (Fig. 19.4A).

First-set Rejection

When an allograft is placed for the first time from a donor to a recipient, the type of primary graft rejection that develops is known as, **first-set rejection** (Fig. 19.4B).

- The skin first becomes revascularized between days 3 and 7; as the reaction develops, the vascularized transplant becomes infiltrated with lymphocytes, monocytes, neutrophils, and other inflammatory cells
- There is decreased vascularization of the transplanted tissue by 7–10 days, visible necrosis by 10 days, and complete rejection by 12–14 days.

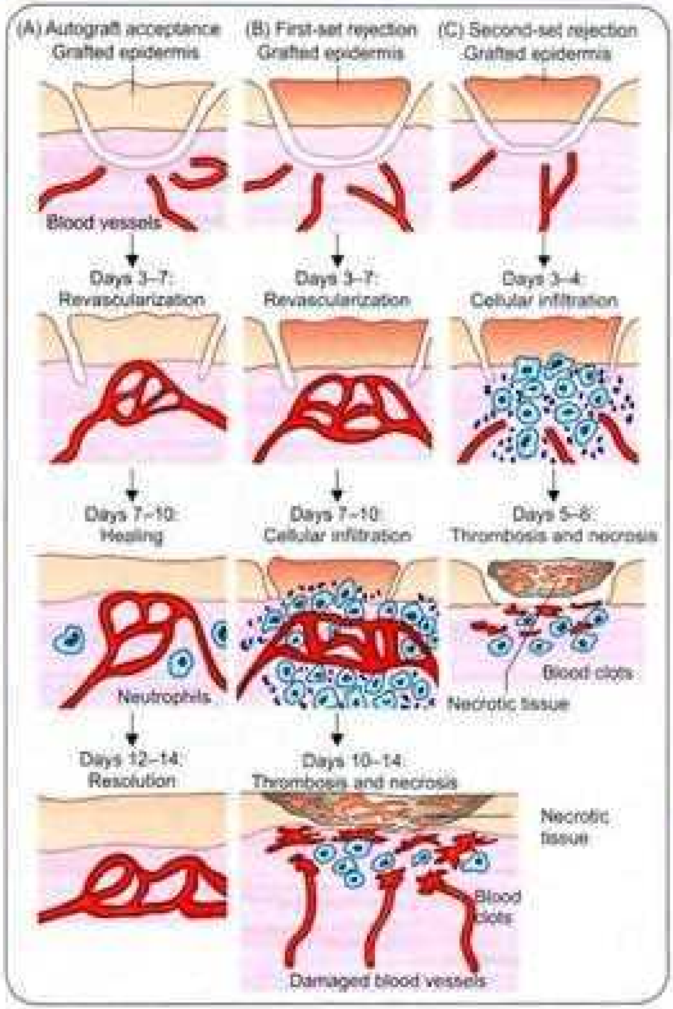
Second-set Rejection

If, in a recipient who has rejected a graft by the first set response, another graft from the same donor is transplanted, it will be rejected in an accelerated fashion.

- Though vascularization starts but is soon interrupted by the inflammatory response
- Necrosis sets in early and the graft sloughs off by the sixth day (Fig. 19.4C).

MECHANISM OF GRAFT REJECTION

Graft rejection is caused principally by a T cell-mediated immune response to alloantigens expressed on the graft cells, primarily the MHC molecules (Fig. 19.5).



Figs 19.4A to C: Graft acceptance and graft rejection

The T cell response to MHC antigens involves recognition of both the donor MHC molecule as well as the associated peptide ligand present in the cleft of the MHC molecule.

- The peptides present in the groove of allogeneic (i.e donor) class I MHC molecules are derived from proteins synthesized within the allogeneic cell
- The peptides present in the groove of allogeneic (i.e. donor) class II MHC molecules are generally proteins taken up and processed by the allogeneic APCs.

The process of graft rejection can be divided into two stages: (1) A sensitization phase-which involves alloantigen (mainly graft MHC molecules) presentation to recipient's T cells and (2) An effector stage, in which immune destruction of the graft takes place due to activation of recipient's T cells.

Sensitization Phase

T cells in the recipient may recognize donor alloantigens in the graft in two different ways: (1) direct pathway and (2) indirect pathway; depending on what cells in the graft are displaying these alloantigens to the recipient T cells (Fig. 19.5).

Direct Pathway of Alloantigen Presentation

Many graft tissues contain antigen presenting cells (APCs, e.g. dendritic cells and macrophages) and when the tissues are transplanted, the APCs are also carried along with the graft to the recipients.

- The allogeneic MHC molecules on graft's APCs are directly presented to the recipient's helper T cells
- This pathway is responsible for most of the acute graft rejections mediated by cytotoxic T cells (described in effector phase).

Indirect Pathway of Alloantigen Presentation

This is similar to that for recognition of any foreign antigen by the host APCs.

- The graft cells are ingested by recipient APCs, donor alloantigens are processed and presented by the MHC molecules present on recipient APCs to recipient's helper T cells
- This pathway is responsible for most of the chronic rejection mediated by helper T cells via specialized form of chronic DTH reaction (described in effector phase).

Effector Phase

A variety of effector mechanisms participate in allograft rejection. The most common are cell-mediated reactions involving delayed-type hypersensitivity T cells and cytotoxic T cells.

- **Delayed-type hypersensitivity:** Activated helper T cells differentiate into T_{DTH} cells. Cytokines secreted from T_{DTH} (e.g. interferon-γ) activate macrophages which destroy the target graft cells by producing lytic enzymes
- **Cytotoxic T cells:** CD8+ T_C cells kill the graft cells by recognizing the allogeneic MHC-I molecules
- **Antibody-mediated mechanisms:** Cytokines produced by helper T cells activate B cells to produce antibodies. Antibodies are also important in mounting immune response against the graft. They take a lead role in mediating hyperacute graft rejections; however, in acute and chronic rejections, they play a minor role. Antibody-mediated destruction of the graft occurs by the following mechanisms:
  - Complement-mediated lysis
  - Antibody-dependent cell-mediated cytotoxicity (ADCC) via NK cell or macrophage mediated destruction.

PREVENTION OF GRAFT REJECTION

Laboratory Tests to Determine Histocompatibility

Prior to transplantation, various laboratory tests should be carried out to assess the histocompatibility between the donor and recipient.

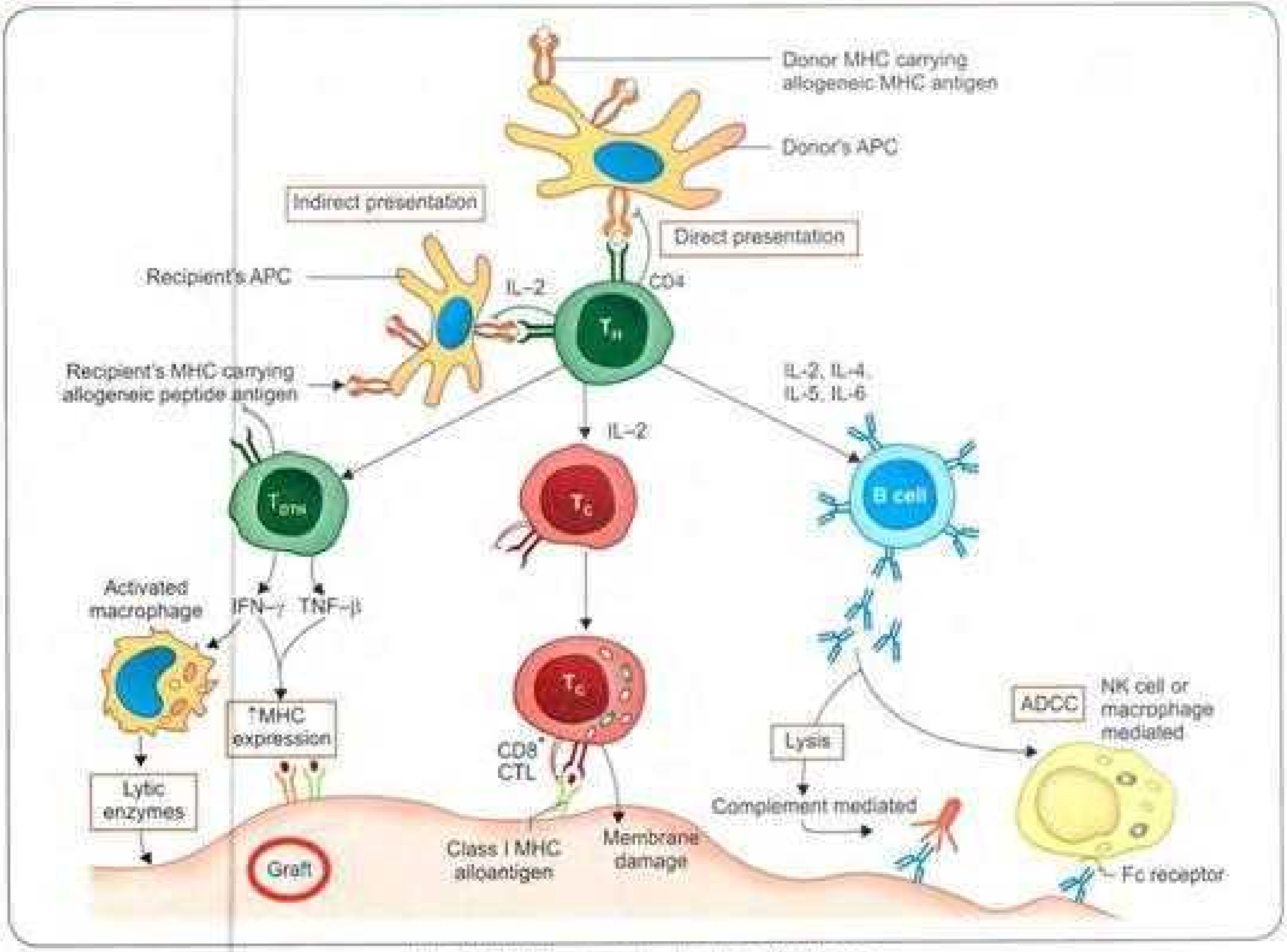


Fig. 19.5: Mechanisms involved in graft rejection

Abbreviations: MHC, major histocompatibility complex; NK, natural killer; APC, antigen presenting cells; IL, interleukin; TNF, tumor necrosis factor; IFN, interferons; CTL, cytotoxic T lymphocytes.

- ❖ ABO blood group compatibility testing by blood grouping and cross matching
- ❖ HLA typing (see highlight box).

Immunosuppressive Therapy

- ❖ Hyperacute rejection manifests severely and within minutes, and so the treatment indicated is immediate removal of the tissue

HLA Typing

In this test, donor's antigens expressed on the surface of leukocytes or their gene to that of recipient are matched. The HLA compatibility is determined by:

- ❑ **Phenotypic method**, such as
  - ➢ Serology: Microcytotoxicity
  - ➢ Tissue typing: Mixed lymphocyte reaction
- ❑ **Genotypic methods**, such as
  - ➢ PCR detecting HLA genes

Contd...

Contd...

  - ➢ PCR-RFLP (restriction fragment length polymorphism)
  - ➢ PCR-SSOP (PCR sequence-specific oligonucleotide probing)
  - ➢ PCR-SSP (PCR-sequence-specific primer)
  - ➢ PCR- DNA sequencing
  - ➢ Conformational analysis.

The phenotypic methods were used widely in the past. But with the advent of molecular methods, they are not preferred now. PCR-SSOP, PCR-SSP and PCR-DNA sequencing are the most reliable methods currently in use; have shown high resolution matching.

- ❖ Chronic rejection is generally considered irreversible and poorly amenable to treatment—only retransplant generally indicated if feasible—though inhaled cyclosporine is being investigated to delay or prevent chronic rejection of lung transplants
- ❖ Acute rejection is treated with therapeutic regimens consisting of one or combination of various immunosuppressive therapies as given in Table 19.2.

Table 19.2: Immunosuppressive agents used to improve graft survival

Immunosuppressive drugs:	
Corticosteroids	Prednisolone, hydrocortisone
Calcineurin inhibitors	Cyclosporine, Tacrolimus
Mitotic inhibitors	Azathioprine Cyclophosphamide Methotrexate
Antiproliferatives	Mycophenolic acid
mTOR inhibitor (mammalian target of rapamycin)	Sirolimus (rapamycin) Everolimus
Monoclonal antibody based:	
mAb to CD2 molecule present on T cell surface	OKT2
mAb to CD3 molecule present on T cell surface	OKT3
mAb to CD4 molecule present on T cell surface	OKT4
Monoclonal anti-IL-2Rα receptor antibodies	Basiliximab Daclizumab
Monoclonal anti-CD20 antibodies	Rituximab
mAb to TNFα	Infliximab
Antithymocyte globulin (ATG)	
Antilymphocyte globulin (ALG)	

Graft-Versus-Host Reaction

Graft-versus-host (GVH) reaction is a condition, where graft mounts an immune response against the host (i.e. recipient) and rejects the host, in contrary to the ususal situation of graft rejections, in which the recepient mounts an immune response against the graft antigens.

The GVH reaction occurs when the following three conditions are present:

1. The graft must contain immunocompetent T cells (e.g. stem cells or bone marrow or thymus transplants).
2. The recipient should possess transplantation antigens that are absent in the graft.
3. The recipient may be immunologically suppressed, therefore cannot mount immune response against the graft.

Types

GVH disease occurs in two forms:

1. Acute or fulminant GVH disease occurs within first 100 days of post-transplantation. It is a major challenge in case of bone marrow transplantation.
2. Chronic GVH disease is less severe form, occurs after 100 days of transplantation.

Clinical Manifestations

- The acute GVH disease is characterized by selective damage to the liver (hepatomegaly), skin (rash), mucosa, and the intestine (diarrhea) mediated by graft's immunocompetent

Contd...

Contd...

T cells. Experimentally, GVH can be produced in mice, called **Runt disease**

- Chronic GVH disease also attacks the above organs, but in addition, it causes damage to the connective tissues and exocrine glands.

Treatment

Glucocorticoids (administrated intravenously) are the standard treatment given for both acute and chronic GVH disease.

CANCER IMMUNOLOGY

Tumor immunology involves the study of antigens on tumor cells and the immune response to these antigens.

TUMOR ANTIGENS

Two types of tumor antigens have been identified on tumor cells:

1. Tumor-specific transplantation antigens (TSTAs).
2. Tumor-associated transplantation antigens (TATAs).

Tumor-specific Transplantation Antigen

Tumor-specific antigens are present only on tumor cells and are absent in normal cells of the body.

They may result from mutations in tumor cells that generate altered cellular proteins; cytosolic processing of these proteins would give rise to novel peptides that are presented with class I MHC molecules, inducing a cell-mediated immune response by tumor-specific cytotoxic T lymphocytes.

TSTAs are induced on tumor cells either by chemical or by physical carcinogens, and also by viral carcinogens.

- In chemically/physically induced tumors, the TSTA is tumor specific. Different tumors possess different TSTA, even though induced by the same carcinogen. Methylcholanthrene and ultraviolet light are the examples of chemical and physical carcinogens that are extensively studied
- In contrast, the TSTA of virus induced tumors is virus specific; all tumors produced by one virus would possess the same antigen. Examples include Epstein-Barr virus which causes nasopharyngeal carcinoma and several types of lymphoma.

Tumor-associated Transplantation Antigens

Tumor-associated antigens (TATAs) are not unique to tumor cells and may also be expressed by normal cells, but at a very low level. Their level gets exponentially high in tumor cells. Examples include (Table 19.3):

Table 19.3: TATAs used as tumor markers for diagnosis of cancers

Tumor markers	Tumor types
Oncofetal proteins	
Alpha-fetoprotein (AFP)	Hepatoma Testicular cancer
Carcinoembryonic antigen (CEA)	Gastrointestinal cancers Lung, ovarian cancers
Secreted tumor antigens	
CA 125	Ovarian cancers Other epithelial cancers
CA 19-9	Various carcinomas
Prostate-specific antigen	Prostate cancer
β2-microglobulin	Multiple myeloma
Hormones	
β subunit of chorionic gonadotropin	Hydatidiform mole Choriocarcinoma Testicular cancers

- **Oncofetal antigens:** They are the proteins that are expressed on normal cells during fetal life, but not expressed in the adult normally
  - Reactivation of the embryonic genes that encode these proteins in tumor cells results in their expression on the fully differentiated tumor cells
  - Examples include alpha-fetoprotein (AFP) and carcinoembryonic antigen (CEA).
- **Non-oncofetal TATAs:** Examples include carbohydrate antigens (CA 125, CA 19-9), prostate specific antigen and macroglobulin.

IMMUNE RESPONSE AGAINST TUMOR CELLS

Both humoral and cell-mediated immune responses are induced by tumor antigens that result in the destruction of the tumor cells. In general, the cell-mediated response appears to play the major role, especially cytotoxic T cell and NK cell.

Cytotoxic T Cells

A number of tumors have been shown to induce tumor-specific T_c cells that recognize tumor antigens presented by class I MHC on the tumor cells. However, as the expression of class I MHC molecules are decreased in a number of tumors, thereby limiting the role of specific T_c cells in their destruction.

Natural Killer (NK) Cells

The recognition of tumor cells by NK cells is not MHC restricted. The activity of NK cells is not compromised; but enhanced by the decreased MHC expression exhibited by some tumor cells.

- This is due to withdrawal of inhibitory receptors induced NK cells suppression. The inhibitory receptors of NK cells will be no longer functional in the absence of MHC I molecules on the target cells so that the activation receptors become active
- The activation receptors can be Fc receptors on NK cells which can bind to antibody-coated tumor cells, leading to ADCC
- The importance of NK cells in tumor immunity is suggested by the mutant mouse strain called **beige** and **Chediak-Higashi syndrome** in humans. In each case, a genetic defect causes marked impairment of NK cells and an associated increase in certain types of cancer.

IMMUNE SURVEILLANCE THEORY

Theory of immunological surveillance (conceptualized by Paul Ehrlich) postulates that the tumor cells may arise frequently in our body but are recognized as foreign and are eliminated by the constant vigilance of our immune system.

Later, Lewis Thomas revived the theory by suggesting the role of cell-mediated branch of the immune system to patrol the body and eliminate cancer cells.

Immune evasion by tumor cells is described in detail in the box given below:

Immune Evasion by Tumor Cells

Though the immune surveillance hypothesis looks attractive, it does not hold always true. In real situation, there are a number of immune evasion mechanisms by which tumor cells bypass the immune surveillance mechanisms.

- **Antitumor antibodies** produced against tumor antigens may have a role in immune evasion
  - Blocking factor: Antitumor antibody may act as a blocking factor. The antibody binds to tumor-specific antigen and masks the antigen from cytotoxic T cells and NK cells
  - Antigenic modulation: Certain tumor-specific antigens have been observed to disappear from the surface of tumor cells in the presence of serum antibodies and then to reappear after disappearance of serum antibodies.
- **Masking the immune cells:** Circulating tumor antigens may act as a 'smokescreen', coating the lymphoid cells and preventing them from acting on the tumor cells
- **Expressing low levels of MHC I:** Many tumor cells down regulate the expression of MHC I molecules; hence preventing their recognition by cytotoxic T cells
- **Poor costimulatory signals:** The costimulatory signal of T cell activation is provided by interaction between the CD28

Contd...

Contd...

molecules on T cell surface with the B7 molecules on the APCs. The poor immunogenicity of many tumor cells may be due to lack of the costimulatory molecules on APCs
- **Secrete soluble factors:** Certain tumor cells secrete soluble factors such as IL-10 and TGF-β that may suppress the immune responses against the tumor cells
- **Expressing Fas ligand:** Some tumor cells express Fas ligand on their surface, which when interacts with Fas (the death receptor) on T cells, causes apoptosis of T cells
- ↑**Opportunistic infections:** Patients with advanced cancers have an increased susceptibility to various opportunistic infections which in turn depresses the T cell responses.

CANCER IMMUNOTHERAPY

Cancer immunotherapy is the use of the immune system to treat cancer. Three main groups of immunotherapy are used to treat cancers: cell-based therapies, antibody therapies and cytokine therapies. They all provoke the immune system to attack the tumor cells by using these cancer antigens as targets.

Cell-based Therapies

Cell-based therapies, also known as **cancer vaccines,** usually involve the removal of immune cells from patients with cancer, either from the blood or from a tumor →immune cells specific for the tumor will be activated, grown and returned to the person with cancer →where the immune cells provoke an immune response against the cancer.

- Cell types that can be used in cancer vaccines include NK cells, cytotoxic T cells and dendritic cells
- The only cell-based therapy currently approved for use is dendritic cells (Provenge) for the treatment of prostate cancer.

Monoclonal Antibodies

Monoclonal antibody (mAb) therapies are currently the most successful form of immunotherapy. Many mAbs are approved for treatments of a wide range of cancers (Table 19.4).

- They are usually targeted against the cell surface molecules, e.g. the epidermal growth factor receptor
- Once bound to the surface receptors on the tumor cells, antibodies can induce tumor cell death by several mechanisms such as ADCC, complement activation, etc.

Table 19.4: Monoclonal antibodies approved for treatment of cancers

Monoclonal antibodies	Target	Approved for treatment of cancers
Alemtuzumab	CD52	Chronic lymphocytic leukemia (CLL)
Bevacizumab	Vascular endothelial growth factor	Colorectal, lung and renal cell cancer
Cetuximab	Epidermal growth factor receptor	Colorectal, head and neck cancer
Ipilimumab	CTLA4	Metastatic melanoma
Rituximab	CD20	CLL
Tositumomab	CD20	Non-Hodgkin lymphoma
Trastuzumab	ErbB2	Breast cancer

- Conjugated mAb: When such mAb conjugated to a toxic molecule is used; the mAb binds to its receptor on tumor cells and delivers the toxic molecule to exert lethal effect on the tumor cells
- The toxic molecule used to conjugate mAb may be either a toxin such as tetanus toxin or diphtheria toxin (immunotoxin) or chemical or radioactive substance.

Cytokine Therapies

Administration of cytokines can regulate and coordinate the behavior of the immune system. Examples include:

- **Interferon α:** It is used in the treatment of hairy-cell leukemia, AIDS-related Kaposi's sarcoma, follicular lymphoma, chronic myeloid leukemia and malignant melanoma
- **Interleukin-2:** It is used in the treatment of malignant melanoma and renal cell carcinoma.

Cancer Vaccine

They are used for treatment of existing cancers or prevention of emergence of new cancers.

- **Preventive cancer vaccines:** Examples of HPV vaccine and hepatitis B vaccine will prevent the emergence cervical and liver cancers, respectively
- **Therapeutic cancer vaccines:** They are used to treat existing cancers. Research is ongoing for preparation of such vaccines. Vaccines against some oncogenic viruses have proven extremely effective.

EXPECTED QUESTIONS

I. Essay:

1. A 55-year-male patient with chronic kidney disease underwent a kidney transplantation donated by a unrelated donor. Patient developed rejection reaction within 3 weeks.
   a. What is the immunological process of rejection?
   b. Mention the pretransplantation investigations to know the suitability of transplant.
   c. How can this be prevented?

II. Write short notes on:

1. Graft-versus-host reaction.
2. Immune evasion by tumor cells.
3. Immunotherapy for tumors.

III. Multiple Choice Questions (MCQs):

1. Application of skin graft for the second time from the same donor will result in:
a. First set rejection
b. Second set rejection
c. Both
d. None

2. Transplantation between members with same genetic constitution is known as:
a. Autograft b. Isograft
c. Allograft d. Xenograft

3. Graft rejection due to preformed antibodies occurs in:
a. Hyperacute rejection
b. Acute rejection
c. Subacute rejection
d. Chronic rejection

4. All of the following are tumor associated transplant antigens, *except*:
a. Prostate-specific antigen
b. Carcinoembryonic antigen
c. CA 125
d. Rituximab

5. Best example of syngeneic graft:
a. Between dizygotic twins
b. Between monozygotic twins
c. Between two members of same or different species
d. Between two sites of same person

6. Grafts placed in anatomically 'abnormal' sites are called as:
a. Orthotopic grafts
b. Heterotopic grafts
c. Vital grafts
d. Static grafts

7. Which of the following is the least important histocompatibility Ag and pose problems of rejection less frequently?
a. MHC molecules (major histocompatibility antigens)
b. Minor histocompatibility antigens
c. ABO blood group systems
d. Rh blood group systems

8. Which is not correct about graft rejection:
a. Hyperacute graft rejection—anti-ABO mediated
b. Acute graft rejection—cytotoxic T cell mediated
c. Chronic Graft rejection—delayed type hypersensitivity (DTH) mediated
d. Acute graft rejection—manifested as fibrosis of the graft

9. Which is a typical example of second-set rejection?
a. Revascularization (day 3–7) → healing (day 7–10) → resolution (day 10–12) → rejection (by day 12–14)
b. Revascularization (day 3–7) → cellular infiltration (day 7–8) → decreased vascularization (day 7–10) → necrosis (day 10–12) → rejection (by day 12–14)
c. In-complete revascularization (day 1–2) → cellular infiltration (day 3–4) → thrombosis and necrosis (day 5–6) → rejection (by day 6)
d. Decreased vascularization (day 3–7) → Revascularization (day 7–10) → necrosis (day 10–12) → rejection (by day 12–14)

10. According to Eichwald-Silmser effect, the rejection risk is greater if graft is transferred:
a. From a male donor to a female recipient
b. From a female donor to a male recipient
c. From a male donor to a male recipient
d. From a female donor to a female recipient

11. Which statement is not true about direct pathway of alloantigen presentation?
a. The allogeneic MHC molecules on graft's APCs are presented to the recipient's helper T cells.
b. Responsible for most of the acute graft rejections mediated by cytotoxic T cells
c. Seen if graft tissues contain macrophages and dendritic cells
d. The donor alloantigens are processed and presented by recipient APCs

12. Which is not an effector mechanism that participates in allograft rejection?
a. Delayed-type hypersensitivity
b. Cytotoxic T cells
c. Antibody-mediated neutralization of graft antigens
d. Complement-mediated lysis

13. Which of the following HLA typing gives high resolution matching?
a. Microcytotoxicity
b. Mixed lymphocyte reaction
c. PCR-RFLP (restriction fragment length polymorphism)
d. PCR – DNA sequencing typing

Answers

1. b **2.** b **3.** a **4.** d **5.** b **6.** b **7.** b **8.** d **9.** c **10.** a **11.** d **12.** c **13.** d

Immunoprophylaxis and Immunohematology

20
CHAPTER

Chapter Preview

- Immunoprophylaxis
  - Active immunoprophylaxis
  - Passive immunoprophylaxis
- Immunohematology
  - Blood group system (ABO, Rh and others)
  - Safe blood transfusion practices
  - Transfusion reactions
  - Transfusion-transmitted infections

IMMUNOPROPHYLAXIS

Immunoprophylaxis against microbial pathogens can be classified into active immunoprophylaxis (or vaccination) and passive immunoprophylaxis (or immunoglobulin administration).

VACCINATION (ACTIVE IMMUNOPROPHYLAXIS)

Vaccine is an immunobiological preparation that provides specific protection against a given disease. Following vaccine administration, the immunogen (active ingredient of the vaccine) stimulates the immune system of the body to produce active immunity in the form of protective antibody and/or immunocompetent T cell response.

- **History:** The terms vaccine and vaccination are derived from '*Variolae vaccinae*' (smallpox of the cow), the term devised by Edward Jenner to denote cowpox. Later, Louis Pasteur proposed the term 'vaccine' to cover all the new protective preparations being developed, in the memory of Edward Jenner
- **Valency:** Vaccines may be **monovalent** that contains single antigen or single serotype of a microorganism) or **polyvalent** (contains two or more strains of the same microorganism), e.g. trivalent vaccines such as influenza vaccine and polio vaccine
- **Homologous and heterologous vaccine:** In most vaccines, the immunizing substance is derived from the same microorganism against which it is used (homologous vaccine). However, there are few exceptions, where the vaccine organism is different from the disease-causing organism. Such vaccines are called as heterologous or "Jennerian" vaccines. Examples include—
  - The classic example is Jenner's use of cowpox to protect against smallpox
  - Use of BCG vaccine made from *Mycobacterium bovis* to protect against human tuberculosis caused by *M. tuberculosis*.
- **Types:** Vaccine may be prepared by live modified organisms, inactivated or killed organisms, extracted or cellular fractions, toxoids or combinations of all these. Preparations that are more recent are subunit vaccines and recombinant vaccine. Vaccines of future prospects include DNA vaccine and edible vaccine.

Live Attenuated Vaccine

Live vaccines, such as BCG (Table 20.1) are prepared from live (usually attenuated) organisms.

- The live attenuated organisms lose their ability to induce full blown disease, but retain their immunogenicity
- Attenuation is achieved by passing the live organisms serially through a foreign host, such as chick embryo/tissue culture or live animals.

  Note: Smallpox vaccine is a live vaccine which is not attenuated. The nonpathogenic cross reactive vaccinia virus or cowpox virus were used to vaccinate against smallpox virus (i.e. variola).

Advantages

Live vaccines in general, are more potent immunizing agent compared to killed vaccines, due to the following reasons:

- The live organisms **multiply** in the host and the resultant antigenic dose would be larger than what is administered

Table 20.1: Example of commonly used vaccines

Bacterial	Viral
Live attenuated vaccines	
BCG vaccine	Measles vaccine
Typhoral vaccine	Mumps vaccine
Epidemic typhus vaccine	Rubella vaccine
	Live attenuated influenza vaccine
	Chickenpox vaccine
	Oral polio vaccine (OPV, Sabin vaccine)
	Rotavirus vaccine
	Yellow fever 17D vaccine
	Hepatitis A vaccine
	Japanese B encephalitis vaccine (14-14-2 strain)
Killed/inactivated vaccine	
Typhoid vaccine	Injectable polio vaccine (IPV or Salk vaccine)
Cholera vaccine	Killed influenza vaccine
Pertussis vaccine	Rabies vaccine
Plague vaccine	Hepatitis A vaccine
	Japanese B encephalitis vaccine (Nakayama strain)
Toxoid vaccine	
DT (Diphtheria toxoid)	–
TT (Tetanus toxoid)	–
Subunit vaccine	
–	Hepatitis B vaccine
–	HPV (Human papillomavirus) vaccine
Cellular fraction	
Meningococcal vaccine	–
Pneumococcal vaccine	–
Haemophilus influenzae type (Hib) vaccine	–
Combined vaccine	
DPT vaccine (Diphtheria, pertussis and tetanus)	Mumps, measles, rubella (MMR) vaccine
Pentavalent vaccine (DPT + Hib + Hepatitis B)	

Note: Details about individual vaccine is discussed in the respective chapters.

- Live vaccines retain all the immunogenic components (major and minor) of the organisms
- They are capable of inducing mucosal immunity by stimulating secretory IgA antibody production at the local mucosal sites.

Precautions While Using Live Attenuated Vaccines

- **Contraindications:** Live vaccines should not be administered in individuals with immunodeficiency diseases or any conditions that supresses the immunity, such as leukemia, lymphoma, malignancies, on corticosteroid or any other immunosuppressive drug therapy
- **Pregnancy** is another contraindication, unless the risk of infection exceeds the risk of harm to the fetus by giving the live vaccine
- When two live vaccines are required to be given; they should be administered with an interval of at least 4 weeks. Exception is yellow fever vaccine which is given less than 4 weeks after MMR vaccine
- **Dosage:** Most live vaccines are given in single dose format as effective immunity is achieved with a single dose. Exception is oral polio vaccine (OPV) which is given as multiple doses at spaced intervals to achieve effective immunity
- **Risk of gaining the virulence:** The attenuation of the live vaccine has to be done in an effective way otherwise there is always a risk of gaining the virulence back again
- **Storage:** Live vaccines must be stored cautiously to retain effectiveness, especially the OPV and measles vaccine.

Inactivated or Killed Vaccine

It consists of organisms, which are grown in culture under controlled conditions and then killed using methods, such as heat or formaldehyde.

- They are generally safer but less efficacious than live vaccines
- Compared to the live vaccines, killed vaccines require large doses, adjuvants, and multiple doses to confer immunity. In most cases, a booster dose is also needed
- Adjuvants increase the immunogenicity of the vaccine antigen (e.g. alum is used as adjuvant in DPT vaccine)
- Killed vaccines are usually administered in subcutaneous or intramuscular routes. The only absolute contraindication is a severe local or general reaction to the previous dose.

Various characteristics of killed and live vaccines are given in Table 20.2.

Toxoid Vaccine

The exotoxins produced by certain bacteria can be detoxicated to form toxoid by treating with acidic pH, formalin or by prolonged storage.

- Toxoid is a form of toxin that loses its virulence property but retains immunogenicity
- When a toxoid preparation is given as vaccine, it induces formation of neutralizing antibodies that are capable of neutralizing the toxin moiety produced during an infection; rather than acting upon the organism

Table 20.2: Characteristics of killed and live vaccines

Characteristics	Killed vaccine	Live vaccine
Number of doses	Multiple	Single*
Need for adjuvant	Yes	No
Duration of immunity	Shorter	Longer
Effectiveness of protection	Lower	Greater
Mimics natural infection	Less closely	More closely
Immunoglobulins produced	IgG	IgA and IgG
Mucosal immunity	Absent	Induced
Cell-mediated immunity	Poor	Induced
Reverts back to virulent form	No	Possible
Excretion of vaccine virus and transmission to non-immune contacts	No	Possible
Interference by other microorganisms in host	No	Possible
Stability at room temperature	High	Low
Immunodeficiency and pregnancy	Safe	Unsafe

*Exception is oral polio vaccine (OPV), which is given as multiple doses at spaced intervals to achieve effective immunity.

- Examples include diphtheria toxoid (from *Corynebacterium diphtheriae*) and tetanus toxoid (from *Clostridium tetani*).

Extracted or Cellular Fractions Vaccine

Vaccines, in certain instances, are prepared from extracted cellular fractions; examples include meningococcal vaccine, pneumococcal vaccine and *Haemophilus influenzae* type b vaccine—all are prepared from the capsular polysaccharide antigens of the respective organism.

Subunit Vaccines

For certain viruses, only a particular subunit of the virus is necessary to initiate the immunity, e.g. hepatitis B surface antigen (HBsAg) is the immunogenic component of hepatitis B virus. So, this viral component alone can be used as vaccine rather than the whole virus.

- Examples of subunit vaccines include hepatitis B vaccine and human papillomavirus (HPV) vaccine
- DNA recombinant technology is used for the preparation of such sub viral components. For example, in hepatitis B vaccine preparation, the gene coding for HBsAg is inserted into the chromosome of baker's yeast, so that, with the multiplication of the yeast, the gene of interest would also replicate resulting in production of large quantity of HBsAg which can be used as vaccine.

Combinations

If more than one immunizing agents are included in a vaccine preparation, it is called combined vaccine. The aim of the combined vaccine is to—

- Simplify administration and
- Augment the immunogenicity of the immunogen. For example, in DPT vaccine, the pertussis component acts as an adjuvant, which increases the immunogenicity of both diphtheria toxoid and tetanus toxoid.

Newer Vaccine Approaches

DNA Vaccine

DNA vaccines are experimental at present, have many advantages such as cost effectiveness and mounting a stronger and wider range of immune response.

The small pieces of DNA containing genes from the pathogenic microorganism are injected into the host. The gene of interest gets integrated with the host cell genome and starts transcribing the proteins against which the host mounts an immune response. Several vaccine trials are going on based on DNA vaccines.

Edible Vaccine

The edible vaccine is a new concept introduced recently.

- The gene encoding the orally active antigenic protein is isolated from the pathogen and is transferred to suitable plant bacteria, which are then used to infect a transgenic plant (e.g. banana, potato, etc.)
- The plants infected by the bacteria then start producing the antigen of interest in large scale. The appropriate plant parts having the antigen may be fed raw to animals or humans to bring about immunization
- The advantages of the edible vaccines are—(1) low cost, (2) ability to produce in large scale, (3) administered orally, (4) induces local immunity, and (5) heat stable
- **Applications:** The edible vaccines are still under experimental stage; some formulations available include—
  - Transgenic potatoes and tomatoes against diarrheagenic organisms
  - Edible banana against Norwalk virus.

Cold Chain

"Cold chain" refers to a system of transport, storage, and handling of vaccines, starting at the manufacturer level and ending with the site of administration of the vaccine to the client. The optimum temperature for refrigerated vaccines is between +2°C and +8°C. For frozen vaccines the optimum temperature is −15°C or lower. In addition, protection from light is a necessary condition for some vaccines. Improper cold chain maintenance is one of the most common causes of vaccine failure; especially oral polio vaccine which is the most sensitive vaccine to heat; must be stored at -20°C.

- Vaccines which must be stored in the freezer compartment are polio and measles vaccines
- Vaccines which must be stored in the COLD part but never allowed to freeze are—DPT, TT, Td, BCG, hepatitis B, *H. influenzae* type b and diluents.

Vaccine Vial Monitor

Vaccine vial monitor is a tool to monitor the stability/ potency of a vaccine and to check the efficiency of cold chain.

It is heat sensitive label lining the vaccine vial. It contains an outer blue circle and an inner white square. With time and exposure to higher temperature, the inner square changes its color gradually from white towards blue, whereas the outer circle is not heat sensitive; it remains blue throughout (Table 20.3 and Fig. 20.1).

Table 20.3: Staging of vaccine vial monitor

	Inner square	Outer circle	Vaccine
Stage 1	White	Blue	Can be used
Stage 2	Light blue	Blue	Can be used
Stage 3	Blue	Blue	Discard
Stage 4	Dark blue	Blue	Discard

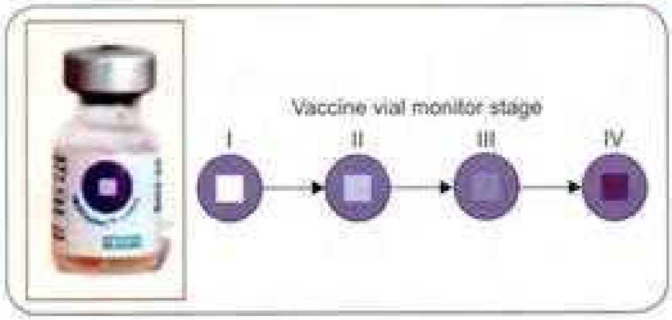


Fig. 20.1: Various stages of vaccine vial monitor (Vaccine is usable up to stages I and II and should be discarded for stages III and IV)
Source: Pondicherry Institute of Medical Sciences (with permission).

National Immunization Schedule 2017 (NIS)

Immunization is one of the most logical and cost effective strategies of any country for the prevention of childhood sicknesses and disabilities and is thus a basic need for all children. The following is the national immunization schedule recommended by the Ministry of Health, Government of India and it includes those vaccines that are given free of cost to all children of our country (Table 20.4).

PASSIVE IMMUNOPROPHYLAXIS (IMMUNOGLOBULINS)

Passive immunoprophylaxis is given in the form of commercially available ready made **immunoglobulins**

Table 20.4: National Immunization Schedule 2017 (NIS) for infants, children and pregnant women

Vaccine	When to give	Maximum age	Dose	Dilution	Route	Site
For pregnant women						
TT-1	Early in pregnancy		0.5 mL	No	IM	Upper arm
TT-2	4 weeks after TT-1*	<36 weeks of pregnancy (if missed, can be given later)	0.5 mL	No	IM	Upper arm
TT- Booster	If received 2 TT doses in a pregnancy within the last 3 years*		0.5 mL	No	IM	Upper arm
For infants						
BCG	At birth or as early as possible	Till 1 year	0.1 mL (0.05 mL for <1 month)	Saline	ID	Left upper arm
Hepatitis B - Birth dose	At birth or as early as possible	Within 24 hour	0.5 mL	No	IM	Anterolateral side of mid-thigh
OPV-0	At birth or as early as possible	Within first 15 days	2 drops	No	Oral	Oral
OPV 1, 2 and 3	At 6 weeks, 10 weeks and 14 weeks	5 years of age	2 drops	No	Oral	Oral
Pentavalent # 1, 2 and 3	At 6 weeks, 10 weeks and 14 weeks	1 year of age	0.5 mL	No	IM	Anterolateral side of mid-thigh
Rotavirus##	At 6 weeks, 10 weeks and 14 weeks	1 year of age	5 drops	No	Oral	Oral
IPV	Two fractional doses at 6 and 14 weeks of age	1 year of age	0.1 mL	No	ID	Right upper arm
Measles /MR 1st Dose$	9 completed months–12 months	5 years of age	0.5 mL	Sterile water	SC	Right upper arm
JE - 1**	9 completed months–12 months	15 years of age	0.5 mL	Phosphate buffer	SC	Left upper arm
Vitamin A (1st dose)	At 9 completed months with measles-Rubella	5 years of age	1 mL (1 lakh IU)	No	Oral	Oral

Contd...

Contd...

Vaccine	When to give	Maximum age	Dose	Dilution	Route	Site
For Children						
DPT booster-1	16–24 months	7 years of age	0.5 mL	No	IM	Anterolateral side of mid-thigh
Measles/MR 2nd dose$	16–24 months	5 years of age	0.5 mL	Sterile water	SC	Right upper arm
OPV Booster	16–24 months	5 years of age	2 drops	No	Oral	Oral
JE-2	16–24 months		0.5 mL	Phosphate buffer	SC	Left upper arm
Vitamin A*** (2nd to 9th dose)	16–18 months. Then one dose every 6 months up to the age of 5 years	5 years of age	2 mL (2 lakh IU)	No	Oral	Oral
DPT Booster-2	5–6 years	7 years of age	0.5 mL	No	IM	Upper arm
TT	10 years and 16 years		0.5 mL	No	IM	Upper arm

*Give TT-2 or Booster doses before 36 weeks of pregnancy. However, give these even if more than 36 weeks have passed. Give TT to a woman in labor, if she has not previously received TT.

****JE Vaccine** is introduced in select endemic districts after the campaign: UP, Bihar, Assam, West Bengal and Karnataka.

*** The 2nd to 9th doses of Vitamin A can be administered to children 1–5 years old during biannual rounds, in collaboration with ICDS (Integrated Child Development Services).

 # Pentavalent vaccine- contains combination of DPT, hepatitis B and *H.influenzae* type b vaccines.

Interval between two doses of pentavalent or OPV should never be less than 1 month.

 # # Rotavirus vaccine: Phased introduction, at present in Andhra Pradesh, Haryana, Himachal Pradesh and Odisha from 2016 and expanded in Madhya Pradesh, Assam, Rajasthan, and Tripura in February 2017 and planned in Tamil Nadu and Uttar Pradesh in 2017.

$ **Measles/ MR 2nd dose:** Phased introduction, at present in five states namely Karnataka, Tamil Nadu, Goa, Lakshadweep and Puducherry. (As of February 2017)

Children who have not been received a single vaccine coming after 1 year: Will be given 3 doses of DPT at an interval of 4 weeks; Measles-1st dose, JE-1st dose (wherever applicable) up to 2 years of age.

Abbreviations: IM, intramuscular; SC, subcutaneous; ID, intradermal.

prepared against the pathogenic microorganism. Unlike vaccines, immunoglobulins act faster, without involvement of host immune apparatus.

Passive immunization is useful in the following circumstances:

- For immunocompromized individuals who cannot synthesize antibodies
- For post-exposure prophylaxis to achieve an immediate effect.

For the treatment of toxin mediated diseases to ameliorate the effect of toxin. Antibiotics cannot neutralize the toxin; hence, they cannot be used for the treatment of toxin mediated diseases.

Passive immunoprophylaxis available against various microbial diseases is given in Table 20.5.

Table 20.5: Passive immunoprophylaxis

Immunoglobulin preparations	Source	Indications
Diphtheria antitoxin	Equine	Treatment of respiratory diphtheria
Tetanus immune globulin (TIG)	Equine, Human	Treatment of tetanus as PEP, for people not adequately immunized with tetanus toxoid
Botulinum antitoxin	Equine, Human	Treatment of botulism
Varicella-zoster immune globulin (VZIG)	Human	PEP for immunosuppressed contacts of acute cases or new born contacts
Cytomegalovirus immune globulin (CMV-IG)	Human	PEP in hematopoietic stem cell and kidney transplant recipients
Rabies immunoglobulin (RIG)	Equine, Human	Treatment of rabies and PEP in people not previously immunized with rabies vaccine
Hepatitis B immunoglobulin (HBIG)	Human	PEP for percutaneous or mucosal or sexual exposure Newborn of mother with HBsAg +ve
Hepatitis A immunoglobulin (HAIG)	Human	Postexposure prophylaxis • Family contacts • Travelers
Rubella	Human	Women exposed during early pregnancy
Measles	Human	Infants or immunosuppressed contacts of acute cases exposed <6 days previously
Rh-isoimmunization (RhIG)	Human	Treatment of Rh-ve mother following delivery of a Rh +ve baby

Abbreviation: PEP, Post-exposure prophylaxis.

IMMUNOHEMATOLOGY

Among the 33 recognized blood group systems, the ABO system is the oldest system to be discovered (Karl Landsteiner, 1900). The other blood group systems include Rh, MN, P, Lutheran, Lewis, Kell, Duffy, Kidd, Diego, Yt, Kg, Dombrock and Colton.

ABO BLOOD GROUP SYSTEM

The ABO blood group system comprises of four blood groups, each is determined by the presence or absence of two antigens A and B on the surface of the red blood cell (RBC) membrane and their corresponding antibodies in

Table 20.6: Distribution of ABO antigens and antibodies in RBCs and serum

Blood group	Antigen on RBC	Isoantibodies in serum
A	A	Anti-B
B	B	Anti-A
AB	AB	None
O	None	Anti-A and Anti-B

serum. The principle followed is if a blood group antigen is present on the RBC then the corresponding antibody would be absent in serum. Examples are given in Table 20.6.

Natural Isoantibodies

Anti-A and anti-B isoantibodies are called natural antibodies because they are seen to arise without any apparent antigenic stimulation.

They are IgM in nature (pentameric), produced by the age of 6 months and persist thereafter.

ABO System in Other Animals

ABO blood types are also present in some other animals, for example rodents and apes, such as chimpanzees, and gorillas.

Distribution of Blood Groups in India

In India, because of diversity of race, religion and creed, the distribution of blood groups within the population is not uniform. A recent study done in North India had shown that group B is the commonest (35%), followed by group O (30%), A (21%) and AB (14%). Previous South Indian studies had shown blood group O to be the most common. However distribution of ABO blood groups may vary between various geographical areas.

RH-BLOOD GROUP SYSTEM

Rh-blood group is the most important blood group system in humans after ABO system. It was so named because the antibody against this Rh-blood group antigen was first prepared in Rhesus monkey by Landsteiner and Wiener.

- At present, the Rh-blood group system consists of 50 defined blood-group antigens, among which the five antigens D, C, c, E, and e are the most important
- The commonly used terms Rh-factor, refer to the D antigen only. Rh-positive and Rh-negative denote presence or absence of Rh-antigen on the surface of RBCs. Unlike ABO system, there are no natural Rh-antibodies in our blood
- In India, about 95% of individuals have Rh-positive blood group; the remainder (5%) are Rh-negative
- Rh-blood group system has two important clinical applications—(1) its role in blood transfusion, (2) its role in causing hemolytic disease of the newborn (or Erythroblastosis fetalis).

Erythroblastosis Fetalis

When the mother is Rh-negative and the father is Rh-positive, the fetus can inherit the Rh-factor from the father. This makes the fetus Rh-positive.

- During delivery, the Rh-positive blood may be passed into maternal circulation
- The mother being Rh-negative, may develop antibodies to an Rh-positive fetal RBCs. However, as anti-Rh-antibodies are produced only after exposure to Rh-antigen (from Rh-positive fetus to Rh-negative mother or mismatched transfusion) and take some time to generate anti-Rh-IgG which can cross the placenta; hence, maternal Rh-antibodies fail to lyse fetal RBCs during the first Rh-incompatible pregnancy
- During the subsequent pregnancies with a Rh-positive fetus, the Rh-antibodies being IgG in nature, can cross the placenta from mother to fetus and can destroy the fetal RBCs
- This condition is called **hemolytic disease of newborn**. It can become severe enough to cause serious illness, brain damage, or even death of the fetus or newborn.

OTHER BLOOD GROUP SYSTEMS

In routine transfusion practice, only the ABO and Rh-antigens are relevant. Proper matching of ABO and Rh-groups should be carried out before transfusing the blood unit. The other blood group antigens are too weak.

SAFE BLOOD TRANSFUSION PRACTICES

Safe blood transfusion practices require that the following conditions are satisfied in choosing a donor:

- The recipient's plasma should not contain any antibodies that will damage the donor's RBCs
- The donor plasma should not have any antibodies that will damage the recipient's RBCs
- The donor red cells should not have any antigen that is lacking in the recipient RBCs. If the transfused cells possess a 'foreign antigen' it will stimulate an immune response in the recipient.

Selection of Blood Group for Blood Transfusion

Ideally, the donor and recipient should belong to the same ABO group. However in emergency situations, O blood group can be used for transfusion for any ABO group individuals and AB blood group individuals can receive blood unit from any blood group donors.

- **Universal donor:** Individuals with 'O' blood group are called as universal donors because they do not possess either A or B antigen; hence, they are generally safe
  - The anti-A and anti-B antibodies in the transfused O blood group are diluted in recipient's serum, do not ordinarily cause any damage to the red cells of the A or B blood group recipients

Contd...

Contd...

- ***Dangerous O group:*** It is observed that if the anti-A and anti-B antibody titers are high (1:200 or above) in the serum of the individuals with O blood group; then transfusion of such blood may result in damage to recipient's RBCs. This type of O blood group is called as the dangerous O blood group and it should not be used for transfusion.

- **Universal recipients:** Individuals with AB blood group do not have both A and B antibodies in serum; therefore, they can receive any other blood group. Hence, they are called as universal recipients.

TRANSFUSION REACTIONS

Transfusion reactions are the complications arising following blood transfusion; which may be of two types—(1) immunological and (2) nonimmunological.

Immunological Complications

Following are the immunological reactions that can take place after an incompatible blood transfusion:

- **Acute hemolytic reactions:** They are rare, occur as a result of mismatched blood transfusion. The RBCs undergo intravascular hemolysis or they may be coated by antibodies and engulfed by phagocytes, removed from circulation and subjected to extravascular lysis
  - Symptoms include fever, chills, chest pain, back pain, hemorrhage, increased heart rate, dyspnea, hypotension and rarely kidney injury
  - When transfusion reaction is suspected, transfusion should be stopped immediately, and blood should be sent for tests to evaluate for presence of hemolysis. Treatment is supportive.
- **Delayed hemolytic reactions:** They may occur in some cases of mismatched blood transfusion involving minor antigens. The mechanism is similar to that seen in acute hemolytic reactions, but they are milder and occur late.
- **Febrile nonhemolytic reactions:** They are the most common type of transfusion reactions and occur due to the release of inflammatory chemical signals by WBCs present in stored donor blood
- **Allergic reactions:** They occur when the recipient has preformed antibodies to certain chemicals in the donor blood, and they do not require prior exposure to blood transfusions
- **Post-transfusion thrombocytopenia:** It may occur following transfusion containing platelets possessing surface protein human platelet antigen-1a (HPA-1a)
- **Transfusion-associated acute lung injury (TRALI):** It is a rare complication, characterized by acute respiratory distress; probably mediated by anti-HLA antibodies. It is more common in pregnancy.

Nonimmunological Complications

These include—iron overload and various transfusion-transmitted infections.

Transfusion-transmitted Infections

The most important nonimmunological complication is transmission of infectious agents during blood transfusion. These may include viruses, bacteria and protozoa. HIV and hepatitis viruses are of great concern among all the infectious agents associated with blood transfusion. List of various infectious agents transmitted via blood transfusion is as follows:

- **Viruses**
  - Human immunodeficiency viruses (HIV)
  - Hepatitis B, C and rarely D viruses
  - Cytomegalovirus (CMV)
  - Human T-lymphotropic virus.
- **Bacteria**
  - *Treponema pallidum*
  - *Leptospira interrogans*
  - *Borrelia burgdorferi.*
- **Protozoa**
  - *Plasmodium* species
  - *Babesia* species.
  - *Leishmania donovani*
  - *Toxoplasma gondii*
  - *Trypanosoma cruzi.*

EXPECTED QUESTIONS

I. Write short notes on:

1. Live vaccines vs. killed vaccines.
2. National Immunization Schedule.
3. Passive immunoprophylaxis.
4. Transfusion reactions.

II. Multiple Choice Questions (MCQs):

1. **All of the following are live attenuated vaccines, except:**
   a. MMR b. Yellow fever 17D
   c. Salk polio d. Sabin polio
2. **Which of the following infectious agents can be transmitted through blood transmission?**
   a. HIV b. *Treponema pallidum*
   c. *Toxoplasma gondii* d. All of the above
3. **All the following vaccines are given at birth, except:**
   a. BCG b. Hepatitis B
   c. DPT d. OPV

Answers

1. c **2.** d **3.** c

Systematic Bacteriology

SECTION 3

Section Outline

Staphylococcus

21 CHAPTER

Chapter Preview

- Introduction
- *Staphylococcus aureus*
- Coagulase-negative staphylococci (CoNS)

INTRODUCTION

Gram-positive cocci are classified into two families—Micrococcaceae and Streptococcaceae, differentiated by the catalase test. Micrococcaceae are catalase positive, gram-positive cocci arranged in tetrads or clusters; whereas Streptococcaceae are catalase negative gram-positive cocci, arranged in pairs or chains.

Family Micrococcaceae comprises of four genera—*Micrococcus, Stomatococcus, Planococcus* and *Staphylococcus*.

- *Micrococcus* species are skin commensals, usually not associated with human infections. They are 1-1.8 µm size, arranged in tetrads. As they are obligate aerobes, they show oxidative pattern in Hugh and Leifson's oxidative-fermentative (OF) test
- *Planococcus* and *Stomatococcus* are not pathogenic to man
- *Staphylococcus* species are arranged in clusters, show fermentative pattern in oxidative fermentive test.
    - Among *Staphylococcus* species, *S. aureus* is the most pathogenic; it produces an enzyme coagulase which forms the basis of coagulase test
    - Whereas, other species do not produce coagulase and are called as coagulase-negative staphylococci (CoNS). They are rarely pathogenic to man; may cause infections in immunocompromised patients
    - *S. epidermidis* is the most common CoNS infecting man, followed by *S. saprophyticus, S. lugdunensis, S. schleiferi, S. haemolyticus* and *S. warneri*.

History

Staphylococcus was first observed in pus by von Recklinghausen (1871) and was first cultured in liquid medium by Louis Pasteur (1880).

It was named as *Staphylococcus* (in Greek, *Staphyle* means 'bunch of grapes' and *kokkos* means berry) by Sir Alexander Ogston (1880). Rosenbach (1884) named two species of staphylococci based on pigmentation of colonies as *S. aureus* (golden yellow colonies) and *S. albus* (white colonies). Later Passet (1885) named a third species as *S. citreus* (lemon yellow colonies).

STAPHYLOCOCCUS AUREUS

Staphylococcus aureus is catalase positive, coagulase positive, facultative anaerobe, non-motile, non-sporing and occasionally capsulated.

- They are spherical cocci, about 1 µm in diameter, arranged in grape-like clusters. This arrangement is due to cell-division in *S. aureus*; which occurs in multiple planes with daughter cells remain attached together
- It produces golden yellow pigmentation on nutrient agar and β hemolytic colonies on blood agar
- *S. aureus* is the most virulent species among staphylococci; produces infections which range from localized pyogenic infections to life-threatening systemic infections in man
- Its importance as human pathogen is greatly enhanced especially in hospital environment because of its ability to develop drug resistance.

Virulence Factors

S. aureus possesses an array of virulence factors as listed in Table 21.1.

Cell Wall Associated Factors

Like most gram-positive bacteria, the cell wall of *Staphylococcus* consists of a thick peptidoglycan layer and teichoic acid. *S. aureus* has additional factors in the cell wall, such as protein A and clumping factor.

Table 21.1: Virulence factors of *Staphylococcus aureus*

Cell wall associated factors
Peptidoglycan Teichoic acid Cell surface adhesins, e.g. clumping factor Protein A
Toxins
Membrane active toxins • Hemolysins—α, β, γ, δ • Leukocidin (or panton valentine toxin) Epidermolytic toxin (exfoliative toxin) Enterotoxins Toxic shock syndrome toxin
Extracellular enzymes
Coagulase Heat stable thermonuclease Deoxyribonuclease Staphylokinase (fibrinolysin) Others—hyaluronidase, lipase, and protease

Table 21.2: Hemolysins of *Staphylococcus aureus* and their activity

Hemolysins	Activities
α-hemolysin	It is inactivated at 70°C but again reactivated paradoxically at 100°C (This is because at 60°C α-hemolysin combines with a heat labile inactivator which gets denatured at 100°C) It possesses lethal, leukocidal, dermonecrotic, cytotoxic and neurotoxic activities
β-hemolysin	*It is sphingomyelinase in nature:* • Lyses sheep RBC, but not human or rabbit RBC; this explains why hemolysis of *S. aureus* is better in sheep blood agar than human blood agar • Exhibits **hot-cold phenomenon**, i.e. hemolysis starts at 37°C but becomes evident only after chilling
γ-hemolysin	It has three protein fragments which act together along with leukocidin to exhibit hemolytic activity
δ-hemolysin	It has detergent like (surfactant) action It is also lethal, leukocidal and dermonecrotic

Peptidoglycan

Similar to other gram-positive bacteria, the peptidoglycan layer of *Staphylococcus* is thicker (15-80 nm, up to 100 layers thick).

- It confers rigidity to the cell wall and maintains the shape
- It induces inflammatory response and also has endotoxin-like activity.

Teichoic Acid

It is made up of ribitol phosphate polymers, helps in adhesion of cocci to mucosal surfaces and inhibits opsonization.

Cell Surface Adhesins

- Clumping factor/bound coagulase—it is a fibrinogen binding adhesin; responsible for slide coagulase reaction
- Fibronectin binding adhesin
- Collagen-binding adhesin.

Protein A (SpA)

It is a 42 kDa polypeptide, encoded by *spa* gene. It is present in 90-99% of human *S. aureus* strains (especially the Cowan I strains).

- Protein A has many **biological properties**, such as anti-complementary, chemotactic, mitogenic, inhibition of opsonization and induction of platelet damage
- **Mediates coagglutination reaction:** Protein A binds to Fc region of any IgG antibody, leaving Fab region free which binds to the corresponding antigen present in clinical samples (Detail described in Chapter 12).

Microcapsule

Some strains of *S. aureus* have polysaccharide microcapsule, which inhibits phagocytosis by neutrophils. The capsular polysaccharides are zwitterionic, i.e. they have both negative and positive charges, which is a feature that is critical for abscess formation.

Toxins

Membrane Active Toxins

Hemolysins

S. aureus produces four distinct hemolysins—α, β, γ and δ hemolysins. They are membrane damaging toxins, act on red blood cells (RBCs) leading to hemolysis. They differ from each other by their action on RBCs of different animals, their lethal, dermonecrotic and leukocidal activity (Table 21.2).

Leukocidins/Panton Valentine Toxin

It is also called as Panton Valentine (PV) toxin; named after its discoverers.

- It has two components F (fast) and S (slow) based on their migration on carboxymethyl cellulose column
- Both the fragments act synergistically with γ-hemolysin to damage leukocytes, RBCs and macrophages
- **Synergohymenotropic toxins:** γ-hemolysin and PV toxin are called as synergohymenotropic toxins. Because they are not active individually, but in combination, they are capable of producing hemolytic and leukocidal activity. There are six combinations possible by the interaction between three fragments of γ-hemolysin with the two fragments of PV toxin

- PV toxin is expressed on MRSA (methicillin-resistant *Staphylococcus aureus*) strains, which are associated with the community acquired infections.

Epidermolytic/Exfoliative Toxin (ET)

This toxin is responsible for staphylococcal scalded skin syndrome (SSSS).

- **It comprises of two proteins:** ET-A (chromosomal, heat stable) and ET-B (plasmid coded, heat labile)
- **Staphylococcal scalded skin syndrome (SSSS)** often occurs in newborns and children, more often than adults
- Illness may vary from localized tender blisters and bullae formation to exfoliation and separation of outer epidermal layer leaving denuded underlying skin (the later is called as **Nikolsky's sign**)
- The mucous membranes are usually spared
- Severe form in a newborn is called as Ritter's syndrome; characterized by fever, lethargy, and irritability with poor feeding
- Milder forms—pemphigus neonatorum and bullous impetigo
- Epidermolytic toxin producing strains belong to *S. aureus* bacteriophage group II.

Enterotoxin

Enterotoxin is expressed by nearly 50% of *S. aureus* strains and is responsible for staphylococcal food poisoning.

- It is a **preformed toxin** (secreted in food before consumption) so that it can act rapidly. As a result, the **incubation period** is short (1–6 hours)
- **Site of action:** The toxin stimulates the vagus nerve and the vomiting center of the brain. It also appears to stimulate the intestinal peristaltic activity
- **Symptoms:** Staphycoccal food poisoning is characterized by nausea, vomiting, occasionally diarrhea, hypotension, and dehydration; however, there is no fever. Symptoms generally resolve within 8–10 hours
- **Most common source** of infection is a food handler, who is a carrier of *S. aureus*. There is no secondary spread
- **Most common food items** involved are milk products, bakery food, custards, potato salad, or processed meat
- It is a heat stable toxin and is resistant to gastric juice
- **Serotyping:** Enterotoxins can be typed into many serotypes (A-E, G-I, R-T and V)
  - Type A is most common to cause food poisoning
  - Serotype-F does not cause food poisoning; but causes toxic shock syndrome
  - Serotype-J, Q and U are enterotoxin—like toxins.
- *S. aureus* enterotoxins are also responsible for some cases of pseudomembranous colitis following use of broad spectrum antibiotics
- **Detection of enterotoxin** in food is carried out by ELISA or latex agglutination test or by detecting enterotoxin gene by multiplex PCR (polymerase chain reaction)
- Treatment is entirely supportive by correcting fluid and electrolyte imbalance.

Toxic Shock Syndrome Toxin (TSST)

This toxin is responsible for toxic shock syndrome (TSS). It has two subtypes—TSST-1 and TSST-2.

- TSST-1 is actually a staphylococcal enterotoxin. Enterotoxin F or pyrogenic exotoxin C is the most common type of TSST-1; rarely enterotoxin-B or C may also be associated
- TSST producing strains belong to *S. aureus* bacteriophage group I
- **Risk factors:** Initially, toxic shock syndrome was reported from women using highly absorbent vaginal tampons during menstruation. Subsequently, TSS has been reported from both men and non-menstruating women as a complication of staphylococcal abscesses, osteomyelitis and post-surgical, traumatic or burn wound infections
- **Pathogenesis:** TSST-1 gets absorbed into circulation from the tampons; then being a superantigen it stimulates the T-cells non-specifically (by binding to Vβ region of T-cell receptor) causing excessive production of cytokines which leads to a potentially fatal multisystem disease. (Both TSST and enterotoxin are examples of superantigens, described in detail in Chapter 10)
- **Clinical features:** Patients present with fever, hypotension, mucosal (conjunctival) hyperemia, vomiting, diarrhea, confusion, myalgia, abdominal pain and erythematous rashes which desquamate later. Subsequently, there is rapid involvement of the other organs such as liver, kidneys, lungs, gastrointestinal tract (GIT) and/or central nervous system (CNS)
- Anti-TSST antibodies usually appear in the convalescent stage, they are protective in nature. TSS is more severe if anti-TSST antibodies fail to appear
- **Diagnosis:** Detection of TSST can be done by latex agglutination test and enzyme immunoassay. PCR-based assays are available for detection of TSST genes 1 and 2. Other findings may include altered liver/kidney function tests and low platelet count.

TREATMENT **Toxic shock syndrome**

- As toxin causes capillary leak; aggressive parenteral fluid replacement should be initiated at the earliest
- Examine for and remove any colonized foreign body, e.g. vaginal tampon
- Clindamycin is the preferred drug for TSS (as it reduces the toxin synthesis). It is given along with anti-staphylococcal penicillin (e.g. cloxacillin) for MSSA or vancomycin for MRSA
- For clindamycin resistant cases: Linezolid can be used for toxin supression. In such case, addition of vancomycin is not required.

Table 21.3: Differences between tube and slide coagulase tests

Tube coagulase	Slide coagulase
Due to coagulase enzyme	Due to clumping factor
Requires CRF in plasma	Does not require CRF in plasma
Test performed on tube	Test performed on slide
Positive if clot is formed	Positive if clumps are formed
Coagulase enzyme has eight serotypes	Clumping factor has one serotype
S. lugdunensis gives a negative result	*S. lugdunensis* gives a positive result
Both tube and slide coagulase positive for *S. aureus*, *S. hyicus* and *S. intermedius*	

Abbreviation: CRF, coagulase reacting factor.

Extracellular Enzymes

Coagulase

The unique feature of *S. aureus* is that, it secretes coagulase enzyme which brings about clotting or coagulation of plasma.

- Coagulase enzyme combines with a plasma protein called CRF (coagulase reacting factor), and together they activate prothrombin, which in turn, converts fibrinogen to fibrin
- This is the basis of tube coagulase test. This has to be differentiated from slide coagulase test, which is mediated by clumping factor (Table 21.3)
- Coagulase can react with rabbit or human plasma; but does not clot with guinea pig plasma as it lacks CRF
- **Subtypes:** Coagulase enzyme has 8 antigenic types (A-H). **Type-A** is the most common type; secreted by human strains of *S. aureus*.

Other Enzymes

- Heat stable thermonucleases and DNase (deoxyribonuclease) are the enzymes that are specific to *S. aureus*; not produced by any other staphylococcal species
- Staphylokinase (fibrinolysin) breaks down fibrin clots and may facilitate the spread of infection
- Hyaluronidase breaks down the connective tissue network
- Lipases and phospholipases breakdown the lipids.

Pathogenesis

Pathogenesis of *S. aureus* involves the following steps:

- **Colonization:** *S. aureus* colonizes on various body surfaces, such as anterior nares, axilla and perineal skin
- **Introduction into the tissue:** Organisms are introduced into the tissues as a result of minor abrasions or instrumentation. Then they adhere to the tissue surfaces; which is mediated by various adhesins, e.g. clumping factor
- **Invasion**: *S. aureus* can invade into the tissues by elaborating enzymes, such as serine proteases, hyaluronidases, thermonucleases and lipases
- **Evasion of host defense mechanisms:** *S. aureus* exhibits various immune evasion mechanisms, such as:
  - Anti-phagocytic activity mediated by microcapsule and protein A
  - Inhibition of leukocyte migration (by chemotaxis inhibitory protein of staphylococci)
  - Intracellular survival inside the endothelial cells (by formation of small colony variants).
- **Metastatic spread:** Finally, *S. aureus* spreads to various distant sites by hematogenous spread.

Clinical Manifestations

Staphylococcus aureus is a pluripotent pathogen, causing various diseases through both toxin-mediated and non-toxin-mediated mechanisms. It is responsible for both nosocomial and community-based infections that range from relatively minor skin and soft tissue infections to life-threatening systemic infections (Table 21.4).

Epidemiology

Staphylococcus aureus is a part of normal human flora. About 25-50% of healthy population are carriers of *S. aureus*, colonizing the organism persistently or transiently.

- **Most common site(s)** of colonization are anterior nares followed by skin (abraded), vagina, axilla, perineum, and oropharynx. These colonization sites serve as a reservoir for future infections
- The rate of colonization is higher among insulin-dependent diabetics, HIV-infected patients, patients undergoing hemodialysis, and individuals with skin damage
- Overall, *S. aureus* is a leading cause of nosocomial infections. In hospitals, the health care professionals are the potential carriers of *S. aureus*. Hospital strains are

Fig. 21.1: Staphylococcal cellulitis

Source: Public Health Image Library, ID# 4647/Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

Table 21.4: Clinical spectrum of *Staphylococcus aureus* infections

Skin and soft tissue infections
S. aureus is one of the most common cause of various skin and soft tissue infections such as: • **Folliculitis** (infection of hair follicles) • **Furuncle (boil):** Painful pustular lesion in moist regions due to infection of the hair follicle • **Carbuncle:** Severe, painful lesion in the lower neck region, extending to the deeper subcutaneous tissue • Mastitis and breast abscess (in nursing mothers) • **Impetigo:** It mainly occurs in children, usually appears as red sores on the face, that bursts and develops into **honey-colored crusts** • Surgical site wound infections (most common cause) • Cellulitis (inflammation of skin and subcutaneous tissue) (Fig. 21.1) • **Hidradenitis suppurativa:** A recurrent follicular infection in areas rich in apocrine glands, such as the axilla • **Botryomycosis:** It is mycetoma-like condition, characterized by subcutaneous swelling, sinuses, and discharge containing granules (Described in Chapter 52). Predisposing factors to *S. aureus* cutaneous infections are—chronic skin conditions (e.g. eczema), skin damage (trauma, injections) or poor personal hygiene
Musculoskeletal infections
S. aureus is the most common cause of various conditions such as: • Septic arthritis (most commonly involved joints are knees, shoulders, hips, and phalanges) • Osteomyelitis (most commonly affected site in children is long bones and in adults is vertebrae) • **Pyomyositis (skeletal muscle infection):** In tropics and HIV infected people • **Abscess:** Psoas abscess and epidural abscess.
Respiratory tract infections
• Ventilator-associated pneumonia in adults • Septic pulmonary emboli • Postviral pneumonia (e.g. influenza) • Empyema and pneumothorax • Pneumatocele (shaggy, thin-walled cavities in lungs) in neonates: *S. aureus* is the most common cause
Bacteremia and its complications
• Sepsis, septic shock • Central line associated blood stream infection (CLABSI) • Metastatic foci of infection involving kidney, joints, bone and lung • Infective endocarditis: ➢ Native-valve endocarditis—*S. aureus* is the most common cause ➢ Prosthetic-valve endocarditis ➢ Intravenous drug use associated endocarditis—*S. aureus* is the most common cause
UTI (Urinary tract infection)
• Staphylococcal UTI and pyelonephritis usually occur secondary to bacteremia • Rarely UTI is seen following instrumentation and insertion of catheter or implants
Toxin-mediated illnesses (Described earlier)
S. aureus causes the following toxin mediated diseases (as described earlier): • Toxic shock syndrome • Food poisoning • Staphylococcal scalded-skin syndrome
Infections associated with CA-MRSA (Community associated methicillin-resistant *Staphylococcus aureus*)
Skin and soft tissues are the most common sites for colonization of CA-MRSA strains; about 5–10% of strains are invasive and can cause various invasive infections, such as: • Necrotizing pneumonia • Sepsis with Waterhouse-Friderichsen syndrome or purpura fulminans (*S. aureus* is rare cause; most commonly caused by meningococcus). • Necrotizing fasciitis (*S. aureus* is a rare cause, *Streptococcus pyogenes* is the most common cause)

often multidrug resistant, spread to patients either from hospital staff/other patients/environment or also from patient's own endogenous flora.

LABORATORY DIAGNOSIS *Staphylococcus aureus*

- **Direct smear microscopy:** Gram-positive cocci in clusters and pus cells.
- **Culture**
  - Nutrient agar—golden yellow pigmented colonies
  - Blood agar—colonies with narrow zone of β-hemolysis
  - Selective media—such as mannitol salt agar, salt milk agar and Ludlam's medium.
- **Culture smear microscopy:** Gram-positive cocci in clusters.
- **Biochemical identification:** Catalase test-positive.
  Tests differentiating S. aureus (gives a positive result) from CoNS (gives a negative result):
  - Coagulase test (slide and tube)—positive
  - Heat stable thermo nuclease test—positive
  - DNase test—positive
  - Phosphatase test—positive
  - Mannitol sugar is fermented
  - Black-colored colonies on potassium tellurite agar
  - Gelatin liquefaction—positive
  - Protein A detection.
- **Typing methods**
  - Phenotypic methods such as bacteriophage typing and antibiogram typing
  - Genotypic methods such as PCR-RFLP.
- **Antimicrobial susceptibility testing.**

Laboratory Diagnosis

Sample Collection

It depends on the nature of the lesion (Table 21.5).

Direct Smear Microscopy

Gram staining of pus or wound swab reveals pus cells with gram-positive cocci in clusters (Fig. 21.2A). However, direct microscopy is of no value when *S. aureus* is a part of normal flora in the sample (e.g. sputum or feces).

Culture

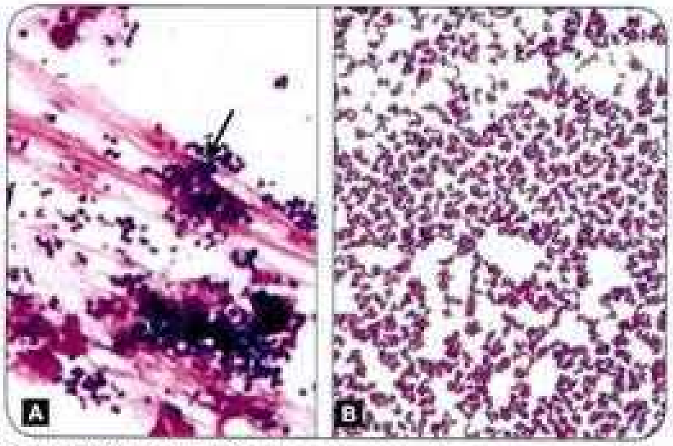
Specimens are inoculated onto various media and incubated overnight at 37°C aerobically. The colony morphology is observed as follows:

- **Nutrient agar:** Colonies are 1–3 mm in size, circular, smooth, convex, opaque and easily emulsifiable. Most strains produce golden yellow non-diffusible **pigments** (made up of β carotene) (Fig. 21.3A)
- **Nutrient agar slope:** It produces golden yellow colonies of confluent growth, looks like oil paint appearance
- **Blood agar:** Colonies are similar to that on nutrient agar, in addition surrounded by a narrow zone of β hemolysis (best observed in sheep blood agar) (Fig. 21.3B)

Table 21.5: *Specimen collection for Staphylococcus aureus infections*

Infection	Specimen
Suppurative lesions	Pus, wound swab
Respiratory infections	Sputum
Urinary tract infection	Midstream urine
FUO, bacteremia	Blood
Food poisoning	Feces, vomitus and food
Carriers	Nasal and perianal swab

Abbreviation: FUO, fever of unknown origin.



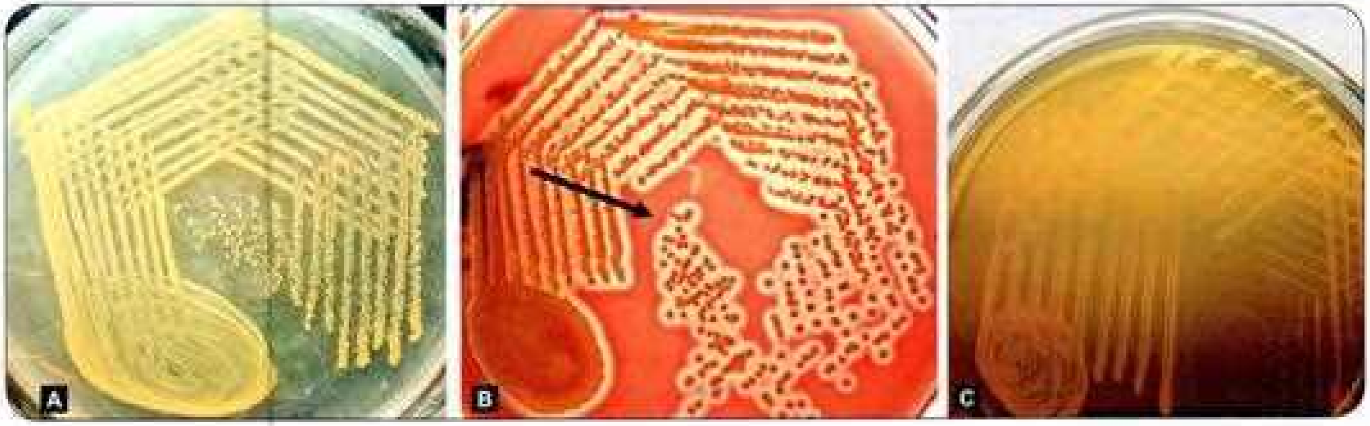
Figs 21.2A and B: A. Direct smear: arrow showing gram-positive cocci in clusters with pus cells; **B.** Culture smear showing gram-positive cocci in clusters

Source: Department of Microbiology, JIPMER, Puducherry (with permission).

- **MacConkey agar:** Small pink colonies are produced due to lactose fermentation
- **Liquid medium (e.g. peptone water):** It produces uniform turbidity
- **Selective media:** They are useful when staphylococci are expected to be scanty or outnumbered by other bacteria in the sample (e.g. swabs from carriers, feces). Salt is added to the media, as it is inhibitory to other bacteria but not to staphylococci. Examples include:
  - Mannitol salt agar contains nutrient agar with 7.5% NaCl and phenol red as an indicator. All staphylococci can grow at 7.5% salt; however, *S. aureus* produces yellow-colored colonies due to mannitol fermentation (Fig. 21.3C)
  - Salt milk agar contains nutrient agar, 6.5% NaCl and 10% skimmed milk
  - Ludlam's medium contains lithium chloride and tellurite.

Culture Smear Microscopy

Gram staining from the colonies shows Gram-positive cocci (1 μm), arranged in clusters (Fig. 21.2B). Hanging drop reveals non-motile cocci.



Figs 21.3A to C: Colonies of *S. aureus* **A.** Nutrient agar—shows golden-yellow pigmented colonies; **B.** Blood agar—arrow shows narrow zone of beta hemolysis surrounding the colonies; **C.** Mannitol salt agar shows yellow-colored colonies of *S. aureus* due to fermentation of mannitol

Source: Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (*with permission*).

Biochemical Tests for Identification

Catalase Test

All members of Micrococcaceae (staphylococci and micrococci) are catalase positive, which differentiates them from Streptococcaceae (catalase negative).

Hugh and Leifson Oxidative Fermentative Test

This test differentiates staphylococci (shows fermentative pattern) from micrococci (shows oxidative pattern).

Tests to Differentiate *S. aureus* from CoNS

S. aureus can be differentiated from CoNS (coagulase-negative staphylococci) by various tests (as described in the laboratory diagnosis box), of which the coagulase test is most important (see Table 21.3).

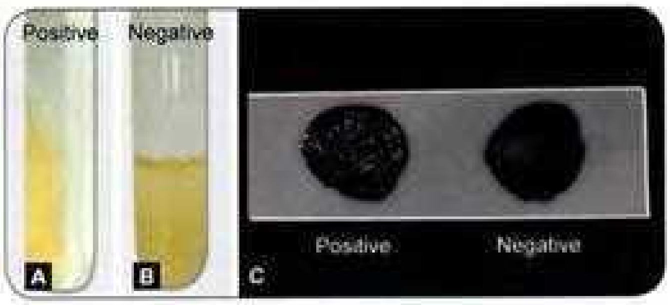
Coagulase Test

It is the most commonly used biochemical reaction for identification of *S. aureus*.

Tube Coagulase Test

It detects free coagulase secreted by *S.aureus*.

- **Procedure:** Colony of *S. aureus* is emulsified in 1 mL of diluted plasma (1:6) in a test tube and incubated at 37°C, preferably in a water bath for up to 4 hours
- **Positive test** is indicated by formation of a clot that does not flow when the test tube is tilted (Fig. 21.4A). Any amount of clot formation is considered as positive
- The **negative tubes** (no clot formation) should be incubated overnight and re-examined as some strains may produce a delayed clot (Fig. 21.4B)
- **False-positive**: Citrated plasma should not be used as some bacteria (e.g. *Pseudomonas*) may utilize citrate and give a false positive result. Heparin or EDTA are the preferred anticoagulants.



Figs 21.4A to C: Coagulase test: **A.** Tube coagulase test (positive); **B.** Tube coagulase test (negative); **C.** Slide showing coagulase test

Source: Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (*with permission*).

Slide Coagulase Test

It detects clumping factor (i.e. bound coagulase).

- **Procedure:** A colony of *S. aureus* is emulsified with a drop of normal saline on a clean slide to form a milky white suspension. Then a loopful of undiluted plasma is added and mixed properly
- Positive result is indicated by formation of coarse clumps (Fig. 21.4C)
- Results should be confirmed by the tube coagulase test as ≥15% of *S. aureus* strains (including some MRSA) give false-negative results. At the same time, few CoNS, such as *S. lugdunensis* give a positive result.

DNase Test

On DNA agar, a clear halo is produced surrounding the colonies of *S. aureus*, due to its ability to digest DNA.

Phosphatase Test

This test is positive for *S. aureus*, *S. epidermidis* and *S. xylosus*. Organism is inoculated on phenolphthalein diphosphate containing media and later the colonies grown are exposed to ammonia vapor (see below).

S. aureus → splits phenolphthalein diphosphate in the media → releases free phenolphthalein → reacts with ammonia vapors → colonies turn pink.

Typing of S. aureus

Typing of *S. aureus* to subspecies level is done for epidemiological purpose to trace the source of infection. It is especially useful in outbreaks such as food poisoning affecting a larger community. Typing methods include both:

- Phenotypic methods such as bacteriophage typing and antibiogram typing
- Genotypic methods such as PCR-RFLP (restricted fragment length polymorphism), ribotyping, PFGE (pulse field gel electrophoresis) and sequence based typing.

Bacteriophage Typing

Strains of *S. aureus* can be further differentiated into subspecies level based on their susceptibility to bacteriophages (pattern method of phage typing).

- **Phage type 80/81** is most commonly associated with outbreaks in hospitals. It is known as epidemic strain of *S. aureus*
- With the advent of molecular typing methods, phage typing has become obsolete nowadays
- Refer author's first edition for detail procedure.

Antimicrobial Susceptibility Test

As *S. aureus* develops resistance to antibiotics readily, drugs should be prescribed according to the antimicrobial susceptibility test done on Mueller Hinton agar.

Drug Resistance in *S. aureus* (Resistance to β lactam antibiotics)

Staphylococcus aureus shows resistance to β lactam antibiotics in various way.

Production of β Lactamase Enzyme

β lactamase or penicillinase enzymes cleave the β lactam rings, and there by organisms producing these enzymes develop resistance to β lactam antibiotics.

- This resistance is **plasmid coded**, can be transferred between *S. aureus* strains by **transduction**
- It is produced by >90% of strains of *S. aureus*
- This resistance can be overcome by addition of β lactamase inhibitors such as clavulanic acid or sulbactam.

By Alteration of Penicillin-Binding Protein (PBP)

It is shown by MRSA strains of *S. aureus*.

TREATMENT ***Staphylococcus aureus***

Since *S. aureus* rapidly develops drug resistance, antibiotics should be cautiously chosen.

Parenteral therapy for serious infections

Sensitive to pencillin	**DOC:** Penicillin G
Methicillin sensitive *S. aureus* (MSSA)	**DOC:** Anti-staphylococcal penicillins such as nafcillin and cloxacillin *Note:* Vancomycin is inferior in terms of efficacy against MSSA, when compared to anti-staphylococcal penicillins; hence should not be prescribed for MSSA
Methicillin resistant *S. aureus* (MRSA)	**DOC:** Vancomycin (15–20 mg/kg bd) **Alternate drugs:** ❑ Teicoplanin ❑ Daptomycin (for endocarditis and complicated skin infections, not used for pneumonia) ❑ Linezolid and telavancin ❑ Quinupristin/dalfopristin *Note:* All β lactam drugs should be avoided except newer cephalosporins (e.g. ceftobiprole)

Empirical therapy (if MRSA status not yet known): Vancomycin with/without an aminoglycoside (vancomycin is indicated only if MRSA risk is high or condition is serious, e.g. cardiac implant).

Oral therapy for skin and soft tissue infections

Sensitive to methicillin	❑ Dicloxacillin ❑ Cephalexin/cefazolin
Resistant to methicillin (MRSA)	Clindamycin **Alternate drugs:** Cotrimoxazole, doxycycline, Linezolid

Abbreviations: DOC, drug of choice; bd, twice a day.

Methicillin-resistant Staphylococcus aureus (MRSA)

Methicillin resistance in *S. aureus* is mediated by a chromosomally coded gene called ***mec A gene***, which alters penicillin-binding protein (PBP) present on *S. aureus* cell membrane to PBP-2a.

- PBP is an essential protein needed for cell wall synthesis of bacteria. β lactam drugs bind and inhibit this protein, there by inhibit the cell wall synthesis
- The altered PBP2a of MRSA strains has less affinity for β lactam antibiotics; hence, MRSA strains are resistant to all β lactam antibiotics
- Recently, *mec C* gene (coding for PBP-2c) has also been found to be associated with MRSA
- **Borderline oxacillin resistant *S. aureus* (BORSA) strains:** Occasionally a non-*mec A* gene mediated low level resistance to oxacillin is observed in some strains of

Table 21.6: Types of MRSA

Community-associated MRSA (CA-MRSA)	Hospital-associated MRSA (HA-MRSA)
These strains express *mecA* gene subtype IV, V, VI	These strains express *mecA* gene subtype I, II, III
They are usually more virulent and express several toxins such as Panton Valentine (PV) toxin	They are multidrug resistant (but their virulence is relatively low)
They cause invasive skin and soft tissue infections such as necrotizing fasciitis (see Table 21.4)	They cause perioperative wound infections in hospitals and nosocomial outbreaks (hospital staff are the major carries)

Note: CA-MRSA and HA-MRSA terminologies are losing their relevance; as many CA-MRSA strains have been isolated in hospitals and vice versa.

S. aureus. This is believed to be due to hyperproduction of β lactamase

- **Prevalence:** MRSA infection rate has been increasing over last few decades, though it varies from place to place. MRSA rates are higher (>50%) in America, some Asian and European countries and Malta. Countries with lowest MRSA rates are Netherlands and Scandinavia (<1%). In India, the MRSA rate is around 30–40%; though varies between years and place.

Types of MRSA

MRSA are either community or hospital associated (Table 21.6).

Detection of MRSA

- **Antimicrobial susceptibility test:** Disk diffusion test can be done by using **cefoxitin** disc
- **Oxacillin screening agar:** Adding oxacillin 6 µg/mL and NaCl (2–4%) to the Mueller Hinton agar and incubated at 30°C for full 24 hours
- PCR detecting *mecA* gene
- Latex agglutination test detecting PBP-2a.

TREATMENT — MRSA

- Vancomycin is the drug of choice for MRSA
- Alternate drugs include—teicoplanin, linezolid, daptomycin, telavancin and quinupristin/dalfopristin
- However, even drugs such as tetracycline, erythromycin or cotrimoxazole may also be effective in non life-threatening infections
- Antimicrobial susceptibility testing is necessary before an alternative drug is used
- For nasal carriers of MRSA, mupirocin (2%) ointment is used.

All β lactam drugs should be avoided. However, 5th generation cephalosporins, such as ceftobiprole, ceftaroline and ceftolozane have shown some activity against MRSA.

Resistance to Vancomycin (VRSA and VISA)

Erroneous and overuse of vancomycin has lead to the emergence of resistance to vancomycin. It may be of low grade resistance, known as VISA (vancomycin intermediate *S. aureus*) or high-grade resistance, known as VRSA (vancomycin-resistant *S. aureus*).

- The MIC (minimum inhibitory concentration) of vancomycin to VISA and VRSA isolates are 4–8 and >8 µg/mL respectively
- **MIC creep:** It is observed that the vancomycin MIC for susceptible strains of *S. aureus* has been gradually increasing over time (known as MIC creep); which indicates that the frequency of VISA and VRSA is likely to increase in future. Current guidelines recommend consideration of alternative drugs if vancomycin MIC is >1 µg/mL as treatment failure has been frequently observed beyond this MIC level
- VRSA is very rare. In India, it is reported from few places such as Hyderabad, Kolkata and Lucknow. However, VISA is more frequently reported than VRSA
- **Mechanisms:** VRSA is mediated by *van A* gene; whereas VISA is due to increase in cell wall thickness of *S. aureus*. The *van A* gene is believed to be acquired from a vancomycin-resistant strain of *Enterococcus faecalis* by horizontal conjugal transfer
- **Fitness cost:** Acquisition of a *van* gene is often associated with compensatory mutations in the genes responsible for survival which results in a reduced fitness of *S. aureus*. In contrast, 'fitness cost phenomena' is not commonly observed in MRSA. This explains why VRSA is seen very rarely (<0.1%), whereas MRSA prevalence is so common (30–40%)
- **Treatment** of VRSA should be based on antimicrobial susceptibility report. Linezolid, telavancin, daptomycin and quinupristin/dalfopristin are the effective drugs.

Control Measures

Prevention of spread of *S. aureus* infections in hospitals involves:

- **Screening of MRSA carriers** among hospital staff should be done when there is an outbreak. Mannitol oxacillin agar is the preferred media for this purpose
- **Treatment of carriers** is done by use of topical mupirocin (for nasal carriers) and chlorhexidine (for skin carriers)
- **Stoppage of antibiotic misuse** in hospitals
- Ensure proper infection control measures such as **hand hygiene** (most efficient way to prevent hospital spread), isolation of the patients and all other measures of contact precautions (described in detail in Chapter 53).

COAGULASE-NEGATIVE STAPHYLOCOCCI

Most of the coagulase-negative staphylococci (CoNS) are harmless commensals and less virulent than *S. aureus*; however, recently their role as pathogen is increasingly been reported.

Table 21.7: Tests for identification of common *Staphylococcus* species

Properties	*S. aureus*	*S. epidermidis*	*S. saprophyticus*	*S. lugdunensis*
Coagulase (tube)	+	–	–	–
Clumping factor	+	–	–	+
Heat stable thermonuclease	+	–	–	–
Phosphatase	+	+	–	–
Novobiocin	S	S	R	S
Urease	V	+	+	V
PYR	–	–	–	+
Ornithine decarboxylation	–	–	–	+

Abbreviation: PYR, pyrrolidonyl-beta-naphthylamide.

Staphylococcus epidermidis

It is the most common CoNS (75–80%), isolated from clinical samples. It is present as normal flora on the skin, oropharynx and vagina; however, its pathogenic role is greatly enhanced in presence of prosthetic-devices.

- **Pathogenesis:** *S. epidermidis* involves a two-step process:
  1. **Initial adhesion to the prosthetic device:** The surface adhesins of the organism bind to host serum or tissue constituents, such as fibrinogen or fibronectin, coated on the implanted prosthetic surfaces
  2. **Colonization:** *S. epidermidis* can produce the extracellular polysaccharide material (glycocalyx or slime) that facilitates formation of a protective **biofilm** on the device surfaces. Biofilm appears to act as a barrier, protecting bacteria from host defense mechanisms as well as from antibiotics
- **Manifestation:** *S. epidermidis* is the most common cause of prosthetic-device related infections, such as endocarditis with insertion of valvular prosthesis and ventricular shunt infections. It is also a common cause of stitch abscess.
- It is coagulase negative, but positive for phosphatase test.

Staphylococcus saprophyticus

It causes urinary tract infection (UTI) in sexually active young women. This is due to expression of a 160 kDa hemagglutinin/adhesin protein that can adhere to uroepithelial cells. It can be differentiated from other staphylococci in being **resistant to novobiocin disk** (5 µg).

Staphylococcus lugdunensis and Staphylococcus schleiferi

Recently, these organisms have been associated with more serious infections such as native-valve endocarditis and osteomyelitis. Their enhanced pathogenesis may be due to expression of virulence factors such as clumping factor and lipase which are usually absent in other CoNS.

Laboratory Diagnosis of CoNS

Various species of CoNS can be differentiated from each other and also from *S. aureus* by various biochemical tests (Table 21.7). Treatment is same as that of *Staphylococcus aureus*.

EXPECTED QUESTIONS

I. Essay:

1. A 55-year-old male was admitted to the hospital with complaints of severe pain in the lateral aspect of his left calf and small amount of pus discharge from the ingrown hair. On physical examination, the local area was found to be red, warm and tender. Pus was aspirated and was subjected to Gram stain (showed gram-positive cocci in clusters), culture on blood agar (showed golden yellow pigmented beta hemolytic colonies).
   a. What is the clinical diagnosis and its causative organism?
   b. List the infections caused by this organism.
   c. List the virulence factors of this organism.
   d. Briefly discuss the laboratory diagnosis.

II. Write short notes on:

1. Toxic shock syndrome
2. Staphylococcal food poisoning
3. MRSA (Methicillin-resistant *Staphylococcus aureus*)

III. Multiple Choice Questions (MCQs):

1. **Scalded skin syndrome is mediated by:**
   a. Hemolysin b. Coagulase
   c. Enterotoxin d. Epidermolytic toxin
2. ***Staphylococcus aureus* causes vomiting in 6–8 hours. The mechanism of action is by:**
   a. Stimulation of cAMP
   b. Vagal stimulation
   c. Stimulation of cGMP
   d. Acts through ganglioside GM receptor

3. **A patient has prosthetic valve replacement and he develops endocarditis 8 months later. Organism responsible is?**
   a. *Staphylococcus aureus*
   b. Viridans streptococci
   c. *Staphylococcus epidermidis*
   d. HACEK
4. **Which hemolysin of *S. aureus* shows hot-cold phenomenon?**
   a. α b. β
   c. γ d. δ
5. **Which hemolysin of *S. aureus* is inactivated at 70°C but again reactivated paradoxically at 100°C?**
   a. α b. β
   c. γ d. δ
6. **Synergohymenotropic toxins includes:**
   a. α-hemolysin and panton valentine toxin
   b. β-hemolysin and panton valentine toxin
   c. γ-hemolysin and panton valentine toxin
   d. α-hemolysin and γ-hemolysin
7. **Coagglutination reaction is mediated by which component of *S. aureus*?**
   a. *Mec A* b. Protein A
   c. Coagulase d. Clumping factor
8. ***Staphylococcus aureus* enterotoxin, all are true, *except*:**
   a. Preformed toxin
   b. Incubation period is short (1–6 hours)
   c. The toxin stimulates the vagus nerve and the vomiting center of the brain
   d. Treatment is mainly by early institution of antibiotics
9. **Tube coagulase differs from slide coagulase by all, *except*:**
   a. Requires coagulase reacting factor in plasma
   b. *S. lugdunensis* gives a positive result
   c. Positive result is indicated by clot formation
   d. Coagulase enzyme has eight subtypes
10. **CA-MRSA strains are increasingly associated with all of the following, *except*:**
    a. Necrotizing pneumonia
    b. Waterhouse-Friderichsen syndrome
    c. Necrotizing fasciitis
    d. Toxic shock syndrome
11. ***S. aureus* is differentiated from CoNS by all, *except*:**
    a. Coagulase test b. DNase test
    c. Catalase test d. Protein A detection
12. **Which sugar fermentation test differentiates *S. aureus* from CoNS?**
    a. Glucose b. Sucrose
    c. Lactose d. Mannitol
13. **All of the above can be given for the treatment of MRSA, *except*:**
    a. Meropenem b. Vancomycin
    c. Cotrimoxazole d. Linezolid
14. **All the following beta lactam drugs can be given for the treatment of MRSA, *except*:**
    a. Ceftaroline
    b. Ceftobiprole
    c. Piperacillin-tazobactum
    d. Ceftolozane
15. **Which is the least preferred antimicrobial for the treatment of methicillin-sensitive *S. aureus* (MSSA)?**
    a. Dicloxacillin b. Cephalexin
    c. Cefazolin d. Vancomycin
16. **About MRSA all are true, *except*:**
    a. In India, the MRSA prevalence in hospital is around 30–40%
    b. MRSA rates are higher in India than in America
    c. Mediated by *Mec A* gene
    d. Cefoxitin disk is superior to oxacillin for detection
17. **About VRSA all are true, *except*:**
    a. VRSA is mediated due to *Van* gene
    b. VISA is due to increased cell wall thickening
    c. VRSA is more common than VISA
    d. Fitness cost phenomena is seen in VRSA
18. **Borderline oxacillin resistant *S. aureus* (BORSA) strains, the mechanism of resistance is due to:**
    a. *Mec A* gene mediated
    b. Alteration of penicillin binding protein
    c. Hyperproduction of β lactamase
    d. *Van* gene mediated
19. **Community-associated MRSA (CA-MRSA) differs from hospital-associated MRSA by all, *except*:**
    a. These strains express *mec A* gene subtype IV, V, VI
    b. Express more Panton Valentine (PV) toxin
    c. They cause more invasive skin and soft tissue infection
    d. Multidrug resistant
20. ***Staphylococcus epidermidis*, all are true, *except*:**
    a. Accounts for 75% of CoNS
    b. Phosphatase negative
    c. Produces biofilm
    d. Causes stitch abscesses
21. **Positive tube coagulase test is a property of all the following species of *Staphylococcus*, *except*:**
    a. *S. aureus* b. *S. hyicus*
    c. *S. intermedius* d. *S. lugdunensis*

Answers

1. d **2.** b **3.** c **4.** b **5.** a **6.** c **7.** b **8.** d **9.** b **10.** d **11.** c **12.** d **13.** a **14.** d **15.** d **16.** b **17.** c **18.** c **19.** d **20.** b **21.** d

Streptococcus, Enterococcus and Pneumococcus

22
CHAPTER

Chapter Preview

- *Streptococcus pyogenes*
- Other β hemolytic streptococci
- *Enterococcus* species
- Viridans streptococci
- *Streptococcus pneumoniae*

INTRODUCTION

Family Streptococcaceae are catalase negative gram-positive cocci, arranged in pairs or chains (due to single plane of division). *Streptococcus*, *Enterococcus* and pneumococcus are the important members of this family. However, according to the molecular structure, *Enterococcus* is now reclassified under separate family Enterococcaceae.

Streptococci are part of normal flora. However, some are important human pathogens, such as *Streptococcus pyogenes* causing pyogenic infections, *S. agalactiae* causing meningitis in newborn and *S. pneumoniae* causing pneumonia and meningitis in all age groups.

History

Billroth coined the term 'streptococci' (*streptos* meaning twisted or coiled), Ogston differentiated them from staphylococci and Rosenbach coined the species *S. pyogenes;* as it causes pyogenic infection.

Classification

Streptococci can be classified into—obligate anaerobes (e.g. *Peptostreptococcus* described in Chapter 26) and aerobes and facultative anaerobes group. The latter can be further classified based on the hemolysis produced on 5% sheep blood agar into α, β and γ hemolytic streptococci (Fig. 22.1).

- **α hemolysis**: It is due to partial lysis of red blood cells (RBCs), producing a small (1-2 mm) zone of greenish discoloration surrounding the colonies. It is observed with viridans streptococci and pneumococci
- **β hemolysis:** It is due to complete lysis of RBCs and zone of lysis is wide (2-4 mm). It is observed with *S. pyogenes* and other β hemolytic streptococci
- **γ hemolysis**: It is a misnomer, there is no hemolysis surrounding the colonies, hence no change in color, e.g. *Enterococcus*.

Lancefield grouping: The β hemolytic streptococci can be further classified by **Rebecca Lancefield** (1933) based on C-carbohydrate antigen present in the cell wall into 20 serological groups named as group A-V (except I and J).

Griffith typing: Majority of streptococci causing human infections belong to group A (*S. pyogenes*), which can be

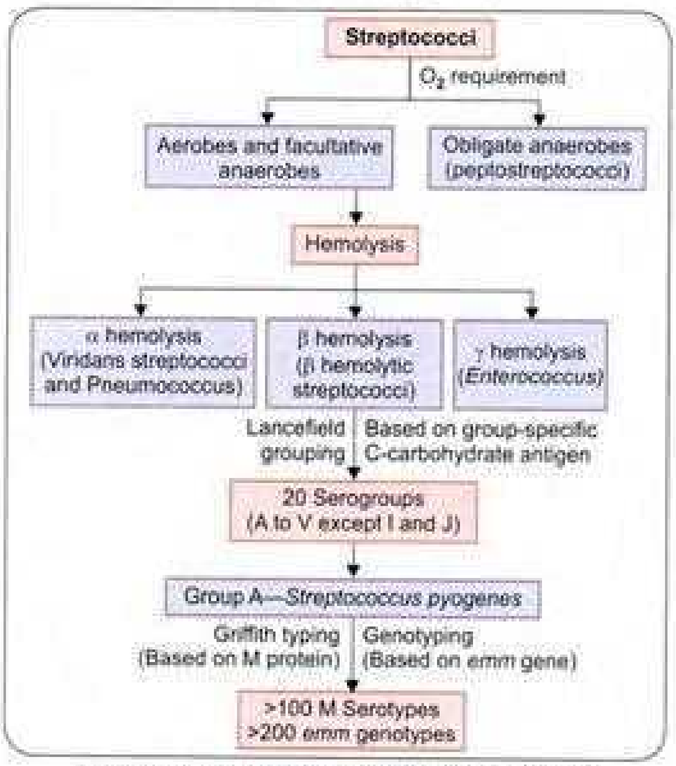


Fig. 22.1: Classification of family Streptococcaceae

further classified into more than 100 serotypes based on M protein present in their cell wall.

Genotyping: Based on *emm* gene (gene encoding M protein); group A streptococci can be typed into >200 genotypes.

STREPTOCOCCUS PYOGENES

S. pyogenes is the only species under Lancefield's group A *Streptococcus* (GAS). It is associated with a variety of suppurative infections and can also trigger post-infectious nonsuppurative complications such as acute rheumatic fever and acute glomerulonephritis.

Virulence Factors and Pathogenicity

Virulence factors of *S. pyogenes* can be categorized into cell wall antigens, toxins and enzymes.

Cell Wall Antigens

Cell wall of *S. pyogenes* is composed of (Fig. 22.2):

- Inner **thick peptidoglycan layer**: It confers cell wall rigidity, induces inflammatory response and has thrombolytic activity
- Middle layer of group specific **C-carbohydrate antigen**
- Outer layer of **protein** and **lipoteichoic acid** (helps in adhesion).

Outer Protein Layer

Several protein antigens such as M, T and R proteins have been identified in the outer protein layer.

M protein: It is the principle virulence factor of group A *Streptococcus*.

- It inhibits phagocytosis (by inhibiting opsonization via alternate complement pathway)
- It binds to fibrinogen which together bind to β2 integrins of neutrophils leading to release of inflammatory mediators that induce vascular leakage; causing streptococcal toxic shock syndrome
- Antibody to M protein is protective in nature and promotes phagocytosis
- Based on M protein (especially, its variable amino terminal end), GAS can be typed into around 100 serotypes
- M protein is further divided into two classes—Class I and Class II. **Antibodies to class I M protein** are responsible for pathogenesis of rheumatic fever.

T and R proteins are other outer proteins of GAS; they are not associated with pathogenesis.

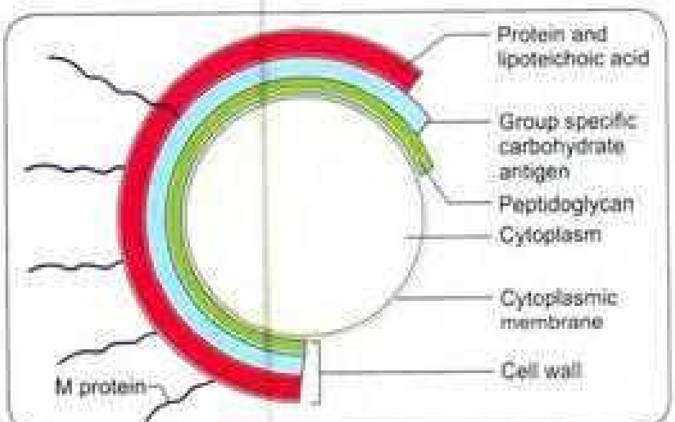


Fig. 22.2: Cell wall of *Streptococcus pyogenes*

Other Cell Wall Proteins

Other cell wall proteins include:

- M associated protein
- Hair-like fimbriae (consist of M protein along with teichoic acid)—project from the cell wall and help in adhesion
- F factor (fibronectin binding protein) helps in adhesion.

Capsule

Some strains of group A *Streptococcus* are capsulated, made up of hyaluronic acid. These strains produce mucoid colonies. Capsule is antiphagocytic, but not antigenic. It helps group A streptococci to colonize the pharynx by binding to CD44, a hyaluronic acid-binding protein expressed on human pharyngeal epithelial cells.

Toxins

Hemolysins

β hemolytic streptococci such as group A, C and G produce two hemolysins—streptolysin-O and streptolysin-S (Table 22.1). They cause RBC membrane lysis, that leads to complete β hemolysis surrounding the colonies.

Streptococcal Pyrogenic Exotoxin (SPE)

It is so named because it induces fever (pyrogenic). It is responsible for the pathogenesis of certain streptococcal infections such as scarlet fever, necrotizing fasciitis and toxic shock syndrome.

- It can be typed into three antigenic distinct subtypes—SPE-A, B and C
- SPE-A and C are bacteriophage coded; whereas SPE-B is chromosomally mediated
- SPE-A and C are superantigens; like staphylococcal toxin (TSST-1), they also act as T cell mitogens which induce a massive release of cytokines causing fever, shock and tissue damage
- **Dick test:** SPE was previously called as **erythrogenic** or **scarlet fever** toxin because its intradermal injection in susceptible children produced local erythema.

Table 22.1: Differences between streptolysin-O and streptolysin-S

Streptolysin (SL-O)	Streptolysin (SL-S)
• Oxygen labile (hence named as streptolysin-O) • Heat labile	• Oxygen stable • Serum soluble (hence named as streptolysin-S)
Hemolysis is seen only in deep colonies (pour plate) as it is inactivated in presence of oxygen	Causes hemolysis on the surface of blood agar plate
It is cytotoxic for neutrophils, platelets and cardiac tissue	It has leukocidal activity
Strongly antigenic	Not antigenic
Antistreptolysin-O antibodies (ASO) are raised in most of the streptococcal infections and are used as a standard marker for retrospective diagnosis of streptococcal infections (except in glomerulonephritis and pyoderma where ASO titer is low)	Not useful for serological diagnosis of streptococcal infections
Streptolysin-O is structurally and functionally similar to: • Tetanolysin of *Clostridium tetani* • Pneumolysin of *S. pneumoniae* • Theta toxin of *Clostridium perfringens* • Listeriolysin O of *Listeria* • Cereolysin of *Bacillus cereus*	

This test was previously used to identify the individuals susceptible to scarlet fever.

Enzymes

Streptokinase (Fibrinolysin)

It activates plasminogen to plasmin, thus breaks down the fibrin barrier around the infected site, there by facilitating the spread of infection.

- Antibodies to streptokinase can be used for retrospective diagnosis of streptococcal infection
- **Therapeutic use:** Being fibrinolytic, this toxin can be used in the treatment of myocardial infarction and other thromboembolic disorders.

Streptodornase (DNase)

It breaks down the DNA, thus helps in liquefying the thick pus (containing large amount of DNA derived from nuclei of necrotic cells) and may be responsible for the serous nature of streptococcal exudates.

- **Therapeutic use:** Preparation containing streptodornase and streptokinase can be used to liquefy the thick exudates in empyema cases
- **Subtypes:** Streptodornase has four distinct subtypes DNase-A, B, C, and D; of which type-B is most antigenic
- **Diagnostic use:** Anti-DNase B antibodies can be used for retrospective diagnosis of the infection, particularly the skin infections (pyoderma) and acute glomerulonephritis where ASO titer is low.

Other Enzymes

- **Hyaluronidase (spreading factor):** It breaks down the hyaluronic acid present in tissues; thus helps in the spread of infection along the intercellular space. It is usually secreted by noncapsulated strains (such as M type 2 and 22)
- **NADase:** It acts on the coenzyme NAD (nicotinamide adenine dinucleotide). It is produced by group A, C and G. It is antigenic and leukotoxic
- **Serum opacity factor:** It is a lipoproteinase produced by a few M serotypes of *S. pyogenes*, causes opacity when applied on agar gel containing serum
- **SpyCEP:** It is a serine protease that inactivates interleukin 8, which is a neutrophil chemoattractant
- **C5a peptidase:** It is a serine protease that cleaves C5a; which is also a neutrophil chemoattractant
- Others include neuraminidase, N-acetyl glucosaminidase, esterase and phosphatase. Their pathogenic role is uncertain.

Clinical Manifestations

Group A *Streptococcus* (GAS) produces both suppurative and non-suppurative manifestations (Table 22.2).

Suppurative Complications

Respiratory Infections

Throat is the primary site of invasion by GAS. Infection occurs through respiratory droplets.

Pharyngitis (Sore Throat)

Sore throat is the most common streptococcal disease; may be localized (tonsillitis) or diffuse (pharyngitis).

- GAS is the most common cause of pharyngitis in children (20–40% of all cases)
- It is characterized by erythema and swelling of pharyngeal mucosa with purulent exudate formation
- Younger children (<3 years) manifest with a syndrome of fever, malaise, and lymphadenopathy without exudative pharyngitis
- Complications occur due to spread of infection from the pharynx to deeper tissues by direct extension, hematogenous or lymphatic routes which may lead to quinsy (peritonsillar abscess), sinusitis, otitis media, meningitis, bacteremia and postviral pneumonia.

Scarlet Fever

Scarlet fever is mediated due to streptococcal toxins such as SPE-A, B, and C. It is characterized by pharyngitis, with:

Table 22.2: Suppurative and non-suppurative manifestations of *Streptococcus pyogenes*

Suppurative	Non-suppurative
Respiratory infections: • Pharyngitis/sore throat • Pneumonia • Empyema	Acute rheumatic fever
Scarlet fever	Acute glomerulonephritis
Skin and soft tissue infections: • Impetigo (pyoderma) • Cellulitis and erysipelas	Guttate psoriasis Reactive arthritis
Deep soft tissue infections: • Necrotizing fasciitis • Streptococcal myositis • Toxic shock syndrome	PANDAS (Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal infections)
Bacteremia leading to toxic shock syndrome, osteomyelitis, meningitis, etc.	
Complications: Puerperal sepsis, otitis media, quinsy, Ludwig's angina, pneumonia (postviral), etc.	

- **Characteristic rash with sandpaper feel:** Rashes may be due to direct action of the circulating toxin or as a result of hypersensitivity reaction
- Strawberry tongue (enlarged papillae on a coated tongue)
- Rash in skin folds (called Pastia's lines).

Scarlet fever has become less common now, although strains producing SPE continue to be prevalent in the community. Reasons are not clear.

Skin and Soft Tissue Infections

Impetigo

It is a superficial infection of the skin, caused primarily by group A *Streptococcus* and occasionally by other streptococci or *S. aureus*.

- Risk factors include young children, warmer months, tropical climates, poor hygiene, colonization of group A *Streptococcus* and minor trauma
- Most common sites involved are face (nose and mouth) and legs
- Individual lesions begin as red papules, which evolve quickly into vesicles and then pustular lesions that break down and coalesce to form characteristic thin papery **honeycomb-like crusts** (Fig. 22.3A). Lesions are painless and not associated with fever.

Cellulitis

It is an infection involving the skin and subcutaneous tissue.

Erysipelas: It is a form of cellulitis, characterized by a tender, bright red, swollen and indurated peau d'orange texture of involved skin (due to involvement of the superficial

Figs 22.3A and B: Streptococcal skin infections. **A.** Impetigo; **B.** Erysipelas on malar area of face (peau d'orange skin)

Source: A. wikipedia/Åsa Thorn, B. Public Health Image Library, Atlanta, ID# 2874/ Dr. Thomas F. Sellers, Emory University/Centers for Disease Control and Prevention (CDC) (with permission).

lymphatics) along with fever and chills. Superficial blebs or bullae may form later.

- Most common sites are malar area of the face and the lower extremities (Fig. 22.3B)
- Recurrences are common, occur after many years, involving the same site.

Deep Soft Tissue Infections

Necrotizing Fasciitis

It is also known as **hemolytic streptococcal gangrene**. It involves the superficial and/or deep fascia invading the muscles (Fig. 22.4).

- **Source of the infection may be of two types:**
  1. Traumatized skin: Most commonly caused by group A *Streptococcus* alone or in mixture with *S. aureus*
  2. Gastrointestinal tract breach: It occurs due to abdominal surgery releasing the bowel flora. It is polymicrobial, involving anaerobic flora and gram-negative bacilli like *E. coli*.
- GAS is the most common cause, accounting for nearly 60% of total cases of necrotizing fasciitis. Common serotypes include M types 1 and 3 which produce streptococcal pyrogenic exotoxins
- The onset is acute and rapid, and is marked by severe pain with minimal erythema at the site of involvement. Patients present with malaise, fever, chills, and a toxic appearance in contrast to cellulitis, where the skin appears more abnormal, but tenderness is mild
- Later on (over several hours), disease tends to be more severe. Skin becomes dusky or develops mottled erythema and anesthetized (due to infarction of the cutaneous nerves induced by spreading inflammatory process) with extensive necrosis of subcutaneous tissue, fascia and muscle (Hence, GAS is also called as **flesh eating bacteria**).

Fig. 22.4: Necrotizing fasciitis of leg

Source: Department of Microbiology, JIPMER, Puducherry (with permission).

TREATMENT **Necrotizing fasciitis**

- It involves early drainage of inflammatory fluid and debridement of involved necrotic area along with antibiotics
- The drug of choice is penicillin G plus clindamycin.

Bacteremia

Streptococcal bacteremia occurs secondary to necrotizing fasciitis, rarely with pharyngitis or cellulitis or pneumonia. It leads to variety of focal infections including endocarditis, meningitis, septic arthritis, osteomyelitis, peritonitis, visceral abscesses and toxic shock syndrome.

Toxic Shock Syndrome (TSS)

Group A *Streptococcus* producing pyrogenic exotoxins may cause TSS secondary to soft tissue infection such as necrotizing fasciitis.

- In contrast to patients with staphylococcal TSS, the majority with streptococcal TSS are bacteremic
- The case definition of TSS includes: (i) isolation of *S. pyogenes* plus (ii) hypotension plus (iii) multiorgan (≥2) involvement.

Puerperal Sepsis

Being colonizer of female vagina, streptococci are often associated with infectious complications of childbirth, usually endometritis and associated bacteremia. Group B streptococci and anaerobic streptococci are more common to cause puerperal sepsis than GAS.

Non-suppurative Complications

Streptococcal antigens show molecular mimicry with human antigens (Table 22.3). Due to antigenic cross reactivity, antibodies produced against previous streptococcal infections cross react with human tissue to produce lesions. This accounts for a number of non-suppurative complications such as:

- Acute rheumatic fever
- Post-streptococcal glomerulonephritis (PSGN)
- Guttate psoriasis
- Reactive arthritis
- Pediatric Autoimmune Neuropsychiatric Disorders Associated with *Streptococcus pyogenes* (PANDAS).

Table 22.3: Antigenic cross reactivity between streptococcal antigens and the corresponding human antigens

Streptococcal antigen	Human antigen
Cell wall **M** protein (of serotypes M1, M5, M6, and M19)	**M**yocardium (tropomyosin and myosin)
Cell wall **C** carbohydrate	**C**ardiac valves
Cytoplasmic membrane	Glomerular vascular intima
Peptidoglycan	Skin antigens
Hyaluronic acid	Synovial fluid

Acute Rheumatic Fever

Acute rheumatic fever (ARF) occurs in people previously infected with streptococcal sore throat.

- **Pathogenesis:** It is unclear. It may be due to:
  - **Autoimmune theory:** Streptococcal antibodies cross react with the human tissue antigens (e.g. heart and joint)
  - **Cytotoxicity theory:** Streptococcal toxins (e.g. SPE) and enzymes (e.g. SL-O) are directly cytotoxic for human cardiac cells.
- **Clinical manifestations and laboratory diagnosis:**
  - It affects heart, joints, and skin. The cardiac lesions include degeneration of heart valves and formation of inflammatory myocardial lesions called as **Aschoff nodules**
  - Acute rheumatic fever is diagnosed by modified Jones criteria (Table 22.4)
  - Prognosis is variable, repeated attacks are common, hence long-term penicillin prophylaxis is indicated
  - The ASO titer is much higher in patients with ARF than that seen in patients with group A *Streptococcus* infections without ARF.

Post-streptococcal Glomerulonephritis (PSGN)

PSGN typically occurs 2–3 weeks following either pyoderma (usually by M serotypes-49, 53-55 and 59-61) or rarely following pharyngitis (caused by M serotypes 1 and 12) (Table 22.5).

- PSGN results from lodging of antigen antibody complexes on the glomerular basement membrane, followed by complement activation. As a result urine retention and renal insufficiency occurs that leads to edema, hypertension, hematuria and proteinuria

Table 22.4: Revised Jones criteria for acute rheumatic fever

Criteria	Manifestations
Major manifestations	Subcutaneous nodules Pancarditis Arthritis (migrating polyarthritis) Chorea (CNS manifestation) Erythema marginatum (skin lesion)
Minor manifestations	• Clinical: Fever, arthralgia • Laboratory: Elevated ESR and C-reactive protein • ECG: Prolonged P-R interval
Supporting evidence (of previous streptococcal infection)	Elevated ASO, or A positive throat culture, or Rapid antigen test for GAS, or Recent scarlet fever
Rheumatic fever is diagnosed if:	Two major manifestations or one major and two minor manifestations plus Any one evidence of previous streptococcal infection

Abbreviations: CNS, central nervous system; ESR, erythrocyte sedimentation rate; ECG, electrocardiography; ASO, antistreptolysin O; GAS, group A *Streptococcus*.

- Patients usually have elevated streptococcal anti-DNase B antibodies
- PSGN usually occurs in children (5-12 years) and has a good prognosis.

Epidemiology

Humans are the natural reservoir for group A *Streptococcus*. It is highly communicable, affecting all age groups. Disease in neonates is uncommon, due to protective maternal antibody. Pharyngitis is more common in children of 3-15 years of age. Outbreaks occur commonly in areas with close contacts, such as schools and military barracks, etc.

LABORATORY DIAGNOSIS — *Streptococcus pyogenes*

- **Specimen collection and transport:** Depends on the site of the infection
- **Transport medium:** Pike's medium
- **Direct smear microscopy:** Pus cells with gram-positive cocci in short chains
- **Culture:**
  - Blood agar: Pinpoint colony with a wide zone of β-hemolysis
  - Selective media: Crystal violet blood agar and PNF media
  - Liquid media: Granular turbidity with powdery deposit.
- **Culture smear microscopy:** Gram-positive cocci in short chains
- **Biochemical identification**
  - Catalase negative
  - Bacitracin sensitive
  - PYR test positive.

Contd...

Table 22.5: Differences between acute rheumatic fever and post-streptococcal glomerulonephritis

Features	Acute rheumatic fever (ARF)	Post-streptococcal glomerulonephritis (PSGN)
Prior history of infection with	Pharyngitis strains	Mainly pyoderma, or rarely pharyngitis strains
Serotype	Most of the strains of group A *Streptococcus*	Pyodermal strains-49, 53–55, 59–61 and pharyngitis strains-1, 12
Immune response	Marked	Moderate
Complement level	Unaltered	Low (due to deposition in glomeruli)
Genetic susceptibility	Present	Absent
Repeated attack	Common	Uncommon
Penicillin prophylaxis	Indicated	Not indicated
Course	Progressive	Spontaneous resolution
Prognosis	Variable	Good
Hypersensitivity reaction	Type II	Type III

Contd...

LABORATORY DIAGNOSIS — *Streptococcus pyogenes*

- **Typing:**
  - *Lancefield grouping:* Shows group A *Streptococcus*
  - *Typing of group A Streptococcus:* Griffith and *emm* typing.
- **Serology:**
  - ASO antibodies
  - Anti-DNase B antibodies.
- **Antimicrobial susceptibility testing.**

Laboratory Diagnosis

Specimen Collection and Transport

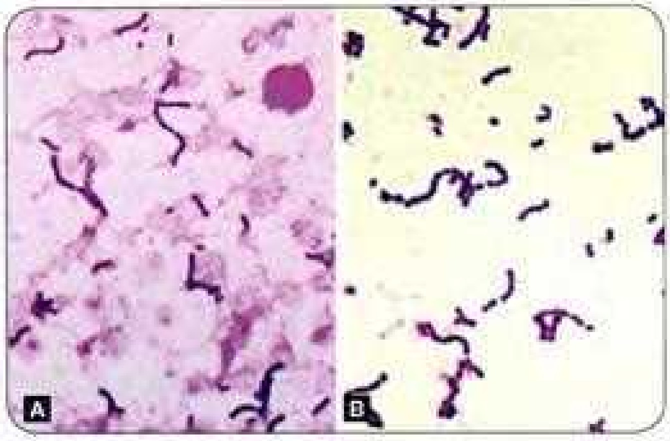
It depends on the site of the lesion. Common specimens are throat swab, pus swab, exudates and blood. Specimens are transported immediately after collection or in **Pike's transport media** (broth containing crystal violet and sodium azide).

Direct Smear Microscopy

Gram staining of pus or wound swab reveals pus cells with gram-positive cocci (0.5-1 μm) in chains (Fig. 22.5A). However, direct microscopy is not useful when *S. pyogenes* is a part of normal flora in the sample (e.g. throat swab).

Culture

The specimens are inoculated onto various media and incubated overnight at 37°C aerobically in presence of 5-10% CO_2. *S. pyogenes* is fastidious, does not grow on MacConkey agar and basal media like nutrient agar or



Figs 22.5 A and B: Streptococci **A:** In Gram stained smear of pus **B:** In culture smear showing gram-positive cocci in chains

Source: Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (with permission).

Table 22.6: Differences between *Streptococcus pyogenes* and *S. agalactiae*

Characters	*S. pyogenes*	*S. agalactiae*
Lancefield grouping	Group A	Group B
Bacitracin sensitivity test	Sensitive	Resistant
PYR test	Positive	Negative
Hippurate hydrolysis test	Negative	Positive
CAMP test*	Negative	Positive
β hemolytic colonies	0.5–1 mm, pin point	Mucoid, slightly larger (2 mm)

*CAMP: Christie, Atkins, and Munch-Peterson test.

peptone water broth. It grows only in media enriched with blood, serum or carbohydrate.

- **Blood agar:** Colonies are small 0.5-1 mm, pinpoint, circular, semitransparent, low convex with a wide zone of β hemolysis (Fig. 22.6A). Stabbing of blood agar plate while inoculating may enhance streptolysin-O induced hemolysis. Colonies of capsulated strains are mucoid
- **Liquid media** can be used such as glucose or serum broth or brain heart infusion broth. Growth appears as granular turbidity with powdery deposit
- **Selective media** used are as follows:
  - **Crystal violet blood agar:** Crystal violet (0.1%) inhibits the growth of staphylococci and other bacteria
  - **PNF media:** This medium is composed of horse blood agar with polymyxin B, neomycin and fusidic acid.

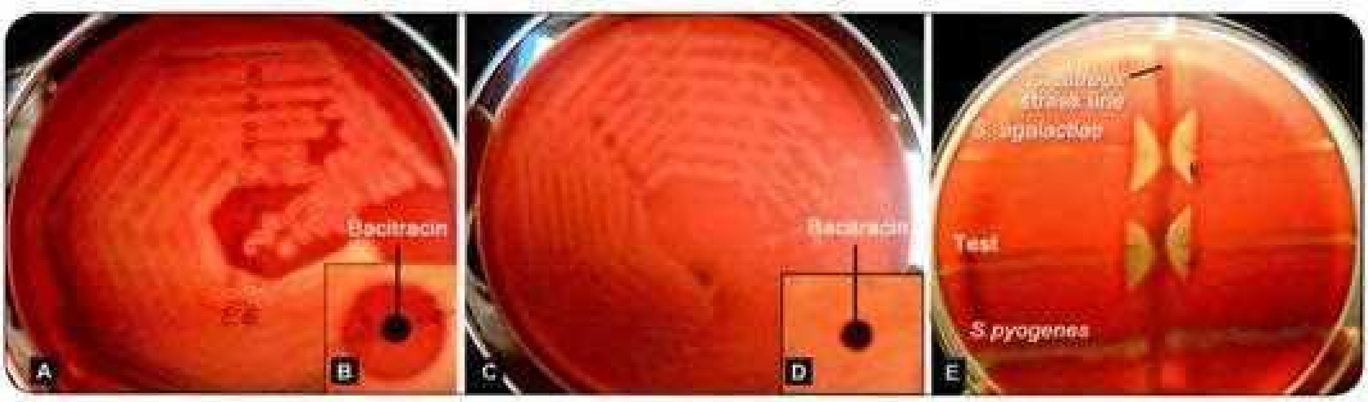
Culture Smear Microscopy

Gram stained smear from the colonies show gram-positive spherical cocci (0.5-1 μm), arranged in chains (Fig. 22.5B). Hanging drop reveals non-motile cocci.

Biochemical Tests for Identification

- **Catalase test:** Streptococci are catalase negative. This test differentiates them from staphylococci which are catalase positive
- **Bacitracin sensitivity testing:** Group A *Streptococcus* is sensitive to bacitracin 0.04 U disk (any zone of inhibition around the disk is considered as positive test), while most of other β hemolytic streptococci are resistant. Hence, it can be used as a rapid diagnostic test for GAS (Fig. 22.6B).

Various tests to differentiate GAS from group B β-hemolytic streptococci are tabulated in Table 22.6.



Figs 22.6A and B: *Streptococcus pyogenes:* **A.** Growth on blood agar with wide zone of beta hemolysis around the pin point colonies; **B.** Bacitracin sensitive

Source: Department of Microbiology, JIPMER, Puducherry (with permission).

Figs 22.6C to E: *Streptococcus agalactiae:* **C.** Growth on blood agar with wide zone of beta hemolysis around the colonies; **D.** Bacitracin resistant; **E.** CAMP test positive

Source: Department of Microbiology, JIPMER, Puducherry (with permission).

Lancefield Grouping

The biochemical identification of Group A *Streptococcus* can be further confirmed by Lancefield grouping. Lancefield grouping is extremely useful in epidemiological studies. Here, the β hemolytic streptococci are grouped serologically based on C-carbohydrate antigen. Test involves extraction of C-carbohydrate antigen followed by testing with group specific antisera.

- C-carbohydrate antigen extraction is done—either by hydrochloric acid (Lancefield's acid extraction), or by formamide (Fuller's method) or by autoclaving (Rantz and Randall method) or by an enzyme produced by *Streptomyces albus* (Maxted's method)
- Extracted antigen is tested with commercially available group specific antisera by latex agglutination test (commonly used) and ring precipitation (used previously).

Typing of Group A Streptococci

Group A *Streptococcus* can further be typed based on two methods; phenotypic method, i.e. serological (Griffith typing) and genotypic method (*emm* typing).

- **Griffith typing:** Based on M protein (especially, its variable amino terminal end), GAS can be typed into around 100 serotypes. M protein can be extracted by Lancefield's acid extraction method and typing is done with type specific antisera
- ***emm* typing:** A few strains of GAS are untypable serologically. Hence, gene coding for M protein (*emm* gene) is widely used for typing. This method is almost replacing the conventional serological typing. More than 200 emm types and >750 subtypes of GAS have been identified so far.

Serology

In rheumatic fever and poststreptococcal glomerulonephritis (PSGN), a retrospective diagnosis of streptococcal infection may be established by detecting antibodies in patient's serum.

- **ASO (Anti-streptolysin O) antibodies:** ASO titer is elevated (>200 IU/mL) in most of the streptococcal infections except pyoderma and PSGN. Previously neutralization test was followed for ASO detection, however, currently it is detected by latex agglutination test
- **Anti-DNase-B antibodies**: Titer more than 300-350 units/mL is diagnostic of PSGN and pyoderma
- Other antibodies elevated are antihyaluronidase antibodies and anti-streptokinase antibodies.

Antimicrobial Susceptibility Test

Antimicrobial susceptibility test is carried out on Mueller Hinton blood agar by disk diffusion test.

Table 22.7: Treatment of streptococcal infection

Conditions	Treatment recommended
Pharyngitis	Benzathine penicillin G, IM single dose; or Oral penicillin V for 10 days
Erysipelas/cellulitis	Mild: Procaine penicillin Severe: Penicillin G
Necrotizing fasciitis	Surgical debridement (most crucial) + Penicillin G + Clindamycin
Pneumonia and empyema	Penicillin G + drainage of empyema
Streptococcal toxic shock syndrome	Penicillin G + Clindamycin + immunoglobulin (to streptococcal pyrogenic exotoxin)
Rheumatic fever	Benzathine penicillin G, IM single dose; or Oral Penicillin V for 10 days Long-term maintenance therapy—with penicillin, once a month for duration: • 5 years or until 21 years of age (without carditis) • 10 years (with carditis) • up to 40 years of age/lifelong (with residual heart disease)
Poststreptococcal glomerulonephritis	Benzathine penicillin G, IM single dose; or oral penicillin V for 10 days
Treatment of asymptomatic carriers	
Pharyngeal carriers	Oral clindamycin for 10 days Penicillin V + rifampicin
Rectal carriers	Vancomycin + rifampicin

Abbreviation: IM, intramuscular.

TREATMENT ***Streptococcus pyogenes***

Penicillin is the drug of choice for pharyngeal infections as well as for suppurative complications. Resistance to penicillin is not reported yet.

- However, failure to penicillin treatment may occur due to: (1) noncompliance, if discontinued before 10 days of full course of oral penicillin V, (2) β lactamases produced by normal throat flora such as *Moraxella*.
- Macrolide, such as erythromycin is given to patients allergic to penicillin. However, resistance to macrolides is common.

Treatment of streptococcal infections is outlined in Table 22.7.

Prophylaxis

Long-term maintenance therapy with penicillin (alternative-sulfadiazine or erythromycin in penicillin allergy) is required for children who develop early signs of rheumatic fever. This prevents streptococcal reinfection and further damage to heart.

Vaccine

There are no licensed vaccines available for *S. pyogenes*. The advanced vaccine trials are the M protein-based 30

Table 22.8: Early and late onset group B *Streptococcus* disease in neonates

Characteristics	Early-onset disease	Late-onset disease
Age of onset	0–6 days of birth	7–90 days of birth
Increased risk following obstetric complications	Prematurity and prolonged labor	Not associated
Mode of transmission to the baby	During or before birth from the colonized maternal genital tract	Contact with a colonized mother and nursing personnel
Common clinical manifestations	Pneumonia and/or respiratory distress syndrome followed by meningitis	Bacteremia and meningitis (most common)
Common serotypes	Ia, III, V, II, Ib	Type III (most common)
Case fatality rate	4.7%	2.8%

valent vaccine (StreptAnova) and conserved M protein vaccines (the J8 vaccine and the StreptInCor vaccine).

OTHER β HEMOLYTIC STREPTOCOCCI

Group B Streptococci (S. agalactiae)

Pathogenesis and Clinical Manifestations

Approximately 30% of women are vaginal or rectal carriers of group B *Streptococcus* (GBS). Hence, the GBS infection is common in neonates and in pregnancy.

- Group B *Streptococcus* has been recognized as a major cause of neonatal sepsis and meningitis. Neonatal sepsis can be of two types—early onset and late onset type (Table 22.8)
- Infections in pregnancy can lead to peripartum fever in women
- Infections in adults generally involve elderly or people with underlying chronic illness, such as diabetes mellitus or malignancy. Common infections are cellulitis and soft tissue infections (including infected diabetic skin ulcers), urinary tract infection, pneumonia and endocarditis
- Group B *Streptococcus* has a capsular polysaccharide which can be typed into nine serotypes.

Laboratory Diagnosis

It is catalase negative like all streptococci, but exhibits the following biochemical properties that differentiate it from group A *Streptococcus* (Table 22.6).

- **CAMP positive:** CAMP factor (named after the discoverers—Christie, Atkins-Munch-Petersen) is a phospholipase produced by GBS that causes synergistic hemolysis with β hemolysin produced by *S. aureus*. When GBS is streaked on blood agar plate perpendicular to *S. aureus*, an enhanced arrowhead-shaped hemolysis is produced at their junction, pointing towards *S. aureus* streak line (see Fig. 22.6E)
- Hippurate hydrolysis test positive
- Bacitracin resistant (see Fig. 22.6D)
- PYR (Pyrrolidonyl-beta-naphthylamide) is negative
- Orange pigment production—enhanced in Islam's medium
- β hemolytic colonies are mucoid and slightly larger (2 mm) then group A streptococci (see Fig. 22.6C).

TREATMENT **Group B Streptococci**

Penicillin is the drug of choice for all GBS infections. GBS is less sensitive to penicillin than GAS, hence a higher dose of penicillin is recommended.

Prevention

Screening for anogenital colonization of GBS is recommended at 35-37 weeks of pregnancy and prophylactic ampicillin or penicillin is given to carrier mothers during delivery to reduce the risk of infection to the newborn.

Group C Streptococci

Group C streptococci commonly cause infection in animals and comprise of four species: *S. equi, S. equisimilis, S. dysgalactiae, S. zooepidemicus*. Human infection is rare.

- *S. equisimilis* can cause pharyngitis especially epidemic food-borne pharyngitis after ingestion of contaminated animal products (milk).
  Other deep infections include skin and soft tissue infections, osteomyelitis, pneumonitis, infective endocarditis, bacteremia, meningitis, epiglottitis, pericarditis, urinary tract infections and puerperal sepsis
- *S. equisimilis* is a common source of streptokinase, which is used for thrombolytic therapy.

Group F Streptococci

They are also called **minute streptococci.** They grow poorly on blood agar, occasionally cause suppurative infection.

Streptococcus MG is an α hemolytic strain belonging to this group. Demonstration of antibodies to *Streptococcus* MG in the patient's sera has been used for diagnosis of primary atypical pneumonia (caused by *Mycoplasma pneumoniae*).

Group G Streptococci

They are throat commensals, occasionally cause puerperal sepsis, neonatal infection, skin and soft tissue infections, tonsillitis, and endocarditis.

Group D Streptococci

Group D streptococci comprise of enterococci (fecal streptococci, described below) and non-enterococci

(*S. gallolyticus* and *S. equinus*). They possess the common group D lipoteichoic acid antigen.

S. gallolyticus (formerly *S. bovis*) is commensal of intestine of animals. It has been occasionally associated with various human infections such as endocarditis, colorectal cancer and spontaneous bacterial peritonitis.

ENTEROCOCCUS

Enterococci were initially grouped under group D *Streptococcus*, but later, it has been reclassified as a separate genus *Enterococcus*. Based on the molecular structure; it is now placed under a new family; Enterococcaceae.

Both enterococci and non-enterococcal group D streptococci give a positive **bile aesculin hydrolysis test** (they grow in the presence of 40% bile and hydrolyse aesculin to aesculetin that combines with ferric chloride to produce black colored complex). However, they differ in many other properties (Table 22.9).

Virulence Factors

Enterococci are part of normal flora of human intestine, biliary tract and to lesser extent vagina and male urethra. At the same time, enterococci are also becoming increasingly important agents of human disease especially in hospitals mainly because of their resistance to antibiotics. *E. faecalis* is the most common species found in clinical specimens, whereas *E. faecium* is more drug resistant than *E. faecalis*. They exhibit a number of virulence factors such as:

- **Cytolysin/hemolysin:** They lyse the sheep and human RBCs
- **Aggregation substances or pheromones:** They help in clumping of adjacent cells to facilitate plasmid exchange (transfers drug resistance)

Table 22.9: Comparing enterococci and Group D streptococci

Features	Enterococci	Non-enterococcal Group D streptococci
Group specific D Ag	Present	Present
Bile aesculin hydrolysis	Positive	Positive
In presence of • 6.5% NaCl • pH 9.6 • at 45°C • at 10°C	Grows	Does not grow
PYR test	Positive	Negative
Drug resistance	Marked	Uncommon
Existence as normal intestinal flora	More common	Less common
Pathogenicity	Marked	Less

Abbreviation: PYR, Pyrrolidonyl-beta-naphthylamide

- **Extracellular surface protein (ESP):** It helps in adhesion to bladder mucosa
- **Common group D lipoteichoic acid antigen:** It induces cytokine release such as tumor necrosis factor α (TNFα)
- **Coccolysin:** It inactivates endothelin, a vasoactive peptide.

Clinical Manifestations

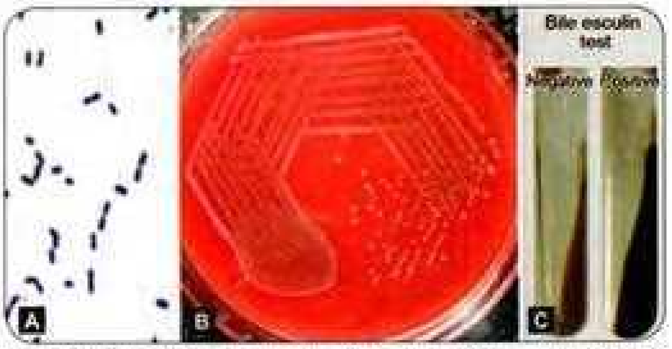
Enterococci are one of the major hospital acquired pathogen, produce various infections such as:

- Urinary tract infections (cystitis, urethritis, pyelonephritis and prostatitis)
- Bacteremia and mitral valve endocarditis (in intravenous drug abusers)
- Intra-abdominal, pelvic and soft tissue infections
- Late-onset neonatal sepsis and meningitis
- Infection on burn surface.

Laboratory Diagnosis

Enterococci show the following characteristics that help in their identification:

- They are gram-positive oval cocci (Fig. 22.7A) arranged in pairs; cocci in a pair are arranged at an angle to each other (**spectacle-shaped** appearance)
- Non-motile cocci (except *E. gallinarum* and *E. casseliflavus*)
- **Blood agar:** It produces non-hemolytic (Fig. 22.7B), translucent colonies (rarely produces α or β hemolysis)
- **MacConkey agar:** It produces minute magenta pink colonies
- **Nutrient agar:** It grows poorly
- Bile aesculin hydrolysis test is positive (Fig. 22.7C)
- PYR (Pyrrolidonyl-beta-naphthylamide) test is positive
- They can grow in presence of extremes of conditions such as—6.5% NaCl, 40% bile, pH 9.6, 45°C and 10°C



Figs 22.7A to C: *Enterococcus*. **A.** Gram-positive oval cocci in pairs; **B.** translucent non-hemolytic colonies on blood agar; **C.** Bile aesculin hydrolysis test (left—negative, right—positive result, black color due to aesculin hydrolysis)

Source: Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (*with permission*).

Table 22.10: Comparison between *Enterococcus faecalis* and *E. faecium*

Features	*E. faecalis*	*E. faecium*
Mannitol	Fermented	Fermented
Arabinose	Not fermented	Fermented
Sorbitol	Fermented	Not fermented
Pyruvate	Fermented	Not fermented

- **Heat tolerance test:** They are relatively heat resistant, can survive 60°C for 30 minutes
- **Groups:** According to Facklam and Collins classification, enterococci can be divided into five groups—group I to V based on mannitol fermentation and arginine dihydrolase test. *E. faecalis* and *E. faecium* belong to group II, which can be further differentiated by several biochemical properties (Table 22.10).

TREATMENT ***Enterococcus***

Enterococci are resistant to penicillins, aminoglycosides, sulfonamides, cephalosporins and cotrimoxazole.

- Resistance to penicillin and aminoglycoside is overcome by combination therapy (e.g. ampicillin plus gentamicin) due to synergistic effect and this remains the standard therapy for life-threatening enterococcal infections
- This combination therapy fails if the isolate is found resistant to either ampicillin or high level aminoglycoside in vitro
- Vancomycin is usually indicated in such cases, but resistance to vancomycin has also been reported
- If resistant to vancomycin: then treatment options available are linezolid, streptogramins (active against *E. faecium*, but not to *E. faecalis*) and daptomycin.

Vancomycin Resistant Enterococci (VRE)

Vancomycin resistance in enterococci has been increasingly reported now a days.

- The prevalence of VRE varies with time and place. A report in 2016 revealed that among hospitalized patients the VRE frequency is high in America (35%) and low in Europe (4%) and moderate (10–15%) in Asian countries. In India, the VRE rate varies from 5–10%
- VRE is mediated by *van* gene, which alters the target site for vancomycin present in the cell wall; i.e. D-alanyl-D-alanine side chain of peptidoglycan layer (which is the usual target site for vancomycin), is altered to D-alanyl-D-serine or D-alanyl-D-lactate. This altered side chains have less affinity for binding to vancomycin
- ***Van* gene has 11 genotypes:** (*van* A, B, C1-C3, D, E, G, L, M and N). The *van* A and *van* B genotypes predominate worldwide; expressed by *E. faecalis* and more commonly by *E. faecium*
- All *van* genes are located on transposons and are inducible; except type C and N (chromosomal and constitutive).
  - Strains with *van* A gene show high level resistance to both glycopeptides vancomycin and teicoplanin

Contd...

Contd...

  - Strains with *van* B gene show low level resistance to vancomycin, but sensitive to teicoplanin
  - *E. gallinarum* and *E. casseliflavus* possess *van* C genes and they show intrinsic resistance to both the glycopeptides.

VRE Carriers

VRE often colonizes the intestine and poses a risk of transmitting to other patients.

- Screening for VRE: It is recommended for high risk patients from ICUs and transplantation units
- Detection: Rectal swab is collected and subjected to (i) Sodium azide agar added with 6 µg/mL of vancomycin or (ii) chromogenic media or (iii) PCR for detection of *van* gene
- Management: Ensure infection control measures such as hand hygiene and isolation precautions (refer Chapter 53). Treatment (i.e. decolonization) is not recommended for VRE carriers.

VIRIDANS STREPTOCOCCI

Viridans streptococci are commensals of mouth and upper respiratory tract. Usually, they are nonpathogenic, however occasionally cause disease such as:

- **Dental caries**: It is mainly caused by *S. mutans* which breaks down dietary sucrose to acid and dextrans. Acid damages the dentine, while adhesive dextran binds together with food debris, mucus, epithelial cells and bacteria to produce **dental plaques**
- **Subacute bacterial endocarditis (SABE):** Viridans streptococci are the most common cause of SABE. The commensal viridans streptococci (*S. sanguis*) in the oral cavity can enter blood to cause transient bacteremia while chewing, tooth brushing and dental procedures that can account for the predilection of these organisms to cause endocarditis
- ***S. milleri* group** (includes *S. intermedius*, *S. anginosus*, and *S. constellatus*): Produce suppurative infections particularly abscesses of brain and abdominal viscera.

Laboratory Diagnosis

- On Gram stain, they appear as long chains of gram-positive cocci (Fig. 22.8A)
- They produce minute α hemolytic green color (rarely non-hemolytic) colonies on blood agar ("*viridis*" means green Fig. 22.8B)
- They can be differentiated from *S. pneumoniae* (which is also α hemolytic) by a number of tests (Table 22.11).

Figs 22.8A and B: Viridans streptococci. **A.** Gram-positive cocci in long chains; **B.** α hemolytic colonies on blood agar

Source: Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (with permission).

Table 22.11: Differences between *Streptococcus pneumoniae* and Viridans streptococci

Features	*S. pneumoniae*	Viridans streptococci
Arrangement	Gram-positive cocci in pairs	Gram-positive cocci in long chains
Morphology	Lanceolate or flame shaped	Round/oval
Capsule	Present	Absent
On blood agar	Draughtsman or carom coin colony	Convex-shaped colony
Liquid medium	Uniform turbidity	Granular turbidity
Bile solubility	Soluble in bile	Insoluble in bile
Inulin fermentation	Fermenter	Non fermenter
Optochin	Sensitive	Resistant
Mice pathogenicity	Pathogenic	Non-pathogenic

TREATMENT **Viridans streptococci**

They are usually sensitive to penicillin except in neutropenic patients with bacteremia, where vancomycin is given.

PNEUMOCOCCUS

Streptococcus pneumoniae (commonly referred to as pneumococcus) is the leading cause of lobar pneumonia, otitis media in children and meningitis in all ages. They are α hemolytic and may present as commensals in human upper respiratory tract. They differ from α hemolytic viridans streptococci in many ways such as their shape (lanceolate-shaped diplococci), bile solubility, optochin sensitivity and presence of a polysaccharide capsule (Table 22.11).

Virulence Factors and Pathogenesis

S. pneumoniae possesses a number of virulence factors such as:

- **Capsular polysaccharide:** It is the principal virulence factor, protects the cocci from phagocytosis. It is type specific (about 95 capsular serotypes are recognized). Being soluble, it diffuses into culture media, tissue and exudates, hence also called **soluble specific substance**
- **C carbohydrate antigen** (C-polysaccharide or C-substance): It is species specific, made up of ribitol, teichoic acid linked to fragments of peptidoglycan. In sera of patients with acute inflammation, a beta globulin appears (synthesized by liver) that precipitates with pneumococcal C-antigen, hence it is named as **C-reactive protein (CRP)**. However, it is not an antibody to C-antigen. CRP is a non-specific acute phase reactant protein, can be raised in many inflammatory conditions (infective as well as noninfective conditions, such as malignancies) and disappears once the inflammation subsides
- **Pneumolysin:** It is a membrane damaging toxin, which inhibits neutrophil chemotaxis and phagocytosis, similar to streptolysin-O
- **Autolysin:** It is an amidase enzyme that cleaves its own peptidoglycan leading to autolysis of cells. The activity is enhanced in presence of bile salts and other surface active agents. This property is responsible for characteristic bile solubility and draughtsman appearance of pneumococcal colonies. Release of cell wall fragments lead to a self-perpetuating inflammatory response that contributes to the pathogenesis
- **Other virulence factors:**
  - **Pneumococcal surface protein A (PspA):** It prevents complement activation. It shows some similarities to the M protein of *S. pyogenes*
  - **IgA protease:** It cleaves IgA, present in the respiratory mucosa, thus facilitates entry
  - **Pneumococcal surface protein C (PspC):** It is also known as *choline-binding protein A* (CbpA). It binds to factor H and accelerates the breakdown of C3 complements
  - **Adhesins:** These include sialidase (neuraminidase) and pneumococcal surface adhesin A (PsaA).
  - Choline-binding protein- helps in adhesion.

Clinical Manifestations

Pneumococci colonize the human nasopharynx at an early age. From the nasopharynx, the bacteria spread either via the bloodstream to distant sites (e.g. brain, joint, bones and peritoneal cavity) or spread locally to cause otitis media or pneumonia.

Various manifestations include:

- **Lobar pneumonia:** *S. pneumoniae* is the most common cause of lobar (alveolar) pneumonia. Though starts as noninvasive illness due to contiguous spread from the nasopharynx, it soon becomes bacteremic and invasive. Patients present with productive purulent cough, fever and chest pain. Important signs are dullness on percussion due to consolidation and crackles on auscultation

- **Empyema and parapneumonic effusion** may occur as complications of pneumococcal pneumonia
- **Invasive pneumococcal disease:** Defined as an infection confirmed by isolation of pneumococci from a normally sterile site. Various examples include:
  - Blood stream infection
  - Pyogenic meningitis: *S. pneumoniae* is the leading cause of meningitis in all ages (except in neonates)
  - Other invasive manifestations: *S. pneumoniae* can cause osteomyelitis, septic arthritis, endocarditis, pericarditis, primary peritonitis, rarely, brain abscess and hemolytic-uremic syndrome.
- **Noninvasive manifestations:** Pneumococci can cause various noninvasive infections such as otitis media and sinusitis (most common cause).

Epidemiology

- **Source** of infection is human upper respiratory tract of carriers (less often patients)
- **Carrier rate:** More than 90% of children of 6 months to 5 years of age harbor *S. pneumoniae* in the nasopharynx
- **Mode of transmission** is by inhalation of contaminated droplet nuclei
- Infection usually leads to colonization and carrier state. Disease results only when the host resistance is lowered due to presence of associated risk factors.

Risk Factors

- **Children (<2 years):** Children are at higher risk to develop pneumococcal infection because of their inability to produce adequate antibodies against the capsular antigen; owing to the immature immune system
- **Splenectomy, sickle cell disease and other hemoglobinopathies:** As spleen is the site of destruction of capsulated bacteria, the conditions where the opsonization and clearance of circulating bacteria by the spleen is hampered, there is increased risk of pneumococcal infection
- **Underlying comorbid diseases:** Such as chronic lung, heart, kidney and liver disease, cochlear implants, diabetes mellitus and immunosuppression (e.g. HIV)
- Underlying viral upper respiratory tract infections (e.g. influenza)
- **Nature of infecting serotypes:** Serotypes vary in their virulence, geographical distribution and age affected.
  - **Most common:** 6 and 19 F are reported universally as major serotypes. In India serotype 1, 6, 19A and 19F are commonly reported.
  - **Age:** In children, seven serotypes (1, 5, 6A, 6B, 14, 19F and 23F) account for nearly 60% of IPD cases in most part of the world. In contrast, the serotypes causing IPD vary widely among adults which may be attributed to the wide variation in the vaccination status in adults.
  - **Virulence:** There is no constant association between serotypes and virulence observed. This is because of expression of various non-capsular virulence factors and also due to competent factor guided uptake of foreign DNA (transformation), which result in wide variation in virulence among the serotypes.

LABORATORY DIAGNOSIS — ***Streptococcus pneumoniae***

- **Specimen collection:** Sputum, CSF, pleural fluid
- **Direct smear microscopy:** Reveals pus cells and lanceolate-shaped gram-positive cocci diplococci, surrounded by a clear halo (due to capsule)
- **Capsular antigen detection in CSF:** By latex agglutination
- **Culture**
  - Blood agar: It forms draughtsman or carrom coin shaped colonies
  - Chocolate agar: It produces greenish discoloration (bleaching effect)
  - In liquid media: It shows uniform turbidity.
- **Culture smear:** Reveals lanceolate-shaped gram-positive cocci in pairs
- **Biochemical identification**
  - Bile soluble
  - Optochin sensitive
  - Inulin fermented.
- **Serotyping:** By Quellung reaction or latex agglutination test
- **Mouse pathogenicity**
- **Molecular methods:** Such as multiplex PCR
- **Non-specific findings:** ↑ acute phase reactant proteins, e.g. C-reactive protein, procalcitonin
- **Antimicrobial susceptibility testing.**

Laboratory Diagnosis

Specimen Collection

Depending on the site of infection, specimens such as sputum, cerebrospinal fluid (CSF), pleural fluid and other sterile body fluids are collected. Blood culture is useful for invasive infection.

Direct Smear Microscopy and Antigen Detection

Direct microscopy of smears made from sputum, pus or CSF show numerous pus cells and **lanceolate or flame-shaped** gram-positive cocci (1 μm) in pairs, surrounded by a clear halo (due to capsule). Direct microscopy is extremely useful especially for meningitis as empirical treatment (antibiotics) can be started early (Fig. 22.9). Capsules can be better demonstrated by India ink staining of CSF.

Detection of capsular antigens in CSF is more sensitive than microscopy. It is done by latex agglutination test using latex beads coated with anticapsular antibodies.

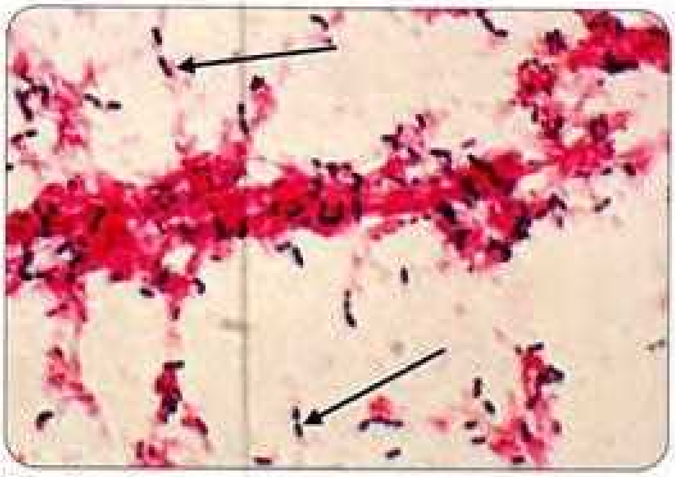


Fig. 22.9: Pneumococci in gram-stained smear of sputum [lanceolate shaped gram-positive cocci in pair surrounded by clear halo (capsule)]

Source: Public Health Image Library, ID#/2896/Dr Mike Miller/Centers for Disease Control and Prevention (CDC), Atlanta (with *permission*).

Culture

S. pneumoniae is fastidious, does not grow in basal media like nutrient agar or nutrient broth. Specimens are inoculated onto enriched media, such as blood agar, and chocolate agar and incubated for 24 hours at 37°C in presence of 5-10% of CO_2.

- **Blood agar:** After 18 hours of incubation, colonies on blood agar are small (0.5–1 mm), dome shaped, glistening surrounded by green discoloration due to α hemolysis. On further incubation, colonies become flat with raised edge and central depression or umbonation due to autolysis of center of the colonies by autolysin enzyme. Colonies appear as concentric rings when viewed from above **(draughtsman-shaped or carom coin-shaped appearance)** (Fig. 22.10A)
- **Chocolate agar:** It produces greenish discoloration (described as bleaching effect) (Fig. 22.10C)
- In liquid media such as glucose broth or BHI broth (brain heart infusion broth): It produces uniform turbidity that readily undergoes autolysis.

Culture Smear

Gram stained smear of the colonies reveals lanceolate or flame-shaped gram-positive cocci (1 μm) in pairs. Motility testing by hanging drop shows non-motile cocci.

Biochemical Identification

Pneumococci are catalase negative and can be differentiated from viridans streptococci (which are also α hemolytic, found as oral commensals in sputum specimens) in various ways (Table 22.11).

- **Bile solubility:** Pnemococci are soluble in bile due to their enhanced autolytic activity in presence of bile. Viridans streptococci are insoluble in bile. Bile solubility can be demonstrated by (adapted from CDC):
  - **Plate method** (drop of 10% sodium deoxycholate is added to pneumococcal colonies on blood agar, incubated at 37°C, colonies disappear leaving behind a zone of green hemolysis within 30 minutes)
  - **Tube method** [0.5 mL of 2% sodium deoxycholate is added to 1 mL of 0.85% saline inoculated with pneumococcal colonies (turbidity of 0.5–1 McFarland standard), incubated at 37°C for 2 hours, look for clearing of turbidity] (Fig. 22.10D).
- **Optochin sensitivity:** Pneumococci are sensitive to optochin disk (5 μg of ethylhydrocupreine hydrochloride) and produce wider zone of inhibition (14 mm or more) (Fig. 22.10B). Viridans streptococci are resistant to optochin. Some strains of pneumococci are optochin-resistant; hence, the result has to be re-confirmed by bile solubility test
- **Inulin fermentation:** Pneumococci can ferment inulin to form acid, but not viridans streptococci. Fermentation test should be carried out in Hiss's serum sugar medium.

Typing of S. Pneumoniae

- **Quellung reaction:** Capsular swelling can be demonstrated by adding type-specific antiserum (omni-serum) raised in rabbit, along with dye such as methylene blue to pneumococcal colonies on a slide and viewed under microscope. In presence of homologous antiserum, capsule becomes swollen, sharply delineated and refractile. Quellung test was routinely done in the past, at bedside directly from sputum samples from acute pneumonia cases (Fig. 22.10E)
- Currently, serotyping is done by latex agglutination test using type specific antisera.

Mouse Pathogenicity

In samples having fewer organisms where culture is negative, isolation can be done by intraperitoneal inoculation into mice, which die in 1-2 days and pneumococci may be demonstrated in the heart blood and peritoneal exudates of the animal.

Molecular Methods

Molecular methods such as PCR are highly sensitive, more useful when organism load is scanty (e.g. CSF), detect earlier than culture and also help in serogroup identification.

- Real time PCR is even more sensitive, specific, takes less time and quantitative
- Multiplex PCR (e.g. BioFire FilmArray, bioMérieux) and Multiplex real time PCR can be used for simultaneous detection of common agents of pyogenic meningitis

Figs 22.10 A to E: Properties of pneumococci **A.** α hemolytic draughtsman-shaped colonies on blood agar; **B.** Sensitive to optochin; **C.** Bleaching effect on chocolate agar; **D.** Bile solubility test (left-viridans streptococci, not soluble in bile; right—pneumococcus, soluble in bile); **E.** Quellung reaction

Source: A: Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry; B to D: Department of Microbiology, JIPMER, Puducherry (with permission).

- Common genes targeted include: *lytA* (autolysin gene), *ply* (pneumolysin) and *psaA* (pneumococcus surface antigen A).

Nonspecific Findings

- Elevated acute phase reactant proteins such as C-reactive protein, procalcitonin
- Leukocytosis
- Chest X-ray shows infiltrates and lobar consolidation. (In children-distinctly spherical consolidation seen in upper part of the lower lobe called round pneumonia).

Antimicrobial Susceptibility Test

It is done for institution of proper antibiotic treatment. Oxacillin (1 μg/mL) disk diffusion on Mueller Hinton blood agar is done for predicting susceptibility to penicillin, ceftriaxone and other β lactams. However, if found resistant to oxacillin, MIC testing for individual β lactams should be performed for confirmation.

TREATMENT ***Streptococcus pneumoniae***

Penicillin G remains the drug of choice, with daily doses ranging from 2,50,000 to 4,00,000 U/kg for meningitis. If resistant to penicillin, then alternative options are:

- Cephalosporins (e.g. ceftriaxone) or vancomycin can be given for meningitis
- Quinolones such as IV levofloxacin may be considered for non-meningeal infections
- Oral amoxicillin is recommended for children with acute otitis media.

Drug Resistance in Pneumococcus

About 30% of IPD, pneumococcus shows resistance to at least one antibiotic or more.

Penicillin resistance in pneumococci has been reported increasingly nowadays.

- This resistance is due to alteration of penicillin-binding protein (PBP) to PBP2b. This altered PBP2b has low affinity for β lactam drugs. The gene coding for altered PBP is acquired by transformation and horizontal transfer of DNA from related streptococcal species.
- Macrolides are given as alternative for penicillin-resistant pneumococci or for patients allergic to penicillin.

Multidrug resistant (MDR) *S. pneumoniae* defined as non-susceptible to ≥3 antibiotic classes is increasingly reported. Resistant to penicillin, tetracycline, erythromycin, sulfonamides and clindamycin have shown to coexist.

- **Serotype 19A** is the most common serotype to exhibit multidrug resistant. As it was not a part of 7-valent conjugate vaccine, it has caused major outbreak. Now, it has been included in 13-valent conjugated vaccine
- Some serotypes can undergo **capsule switching** (change from one serotype to another), which may contribute to the development of antibiotic resistance.

Prevention and Vaccination

Measures to prevent pneumococcal disease include vaccination, treatment of underlying diseases (that increase the risk of pneumococcal disease), infection control measures (droplet precautions, refer Chapter 53), and prevention of antibiotic overuse.

Pneumococcal Vaccines

There are two vaccines available for pneumococcus: (i) 23-valent pneumococcal polysaccharide vaccine (PPSV23) and (ii) pneumococcal conjugate vaccine (PCV13). Detail is given in Tables 22.12 and 22.13.

Table 22.12: Type of pneumococcal vaccines

	PPSV23	PCV13
Name	23-valent pneumococcal polysaccharide vaccine	Pneumococcal conjugate vaccine
Brand	Pneumovax 23	Prevnar13
Serotypes included	**Contains 23 serotypes of *S. pneumoniae*** 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F	**Contains 13 serotypes of *S. pneumoniae*** 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, & 23F. Conjugated with diphtheria toxoid
Coverage	Covers 70–80% of invasive serotypes in adults	Covers only 30–40% of invasive serotypes in adults, however it covers most of the serotypes infecting children
Immunogenicity	**Less immunogenic:** Capsular antigen being T cell independent antigen, is less immunogenic to children, hence not given to children <2 years age	**More immunogenic:** The protein conjugate acts as adjuvant and increases the immunogenicity of capsular antigen. Hence, PCV is effective against children <2 years age
Duration	Provides short-term immunity (3–5 years)	Provides longer immunity
Herd	Promotes herd immunity	Does not promote herd immunity
Effect on carriers	Does not provide mucosal immunity, hence no effect on carriers	Provides mucosal immunity; hence, it can eradicate carriers from nasopharynx
Cost	Less expensive	More expensive
Indication (see Table 22.13)	Adults ≥65 years Adults 19–64 years age with underlying risk factors	Indications in adults same as PPSV23 Children (as primary immunization)

Table 22.13: Indication and schedule of pneumococcal vaccines

Indication	Schedule	
(1) After birth	PCV 13 (4 doses): at 2,4,6 months of age and booster at 12–15 months	
(2) Age ≥65 years	PCV13 followed by PPSV23 at gap of ≥1 year	
(3) **Age 19–64 years with underlying risk factors**	**During 19–64 year age**	**When they reach ≥65 years**
Immunocompetent persons—With alcoholism and smoking, chronic heart, liver and lung disease, diabetes mellitus	PPSV23 (1 dose) is given	PCV13 followed by PPSV23 at gap of ≥1 year
Immunocompetent persons—With cochlear implants and CSF leaks	PCV13 followed by PPSV23 (1 dose) given at gap of ≥8 weeks	
Splenic dysfunction—Congenital or acquired asplenia, sickle cell disease/other hemoglobinopathies *Immunocompromised persons*—Chronic renal failure, Congenital or acquired immunodeficiencies, HIV infection, malignancy and solid organ transplant	PCV13 followed by PPSV23 (1st dose) given at gap of ≥8 weeks; followed by PPSV23 (2nd dose) after ≥5 years	PCV13 followed by PPSV23 given at gap of ≥8 weeks

Note:
- The minimum gap between two doses of PPSV23 is ≥5 years
- The minimum gap between PCV13 and PPSV23 is ≥1 year (≥8 weeks if some underlying risk factors are present).
- If PCV13 is given during 19–64 age; then it is not given again after 65 years age

Abbreviations: PPSV23, 23-valent pneumococcal polysaccharide vaccine; PCV13, 13-valent pneumococcal conjugate vaccine.

Adopted from Centers for Disease Control and Prevention (CDC).

EXPECTED QUESTIONS

I. Essays:

1. Chinu, a 3-year-old girl from Mangaluru has developed sore throat, difficulty in swallowing. On examination, the pharyngeal mucosa was found to be inflamed. A clinical diagnosis of pharyngitis was made. The culture of the throat swab revealed beta hemolytic pin point colonies.
   a. What is the most likely etiologic agent?
   b. Describe the virulence factors and the other clinical manifestations produced by the etiological agent?
   c. Briefly discuss the laboratory diagnosis of the infections caused by this organism.
2. Alisha, A 4-year-old girl from Bhubaneswar was brought to the emergency room by her parents due to an acute onset of fever, neck rigidity and altered sensorium for past 2 days. Physical examination showed that when her neck was passively flexed, her legs also flexed (positive Brudzinski's sign). Direct examination of the CSF showed gram-positive, lanceolate-shaped diplococci surrounded by a halo.
   a. What is your clinical diagnosis of this condition and the most likely etiologic agent?
   b. How will you confirm the etiological agent in the laboratory?

c. Describe the virulence factors and pathogenesis of the etiological agent.

II. **Write short notes on:**
1. Non-suppurative sequelae of *S. pyogenes* infection
2. Group B *Streptococcus*
3. Pneumococcal vaccines
4. Vancomycin resistant Enterococci

III. **Multiple Choice Questions (MCQs):**

1. **Serotyping of *Streptococcus pyogenes* is based on which of the following protein?**
a. M protein b. T protein
c. R protein d. Carbohydrate antigen

2. ***Streptococcus pyogenes* can be differentiated from *S. agalactiae* by testing susceptibility to:**
a. Optochin b. Bacitracin
c. Polymyxin d. Novobiocin

3. **CAMP test is useful in identification of:**
a. *S. pyogenes* b. *S. agalactiae*
c. Viridans streptococci d. *S. pneumoniae*

4. **Which is a poststreptococcal sequelae:**
a. Acute rheumatic fever
b. Cellulitis
c. Pharyngitis d. Impetigo

5. **Neonatal meningitis acquired through infected birth canal is due to:**
a. *S. pyogenes* b. Viridans streptococci
c. *S. agalactiae* d. *S. pneumoniae*

6. **Carrom coin appearance of colonies is seen for:**
a. *S. pyogenes* b. Viridans streptococci
c. *S. agalactiae* d. *S. pneumoniae*

7. **Which is not a property of *S. pneumoniae*?**
a. Bile solubility
b. Animal pathogenicity in mice
c. Growth in presence of 40% bile
d. Optochin sensitivity

8. ***Staphylococcus* is differentiated from *Streptococcus* by:**
a. Catalase test b. Coagulase test
c. Bacitracin susceptibility
d. CAMP test

9. **A child presents with red sores on the face that burst and develop into honey-colored crusts. What is the most common agent?**
a. *S. aureus* b. *S. pyogenes*
c. *Candida* d. *Pseudomonas*

10. ***S.pyogenes* is differentiated from *S. agalactiae* by all, *except*:**
a. C carbohydrate antigen
b. M protein
c. Bacitracin susceptibility
d. CAMP test

11. ***S. pyogenes* is classified at subspecies level by:**
a. M protein
b. C carbohydrate antigen
c. Hemolysis on blood agar
d. Peptidoglycan

12. ***S. pyogenes* is classified at subspecies level by targeting which gene?**
a. *emm* b. *mecA*
c. *van* d. *fir*

13. ***Streptococcus, Enterococcus* and pneumococcus are differentiated by:**
a. M protein
b. C carbohydrate antigen
c. Hemolysis on blood agar
d. Peptidoglycan

14. **Streptococcal pyrogenic exotoxin (SPE)—which types are bacteriophage coded?**
a. A and C b. B
c. A and D d. B and D

15. **All are true about streptolysin O, *except*:**
a. Causes hemolysis on the surface of blood agar plate
b. Strongly antigenic
c. Useful for retrospective diagnosis of streptococcal infection
d. Not useful for diagnosis of poststreptococcal glomerulonephritis

16. **Streptolysin-O is structurally and functionally similar to all, *except*:**
a. Tetanolysin b. Pneumolysin
c. Listeriolysin
d. β-hemolysin of *S.aureus*

17. **Most common cause of pharyngitis in children is:**
a. *S. pyogenes* b. *S. aureus*
c. *Candida*
d. Herpes simplex virus

18. **Scarlet fever, all are true, *except*:**
a. Rash with sandpaper feel
b. Strawberry tongue
c. Agent is *S. pyogenes*
d. Is frequently seen nowadays

19. **In revised Jones criteria for acute rheumatic fever, the major manifestations include all, *except*:**
a. Subcutaneous nodules
b. Pancarditis
c. Arthritis (migrating polyarthritis)
d. Increased ASO antibodies

20. **Poststreptococcal glomerulonephritis: the correct statement is:**
a. Prior history of pharyngitis
b. Complement level is low
c. Genetic susceptibility is present
d. Penicillin prophylaxis is required

21. **Post-streptococcal glomerulonephritis: which antibody level is raised?**
a. Anti-streptolysin O antibodies
b. Anti-DNase-B antibodies
c. Anti-hyaluronidase antibodies
d. Anti-streptokinase antibodies

Answers

1. a **2.** b **3.** b **4.** a **5.** c **6.** d **7.** c **8.** a **9.** b **10.** b **11.** a **12.** a **13.** c **14.** a **15.** a **16.** d
17. a **18.** d **19.** d **20.** b **21.** b

Neisseria and Moraxella

23

CHAPTER

Chapter Preview

- *Neisseria meningitidis*
- *Neisseria gonorrhoeae*
- Commensal *Neisseria* species
- *Moraxella*

Gram-negative cocci include *Neisseria, Moraxella catarrhalis* and *Veillonella* (the latter is a non-sporing anaerobe described in Chapter 26).

Members of genus *Neisseria* are catalase and oxidase positive, non-motile, aerobic gram-negative diplococci. Two species are pathogenic to humans—(1) *N. meningitidis* (causes pyogenic meningitis) and (2) *N. gonorrhoeae* (causes gonorrhea), both differ from each other in various aspects (Table 23.1). Other species are commensals of genital tract or oral cavity, such as *N. lactamica, N. flavescens, N. mucosa, N. sicca, N. subflava*, etc.

NEISSERIA MENINGITIDIS (MENINGOCOCCUS)

Meningococci are capsulated gram-negative diplococci with adjacent sides flattened (lens-shaped/half moon-shaped). Though it is a commensal in nasopharynx of many healthy adolescents, invasive disease such as meningitis or septicemia can occur in susceptible individuals.

Table 23.1: Differences between *Neisseria meningitidis* and *Neisseria gonorrhoeae*

N. meningitidis	*N. gonorrhoeae*
Capsulated	Noncapsulated
Lens-shaped/half moon-shaped (diplococci with adjacent sides flattened)	Kidney-shaped (diplococci with adjacent sides concave)
Ferments glucose and maltose	Ferments only glucose
Rarely have plasmids	Usually possess plasmids, coding for drug-resistant genes
Exist in both intra- and extracellular forms	Predominantly exist in intracellular form
Colony—circular	Colony—varies in size with irregular margin
Habitat—nasopharynx	Habitat—genital tract (urethra, cervix), rarely pharynx

Virulence Factors

Capsular Polysaccharide

It is the principal virulence factor of meningococci; protects the bacteria from complement-mediated phagocytosis.

- Based on the antigenic nature of the capsule, meningococci can be typed into 13 serogroups [A-D, X-Z, 29E, W, H-J and L], among which only 6 serogroups—**A, B, C, X, Y, and W** (formerly W135)—account for the majority of cases of invasive disease
- Other capsular serogroups and noncapsulated meningococci (16% of isolates are not capsulated) commonly colonize the nasopharynx of asymptomatic carriers and are rarely associated with invasive disease.

Other Virulence Factors

- **Outer membrane proteins:** They are the porin proteins present beneath the capsule, embedded in the outer membrane. They are of two types—PorA and PorB; both show antigenic variability and are responsible for serotyping (PorB) and serosubtyping (PorA) of meningococci
- **LPS and endotoxin:** Like other gram-negative bacteria, meningococci possess LPS (lipopolysaccharide) and endotoxins in their cell wall. Endotoxin binds to CD14 molecules on host cell surface, in association with Toll-like receptor-4 (TLR4). This inturn activates the endothelial cells by inducing release of various inflammatory mediators, such as tumor necrosis factor, interleukin (IL)-1, IL-6, IL-8, IL-10, plasminogen-activator inhibitor-1, etc. Endothelial injury is central to many clinical features of meningococcemia, such as:
  - Increased vascular permeability leading to loss of fluid and shock
  - Intravascular thrombosis (due to activation of procoagulants) leading to disseminated intravascular coagulation (DIC)

- Myocardial dysfunction

❖ **IgA proteases:** Cleave mucosal IgA

❖ **Transferrin binding proteins:** They help in uptake of iron from transferrin.

❖ **Adhesins:** Include opacity proteins and pili.

Epidemiology

Worldwide, nearly 5 lakh cases of meningococcal disease occur each year, and 10% of those die.

❖ **Patterns of disease:** Meningococcus causes several patterns of invasive disease ranging from sporadic infection, to endemic, hyperendemic and explosive epidemics

❖ **High prevalence area:** The sub-Saharan belt of Africa (from Ethiopia to Senegal) is the most prevalent area for meningococcal infections. Around 30,000 cases are still reported each year from this area

❖ **World:** The serogroups distribution among various regions of the world has been depicted in Table 23.2
- **Group A:** It was the leading cause of epidemic meningitis worldwide. With the advent of vaccination, the occurrence of group A has been reduced considerably
- **Group B and C** are currently the major serogroups causing invasive disease worldwide
  - Group B can cause hyperendemic disease (>10 cases per 100,000 population)
  - Group C has caused a recent explosive outbreak in Nigeria in 2016-17 (>18,000 cases).

❖ **Group X, Y and W** are less commonly reported worldwide. Group W (formerly W 135) can cause outbreaks in mass gatherings; has caused the global outbreak in 2000 in Hajj pilgrimage

❖ **India:** Sporadic cases have been occurring every year, mainly from North India with occasional outbreaks
- Serogroup A has been reported from few places, though accurate data on serogroup distribution is lacking

Table 23.2: Serogroup distribution of invasive meningococcal disease

World regions	Most frequent	Less frequent
Sub-Saharan Africa	C, W, X	A
Southern Africa	B, W	Y
North America	B, C, Y	W
South America	B, C	W
Europe	B, C, W	Y
Fareast	B, C, W	A
Central Asia	A, C	
South-east Asia	No data	No data
Australia	B, C	W

Source: World health organization, 2018.

- In 2015, >12,000 cases were reported, maximum from Bihar (>8,000 cases)

❖ **Seasonality:** Meningococcal infections are common in winter and spring (cold and dry climate)

❖ **Age:** Meningitis is common in early childhood (3 months to 5 years) with a second peak occurring in adolescents (15-25 years of age)

❖ **Risk factors that promote colonization include:**
- Overcrowding and semiclosed communities, such as schools, military and refugee camps
- Travelers (Hajj pilgrims)
- Smoking
- Viral and *Mycoplasma* infection of respiratory tract.

❖ **Risk factors that promote disease include:**
- Deficiency of terminal complement components (C5-C9)
- Eculizumab therapy—a terminal complement inhibitor
- Hypogammaglobulinemia and hyposplenism.

Pathogenesis

Humans are the only natural host for meningococci. Most common source of infection is nasopharyngeal carriers (mainly children).

❖ **Mode of transmission:** It is by droplet inhalation and the portal of entry is nasopharynx

❖ **Spread of infection:** From nasopharynx, meningococci reach the meninges either by: (1) hematogenous route causing septicemia (most common); or (2) by direct spread along olfactory nerve through cribriform plate; or (3) rarely through conjunctiva.

Clinical Manifestations

Asymptomatic colonization is the most common presentation. Manifestations observed among symptomatic individuals include:

❖ **Rashes:** A non-blanching rash (petechial or purpuric) develops in more than 80% of the cases

❖ **Septicemia:** It is attributed to endotoxin induced endothelial injury leading to increased vascular permeability and intravascular thrombosis

❖ **Waterhouse-Friderichsen syndrome:** It is a severe form of fulminant meningococcemia, characterized by large purpuric rashes (purpura fulminans), shock, disseminated intravascular coagulation (DIC), bilateral adrenal hemorrhage and multiorgan failure (Fig. 23.1)

❖ **Pyogenic meningitis:** It commonly affects young children (3-5 years of age). Presentation includes fever, vomiting, headache, neck stiffness—similar to any other bacterial meningitis, except for the presence of rashes

❖ **Chronic meningococcemia:** It occurs rarely and is characterized by repeated episodes of petechial rash, fever, arthritis, and splenomegaly

Fig. 23.1: Child with gangrene of lower extremities due to meningococcemia

Source: Public Health Image Library, ID# /1335/Mr Gust/Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*).

- **Postmeningococcal reactive disease:** Immune complexes (made up of capsular antigens and their antibodies) develop 4–10 days later, lead to manifestations like arthritis, rash, iritis, pericarditis, polyserositis, and fever
- **Mortality:** It is high (>10%), reaches up to 50% when untreated and high frequency (>10%) of severe sequelae.

LABORATORY DIAGNOSIS — Meningococcus

- **Specimen collection:** CSF, blood, nasopharyngeal swab
- **CSF examination**
  - First portion is centrifuged and used for:
    - Capsular antigen detection
    - Biochemical analysis: ↑CSF pressure, ↑protein and ↓glucose
    - Gram-staining shows pus cells with gram-negative diplococci, lens-shaped.
  - Second portion: Culture on blood agar, chocolate agar
  - Third portion is enriched in BHI broth.
- **Nasopharyngeal swab culture:** on Thayer Martin medium
- **Biochemical tests**
  - Oxidase and catalase positive
  - Ferments glucose and maltose but not sucrose.
- **Serology:** Antibodies to capsular antigens (ELISA)
- **Molecular diagnosis:** By multiplex PCR (*ctrA*, *sodC* genes).

Laboratory Diagnosis

Specimen Collection

Important specimens include cerebrospinal fluid (CSF), blood and skin scrapings from petechial rashes from cases, and nasopharyngeal swabs from carriers.

- Specimens are collected in sterile container and transported immediately without any delay
- CSF should be processed immediately. It should never be refrigerated as suspected agents of meningitis, such as meningococci and *Haemophilus influenzae* may die on refrigeration
- **Blood culture:** Blood should be immediately injected into blood culture bottles (brain-heart infusion broth, automated systems such as BacT/Alert) and incubated; subcultures are made onto blood agar plate and colonies grown are processed (as described for CSF later)
- Nasopharyngeal swabs, pus or scrapings from rashes should be carried in transport media (such as **Stuart's medium**) and inoculated onto selective media, such as **Thayer Martin medium** or **New York City medium** (to suppress the growth of normal flora).

CSF Examination

For bacteriological examination, the CSF is divided into the following three portions:

- **First portion** of CSF is centrifuged
  - The supernatant is used for:
    - Capsular antigen detection by latex agglutination test
    - Biochemical analysis: It reveals elevated CSF pressure, increased protein content and decreased glucose content.
  - The sediment is used for direct Gram staining: Meningococci appear as gram-negative diplococci (0.8 µm in size) with adjacent sides flattened (**lens or half-moon-shaped**), present inside the polymorphs, often extracellular also. This presumptive diagnosis helps to start empirical antibiotics (Fig. 23.2).
- **Second portion of CSF:** It is directly inoculated onto an enriched media, such as blood agar and chocolate agar and incubated for 24–48 hours at 37°C under 5–10% CO_2
  - At 24 hours of incubation, colonies are small (1 mm), round, convex, gray, non-hemolytic, translucent. After 48 hours, colonies become larger with opaque raised center and thin transparent margin
  - Meningococci are very delicate and fastidious; do not grow in basal media.
- **Third portion of CSF:** It is inoculated into enriched broth, such as BHI broth, incubated overnight till granular turbidity is produced and then subcultured onto blood agar and chocolate agar.

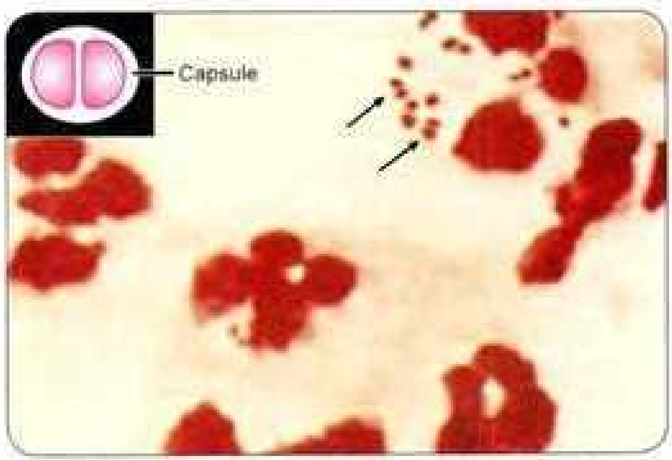


Fig. 23.2: Meningococci in CSF smear (gram-negative diplococci, lens-shaped) (arrows showing)

Source: Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

Biochemical Tests

Biochemical tests should be carried out to differentiate meningococci from other commensal *Neisseria* species.

- Meningococci are catalase and oxidase positive
- They ferment glucose and maltose but not sucrose. This can be tested by using rapid carbohydrate utilization test (RCUT).

Serogrouping

Slide agglutination serogrouping (SASG) test is done for serogrouping of meningococci isolates by using appropriate antisera.

Serology

Antibodies to capsular antigens can be detected by ELISA. This helps:

- In retrospective diagnosis of disease (as antibodies develop later in convalescent phase)
- To know the response to vaccination
- In diagnosis of chronic meningococcemia.

Molecular Diagnosis

- PCR is highly sensitive, detects even few bacteria in CSF, detects earlier than culture and also helps in serogroup identification
- Real time PCR is even more sensitive, specific, takes less time and quantitative
- Multiplex PCR (e.g. BioFire FilmArray) and Multiplex real-time PCR can be used for simultaneous detection of common agents of pyogenic meningitis
- Common genes targeted include: *ctrA* (capsule transport gene) and *sodC* (Cu-Zn superoxide dismutase gene).

TREATMENT **Meningococcus**

- **Drug of choice** is third-generation cephalosporins, such as ceftriaxone or cefotaxime, given for 7 days. Penicillin can also be given; however, reduced meningococcal sensitivity to penicillin has been reported from few countries
- **Symptomatic treatment**, such as aggressive fluid resuscitation (for shock) and measures to decrease intracranial pressure.

Prevention

Chemoprophylaxis

Chemoprophylaxis aims at eradicating the colonization of close (house hold and kissing) contacts of the primary cases.

- Ceftriaxone (single dose, IM) is the drug of choice
- Alternatively, rifampicin and ciprofloxacin can be given.

Vaccine Prophylaxis

Meningococcal polysaccharide vaccines are currently formulated as either bivalent (serogroups A and C) or quadrivalent (serogroups A, C, Y, and W135).

- **Dose:** 50 µg of each polysaccharide antigen per dose is used, administered as two doses, 2-3 months apart to children of 3-18 months of age or a single dose to older children or adults
- **Efficacy:** It has a protective efficacy rate of >95%. The duration of protection lasts for 3-5 years
- **Indication:** It is recommended for high-risk people such as (i) contacts of patients during outbreaks, (ii) splenic dysfunction, (iii) terminal complement component deficiency, (iv) taking eculizumab therapy, (v) laboratory staff at risk
- **Capsular vaccine is not available for serogroup B as:**
  - Capsule of serogroup B (made up of sialic acid) is less immunogenic
  - It is also encephalitogenic due to expression of similar cross reactive antigens on neural cells.
- **Not given below 3 years:** Similar to pneumococcal vaccine, meningococcal capsular vaccine is also an example of T-cell-independent antigen and is poorly immunogenic to children; hence not given to children of less than 2-3 years of age
- **Conjugated vaccine**: However, conjugated meningococcal capsular vaccine is available which can be given to young children. Addition of a protein carrier (adjuvant) increases the immunogenicity of the capsular vaccine.

Vaccine for Group B (*MenB* vaccine)

Recently, recombinant vaccine for Group B meningococcus has been licensed.

- Vaccine contains four recombinant proteins: adhesin A, heparin binding antigen, factor H binding protein and outer membrane vesicles (OMV)
- Schedule: Two doses, given IM route 1 month apart
- Indication: 16-25 years age.

NEISSERIA GONORRHOEAE (GONOCOCCUS)

Neisseria gonorrhoeae is noncapsulated, gram-negative kidney-shaped diplococcus. It causes 'gonorrhea', a sexually transmitted infection (STI) and commonly manifests as cervicitis, urethritis and conjunctivitis.

Virulence Factors

- **Pili or fimbriae:** Pili are the principal virulence factors of gonococci that help in adhesion to host cells and prevent bacteria from phagocytosis
- **Outer membrane proteins:**
  - **Porin (protein I):** This accounts for more than 50% of total outer membrane proteins
    - They form transmembrane channels (pores) which help in exchange of molecules across gonococcal surface
    - There are two major serotypes: PorB.1A and PorB.1B serotypes. PorB.1A strains are often

associated with both local and disseminated gonococcal infections (DGI), while PorB.1B strains usually cause local genital infections only.
- **Opacity-associated protein (Protein II):** It helps in adhesion to neutrophils and other gonococci.

❖ **Transferrin-binding and lactoferrin-binding proteins:** They help in uptake of iron from transferrin and lactoferrin

❖ **IgA1 protease:** It protects the organism from the action of mucosal IgA antibody

❖ **Lipo-oligosaccharide (LOS):** Here, the LPS is modified, consisting of only lipid A and core oligosaccharide but lacks the repeating O side chain which is seen in other gram-negative bacteria. It has marked endotoxic activity.

Typing of Gonococci

❖ **Serotyping:** It is based on protein-I (porin). Up to 24 protein-IA and 32 protein-IB have been identified

❖ **Auxotyping:** It is based on nutritional requirements of the strains, e.g. AHU auxotype needs arginine, hypoxanthine and uracil as growth factors.

Clinical Manifestations

Gonorrhea is a venereal disease reported since ancient times; produces various infections in males, females and also in newborns.

❖ **In males:** Acute urethritis is the most common manifestation
- It is characterized by purulent urethral discharge (the word 'gonorrhea' is derived from flow of seed resembling semen, coined by Galen in 130 AD)
- The usual incubation period is 2–7 days
- Untreated cases may go for complications, such as epididymitis, prostatitis, and balanitis
- Infection may spread to periurethral tissues causing abscess with sinus formation (**water-can perineum**).

❖ **In females:** Gonococcal infection is less severe in females, with more asymptomatic carriage than males
- **Mucopurulent cervicitis:** It is the most common presentation
- **Vulvovaginitis:** It is not seen in adult females as the adult vagina is resistant to gonococcal infection (due to its low pH and thick stratified squamous epithelium). However, gonococcal vaginitis can occur in prepubertal girls and postmenopausal women where the vagina is lined by thinned out mucosa with higher pH
- **Spread:** Infection may spread to Bartholin's gland, endometrium and fallopian tube. Salpingitis and pelvic inflammatory disease may lead to sterility
- **Fitz-Hugh-Curtis syndrome:** It is a rare complication, characterized by peritonitis and associated perihepatic inflammation.

❖ **In both the sexes:** The following manifestations may occur in both the sexes:
- Anorectal gonorrhea (spread by anal sex): Rectal isolates are usually multidrug-resistant
- Pharyngeal gonorrhea (spread by orogenital sex)
- Ocular gonorrhea.

❖ **In pregnant women:** Gonococcal infection causes prolonged rupture of the membranes, premature delivery, chorioamnionitis, and sepsis in infant

❖ **In neonates (Ophthalmia neonatorum):** It is characterized by purulent eye discharge, occurs within 2–5 days of birth. Transmission occurs during birth from colonized maternal genital flora

❖ **Disseminated gonococcal infection (DGI):** It occurs rarely following gonococcal bacteremia. DGI is characterized by polyarthritis and rarely dermatitis and endocarditis. It is most commonly associated with PorB.1A serotypes which are also characterized as AHU auxotypes

❖ **In HIV-infected persons**: Nonulcerative gonorrhea enhances the transmission of HIV by three-to-five folds, possibly because of increased viral shedding.

Epidemiology

The incidence of gonorrhea has come down in developed countries; however, it still remains a public health problem in developing countries, and may play a role in enhancing transmission of HIV. Because of the associated social stigma, it is often under-reported.

❖ **Host:** Gonorrhea is an exclusively human disease, there are no animal reservoirs

❖ **Source:** The source of infection are asymptomatic female carriers or less often a patient

❖ **Transmission:** It is almost exclusively transmitted—(1) by sexual contact (venereal); transmission from males to females is more efficient than in the opposite direction, (2) from mother to baby during birth.

LABORATORY DIAGNOSIS — **Gonococcus**

- **Specimen:** Urethral swab in men and cervical swab in women
- **Transport media:** Stuart's and Amies transport media
- **Microscopy:** Gram-negative intracellular kidney-shaped diplococci
- **Culture**
  - Thayer Martin medium
  - Modified New York City medium.
- **Biochemical tests**
  - Oxidase and catalase positive
  - Ferments only glucose but not maltose.
- **Molecular Method:** PCR targeting 16S or 23S rRNA gene.

Laboratory Diagnosis

Specimen Collection

Urethral swab in men and cervical swab in women are the preferred specimens. Vaginal swab is not satisfactory.

❖ The urethral meatus is cleaned with gauze soaked in saline. The purulent discharge is expressed out by pressing at the base of the penis and collected directly on to slides or swabs

- **Dacron or rayon swabs** are preferred, as cotton and alginate swabs are inhibitory to gonococci
- **In chronic urethritis:** As discharge is minimal, prostatic massage is done to collect the secretion; alternatively, the morning drop of secretion may be collected.

Transport Media

Specimens should be transported immediately. If not possible, then it should be collected in charcoal-coated swabs kept in **Stuart's** transport medium or, alternatively, charcoal containing medium (**Amies** medium) can be used. Currently, various commercial transport devices, such as JEMBEC or Gono-Pak system are available.

Microscopy

Gram staining of urethral exudates reveals gram-negative intracellular kidney-shaped diplococci (Fig. 23.3). Gram staining is highly specific and sensitive in symptomatic men. However in females, it is only 50% sensitive because, the presence of commensal *Neisseria* species may confound with interpretation. Hence, culture is recommended for diagnosis of gonorrhea in women.

Culture

Endocervical culture has a sensitivity of 80–90%. As cervical swabs contain normal flora, hence, selective media are preferred, such as:

- **Thayer Martin medium** is chocolate agar with added vancomycin, colistin and nystatin. It inhibits commensal *Neisseria* species
- **Modified New York City medium:** Lysed blood agar and lincomycin, colistin, trimethoprim and amphotericin B
- **Martin-Lewis medium.**

Inoculated media are incubated at 37°C for 24–48 hours. Colonies on Thayer Martin media are large 1.5–2.5 mm, grey, convex, with crenated margin and raised opaque center. In contrast to meningococci, the colonies of gonococci vary in size with irregular outline.

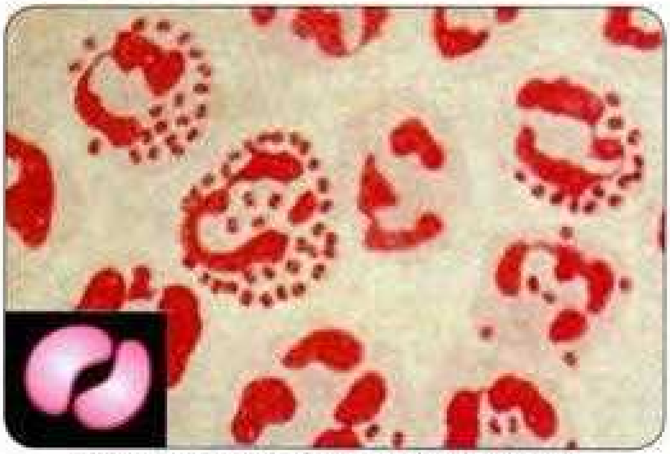


Fig. 23.3: Gonococcus (gram-negative diplococci, kidney-shaped)

Source: Public Health Image Library, ID# /2108, Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

Blood and synovial fluid cultures should be done in suspected cases of DGI on enriched media, such as lysed blood agar (by saponin) and chocolate agar.

Biochemical Tests

Biochemical tests should be carried out to differentiate gonococci from other commensal *Neisseria* species.

- Gonococci are catalase and oxidase positive
- They ferment only glucose, but not maltose and sucrose. This can be tested by rapid carbohydrate utilization test (RCUT).

Molecular Method

PCR assay is available for detection of *N. gonorrhoeae* from the clinical specimens targeting 16s or 23s rRNA gene.

TREATMENT — **Gonococcus**

- **Drug of choice:** Third generation cephalosporins currently are the mainstay of therapy for uncomplicated gonococcal infection. Both the sexual partners should be treated
  - Ceftriaxone (250 mg given IM, single dose)
  - Cefixime (400 mg given orally, single dose).
- If coexisting chlamydial infection is present, then azithromycin or doxycycline can be added to the regimen.

Drug Resistance in *Neisseria gonorrhoeae*

Gonococci were initially susceptible to most antibiotics, such as sulfonamides, penicillins, quinolones, but because of their continual usage, resistance has emerged over the time.

Various resistant strains have evolved in due course of time (Table 23.3). The strains are often resistant to many drugs at a time.

Though third-generation cephalosporins are the drugs of choice for gonococcal infections at present, some strains show reduced susceptibility to ceftriaxone and cefixime (termed as cephalosporin intermediate/ resistant strains), which may be due to altered penicillin-binding protein 2.

Table 23.3: Drug resistance in gonococci

Drug resistance in gonococci	
PPNG	Penicillinase producing strains of *Neisseria gonorrhoeae*: • Originated from Africa and Asia in 1976; later on, spread worldwide • Plasmids coding for β-lactamases are transferred horizontally by conjugation
CMRNG	Chromosomally mediated resistant *N. gonorrhoeae*: • Shows resistance to penicillin and also to tetracycline • It is due to mutations at multiple sites, which decreases the permeability of the cell to antibiotics
TRNG	Tetracycline-resistant *N. gonorrhoeae*; plasmid-borne
QRNG	Quinolone-resistant *N. gonorrhoeae*

Table 23.4: Differences between gonococcal and nongonococcal urethritis

Features	Gonococcal urethritis	Nongonococcal urethritis
Onset	48 hours	Longer (>1 week)
Urethral discharge	Purulent (flow of seed-resembling semen)	Mucous to mucopurulent
Complication	DGI (polyarthritis and endocarditis) Water-can perineum	**Reiter's syndrome:** Characterized by conjunctivitis, urethritis, arthritis and mucosal lesions
Diagnosis	• Gram stain • Culture on Thayer Martin media	• For *Chlamydia*—culture on McCoy and HeLa cell lines • For *Trichomonas*—detection of trophozoite • For *Candida*—detection of budding yeast cells in discharge • For PCR—can be done for HSV or *Chlamydia*
Treatment	Ceftriaxone	• For *Chlamydia*—Doxycycline • For *Trichomonas*—Metronidazole • For *Candida*—Clotrimazole (as vaginal cream or tablet)

Abbreviations: DGI, disseminated gonococcal infection; GU, gonococcal urethritus; NGU, nongonococcal urethritis; HSV, herpes simplex virus; PCR, polymerase chain reaction.

Prophylaxis

There is no vaccination available for gonococci. The general prophylactic measures include:

- ❖ Early detection of cases
- ❖ Treatment of both partners
- ❖ Tracing of contacts
- ❖ Health education about safe sex practices, such as use of condoms.

Nongonococcal (Nonspecific) Urethritis (NGU)

Chronic urethritis where gonococci cannot be demonstrated has been labeled as nongonococcal urethritis. NGU is more common than gonococcal urethritis.

Several agents are implicated in NGU such as:

- ❑ **Bacteria:**
  - ➢ *Chlamydia trachomatis*: Most common agent
  - ➢ *Ureaplasma urealyticum*
  - ➢ *Mycoplasma hominis*
  - ➢ Some cases may be due to gonococcal infection, the cocci persisting as L forms and, hence, undetectable by routine tests.
- ❑ **Viruses:** Herpes simplex virus, and cytomegalovirus
- ❑ **Fungi:** *Candida albicans*
- ❑ **Parasites:** *Trichomonas vaginalis*.

Differences between gonococcal and nongonococcal urethritis are given in Table 23.4.

COMMENSAL NEISSERIA SPECIES

Several species of neisseriae are harmless commensals of human respiratory tract; however, occasionally they cause human disease. They can be differentiated from pathogenic neisseriae by various ways, such as:

- ❖ They can grow on basal media, such as nutrient agar
- ❖ They can grow at 22°C
- ❖ Mostly, they do not grow on selective media (except *N. lactamica*)
- ❖ Not capnophilic (CO_2 is not required)
- ❖ Ferment a number of carbohydrates
- ❖ ONPG test for β-galactosidase is positive by *N. lactamica*
- ❖ Some are pigmented: *N. flava* and *N. flavescens*
- ❖ Some are capsulated: *N. mucosa*
- ❖ Some are rod-shaped: *N. elongata* and *N. weaveri*.

MORAXELLA

Moraxella includes *M. catarrhalis* and *M. lacunata*.

Moraxella catarrhalis

Moraxella catarrhalis (previously classified under *Branhamella* or *Neisseria*) is a harmless commensal of upper respiratory tract and genital tract.

- ❖ **Morphology:** Gram-negative diplococci, 0.6–1 µm oval with flattened adjacent sides
- ❖ **Culture:** It grows on basal medium like nutrient agar
- ❖ **Biochemical reactions:** It is catalase and oxidase positive. It differs from pathogenic *Neisseria* as:
  - ▪ It does not ferment any carbohydrate
  - ▪ Gives a positive tributyrin hydrolysis test
  - ▪ Shows DNase test positive.
- ❖ **Pathogenesis:** It causes opportunistic lower respiratory tract infections, especially in adults with chronic obstructive airway disease
- ❖ It has also been isolated in cases of otitis media, less commonly in meningitis, endocarditis and sinusitis
- ❖ Some strains of *M. catarrhalis* secrete beta-lactamases which destroy penicillin that makes β-lactam antibiotics ineffective to meningococci and other penicillin-sensitive bacteria of the respiratory tract.

Moraxella lacunata

It is also called Morax-Axenfeld bacillus. It is non-fermenting gram-negative rod-shaped and generally present as pairs. It causes catarrhal conjunctivitis, and angular conjunctivitis.

EXPECTED QUESTIONS

I. Essay:

1. Describe the virulence factors, pathogenesis and laboratory diagnosis of *Neisseria meningitidis*.
2. Kallu, a 25-year-old heterosexual male from Chennai came with history of dysuria and noted some 'pus-like' drainage in his underwear and at the tip of his penis. He gave a history of being sexually active with five or six partners in the past 6 months. His physical examination showed yellow urethral discharge and tenderness at the tip of the penis. Examination of the urethral discharge revealed intracellular gram-negative diplococci with plenty of pus cells.
   a. What is the clinical diagnosis and the causative agent?
   b. Describe the pathogenesis of this condition.
   c. How will you confirm the diagnosis?

II. Write short notes on:

1. Meningococcal vaccines.
2. Nongonococcal urethritis (NGU).

III. Multiple Choice Questions (MCQs):

1. The most common mode of transmission of gonorrhea is:
a. Venereal mode b. Injection
c. Blood transfusion d. Inhalation

2. All of the following are causative agents of NGU, *except*:
a. *Chlamydia trachomatis*
b. *Mycoplasma hominis*
c. *Candida albicans*
d. Meningococci

3. All of the following meningococcal serogroups cause invasive disease, *except*:
a. W135 b. A
c. D d. Y

4. Gonococci can be differentiated by meningococci by following sugar fermentation test:
a. Glucose b. Sucrose
c. Mannitol d. Maltose

5. Serogrouping of meningococcus is based on-
a. Outer membrane proteins
b. Endotoxin
c. Capsular polysaccharide
d. Transferrin binding proteins

6. Serotyping and serosubtyping of meningococci are based on:
a. Outer membrane proteins
b. Endotoxin
c. Capsular polysaccharide
d. Transferrin binding proteins

7. Meningococci are divided into how many serogroups?
a. 7 b. 9
c. 11 d. 13

8. Which serogroup of meningococcus can cause hyperendemic disease?
a. A b. B
c. C d. F

9. Which serogroup of meningococcus had caused major outbreak in Hajj pilgrimage?
a. A b. B
c. W d. E29

10. Which is not a risk factor for meningococcus infection?
a. Overcrowding and semiclosed communities
b. Travelers c. Smoking
d. Deficiency of early complement components

11. Waterhouse-Friderichsen syndrome, all are true, *except*:
a. Caused by meningococcus
b. Bilateral adrenal haemorrhage is seen
c. Purpura fulminans is a feature
d. Pathogenesis is due to capsule

12. Most common route of spread of meningococci from nasopharynx to reach the meninges:
a. Hematogenous route
b. Direct spread along olfactory nerve through cribriform plate
c. Through conjunctiva
d. Via lymphatics

13. Drug of choice for chemoprophylaxis for meningococcus is:
a. Ceftriaxone b. Rifampicin
c. Ciprofloxacin d. Penicillin

14. Capsular vaccine for meningococci is available for all serogroups, *except*:
a. A b. B
c. C d. Y

15. Meningococcal vaccine is indicated in all the following conditions, *except*:
a. Contacts of patients during outbreaks
b. Splenic dysfunction
c. Terminal complement component deficiency
d. Children <2 years

16. Vaccine for group B contains all, *except*:
a. Adhesin A
b. Heparin binding antigen
c. Outer membrane vesicles
d. Capsular polysaccharide

17. Gonococcal infection in females, all are true, *except*:
a. Less severe than male
b. Mucopurulent cervicitis is the most common presentation
c. Vulvovaginitis is seen frequently
d. Fitz-Hugh-Curtis syndrome seen

18. Transport Media used for gonococci is:
a. Stuart's medium b. Pike's medium
c. VR medium d. Cary Blair medium

Answers

1. a **2.** d **3.** c **4.** d **5.** c **6.** a **7.** d **8.** b **9.** c **10.** d **11.** d **12.** a **13.** a **14.** b **15.** d **16.** d **17.** c **18.** a

Corynebacterium

24
CHAPTER

Chapter Preview

- *Corynebacterium diphtheriae*
- Other coryneform bacteria

Corynebacteria are gram-positive, non-capsulated, non-sporing, non-motile rods. They are irregularly stained and frequently show **club-shaped swellings** (Fig. 24.1A) (Greek word *koryne*, meaning club). *Corynebacterium diphtheriae*, the causative agent of **diphtheria** is the most important species pathogenic to man; other species are occasionally pathogenic, such as *C. ulcerans* and *C. pseudotuberculosis*.

CORYNEBACTERIUM DIPHTHERIAE

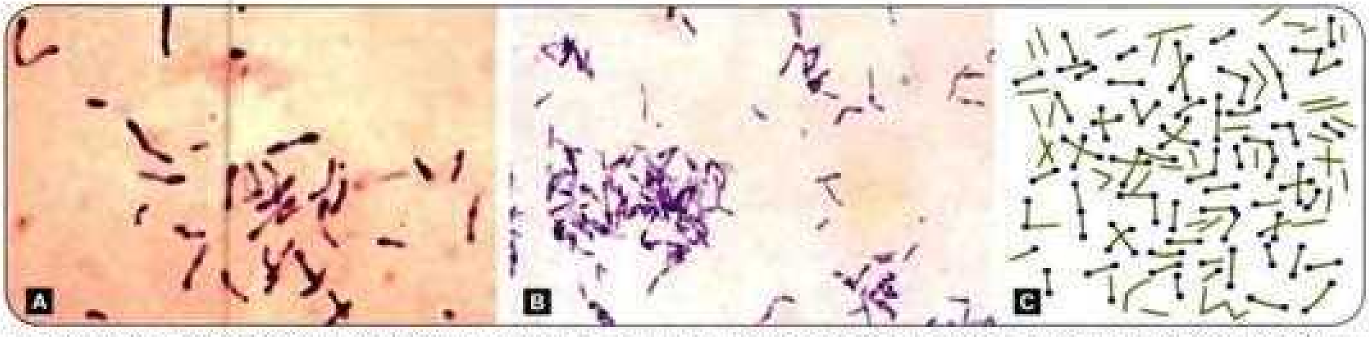
Corynebacterium diphtheriae is club-shaped, irregularly stained gram-positive pleomorphic rod that typically shows two characteristic features:

1. **Chinese letter or cuneiform** arrangement: They appear as V- or L-shaped in smear, because the bacterial cells divide and daughter cells tend to lie at acute angles to each other. This type of cell division is called **snapping type of division** (Fig. 24.1B).
2. **Metachromatic granules:** They are present at ends or poles of the bacilli (also called polar bodies or Babes-Ernst bodies or volutin granules).
   - They are storage granules of the organism, composed of polymetaphosphates
   - Granules are stained strongly gram-positive compared to remaining part of the bacilli. The granules take up bluish purple metachromatic color when stained with Loeffler's methylene blue
   - However, they are better stained with special stains, such as Albert's, Neisser's and Ponder's stain (Fig. 24.1C)
   - Granules are well developed on enriched media, such as blood agar or Loeffler's serum slope
   - Volutin granules can also be possessed by other organisms such as—by *C. xerosis* and *Gardnerella vaginalis*.

History

Diphtheria is an ancient disease, known since the time of Hippocrates.

- Diphtheria was first recognized by Pierre Bretonneau (1826). He used the term diphthérite (Greek word *diphtheros*—meaning leather like) to describe the characteristic manifestation, i.e. leathery pseudomembrane formation over tonsil



Figs 24.1A to C: *Corynebacterium diphtheriae*. **A.** Club-shaped bacilli in methylene blue-stained smear; **B.** Gram-stained smear shows V- or L-shaped bacilli with cuneiform arrangement; **C.** Albert's stain shows dark blue metachromatic granules at the ends of the bacilli (schematic)

Source: Public Health Image Library, A. ID# /7323/P.B. Smith, B. ID# /1943, Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*).

- *C. diphtheriae* was first observed by Klebs (1883) and first cultivated by Loeffler (1884); hence, it is known as **Klebs-Loeffler bacillus**
- Roux and Yersin (1888) established the pathogenic effect of diphtheria toxin; subsequently, its antitoxin was described by Von Behring (1890).

Virulence Factors (Diphtheria Toxin)

Diphtheria toxin (DT) is the primary virulence factor responsible for diphtheria.

- The toxin is synthesized in precursor form of molecular weight 58,700 Da (Daltons) containing a polypeptide made up of 535 amino acids
- Toxin has two fragments; A (active) and B (binding) of molecular weight 21,500 and 37,200 Da respectively
- Fragment B is the binding fragment which binds to the host cell receptors (such as epidermal growth factor) and helps in entry of fragment A
- Fragment A is the active fragment, gets internalized into the cell and then acts by the mechanism given below.

Mechanism of Diphtheria Toxin (DT)

- Fragment A is the active fragment, which causes ADP ribosylation of elongation factor 2 (EF-2) → leads to inhibition of EF-2 → leads to inhibition of translation step of protein synthesis
- Exotoxin A of *Pseudomonas* has a similar mechanism like that of DT.

Factors Regulating Toxin Production

The production of diphtheria toxin is dependent on various factors.

- **Phage coded:** DT is coded by a bacteriophage called β-corynephage, carrying *tox* gene. *C. diphtheriae* remains toxigenic as long as the phages are present inside the bacilli (lysogenic conversion)
- **Iron concentration:** The toxin production depends on optimum concentration of iron (0.1 mg per liter). Higher level of iron inhibits toxin synthesis by upregulating DT repressor gene in the bacterial chromosome
- **DT repressor** gene (DtxR): It is an iron-dependent negative regulator of DT production and iron uptake in *C. diphtheriae*
- **Biotypes:** Among the three biotypes of *C. diphtheriae*, (described later, Table 24.1) all strains of gravis, 95–99% strains of intermedius and 80–85% of mitis strains are toxigenic. However, toxins produced by different biotypes are antigenically similar
- **Other species:** DT is also produced by *C. ulcerans* and *C. pseudotuberculosis*.

Toxoid is used for Vaccination

Diphtheria toxin is antigenic and antitoxins are protective in nature. However, as it is virulent, it cannot be given directly for vaccination.

- Toxin can be converted to toxoid which is used for vaccination. Toxoid is a form of toxin, where the virulence is lost, retaining its antigenicity
- Toxoid formation is promoted by formalin, acidic pH and prolonged storage
- **Park William 8** strain of *C. diphtheriae* is used as a source of toxin for the preparation of vaccine
- **LF unit:** DT is expressed as Loeffler's flocculating (Lf) unit. 1 Lf unit is the amount of toxin which flocculates most rapidly with one unit of antitoxin.

Pathogenicity and Clinical Manifestations

Pathogenesis of diphtheria is toxin mediated.

- Diphtheria is toxemia but never a bacteremia
- Bacilli are noninvasive, present only at local site (pharynx), secrete the toxin which spreads via bloodstream to various organs
- It is the toxin which is responsible for all types of manifestations including local (respiratory) and systemic complications (except the skin lesions, which is caused due to the organism, not toxin).

Respiratory Diphtheria

This is the most common form of diphtheria. Tonsil and pharynx (faucial diphtheria) are the most common sites followed by nose and larynx. Incubation period is about 3–4 days.

- **Faucial diphtheria:** Diphtheria toxin elicits an inflammatory response, that leads to necrosis of the epithelium and exudate formation
  - This leads to formation of mucosal ulcers, lined by a tough leathery greyish white **pseudomembrane coat;** composed of an inner band of fibrin surrounded by neutrophils, RBCs and bacteria (Fig. 24.2A)
  - It is so named as it is adherent to the mucosal base and bleeds on removal, in contrast to the true membrane which can be easily separated.
- **Extension of pseudomembrane:** In severe cases, it may extend into the larynx and bronchial airways, which may result in fatal airway obstruction leading to asphyxia. This mandates immediate tracheostomy
- **Bull-neck appearance:** It is characterized by massive tonsillar swelling and neck edema. Patients present with foul breath, thick speech, and stridor (noisy breathing) (Fig. 24.2B).

Cutaneous Diphtheria

It presents as punched-out ulcerative lesions with necrosis, or rarely pseudomembrane formation; most commonly occurs on the extremities (Fig. 24.3).

- Cutaneous diphtheria is due to the organism itself and is not toxin-mediated. Hence it is possible that, the skin lesions may also be caused by nontoxigenic strains

Figs 24.2A and B: A. Pseudomembrane covering the tonsils classically seen in diphtheria and **B.** Bull neck appearance (arrow showing)

Source: A. Department of Microbiology, JIPMER, Puducherry and B. Public Health Image Library/ID#5325, Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

Fig. 24.3: Cutaneous diphtheria

Source: Public Health Image Library,ID# /1941, Centers for Disease Control and Prevention (CDC), Atlanta (with permission)

- There is increasing trend of cutaneous diphtheria nowadays, especially in vaccinated children; because antitoxins present in vaccinated people cannot prevent the disease.

Systemic Complications

Polyneuropathy and myocarditis are the late toxic manifestations of diphtheria, occurring after weeks of infection. Other complications of diphtheria include pneumonia, renal failure, encephalitis, cerebral infarction, and pulmonary embolism.

- **Neurologic manifestations:** It is a toxin mediated non-inflammatory demyelinating disorder; presented with:
  - Cranial nerve involvement
  - Peripheral neuropathy
  - Ciliary paralysis
- **Myocarditis:** It is typically associated with arrhythmias and dilated cardiomyopathy.

LABORATORY DIAGNOSIS ***Corynebacterium diphtheriae***

Isolation of the *Corynebacterium diphtheriae*

- **Specimen:** Throat swab and a portion of membrane
- **Direct smear**
  - Gram-stain: Club shaped gram-positive bacilli with Chinese letter arrangement
  - Albert's stain: Green bacilli with bluish black metachromatic granules.
- **Culture media**
  - Enriched medium: Blood agar, chocolate agar and Loeffler's serum slope
  - Selective medium: Potassium tellurite agar and Tinsdale medium, produces black colonies.
- **Biochemical identification**
  - Hiss's serum sugar media: Ferments glucose, maltose and/ or starch
  - Urease test negative.

Diphtheria Toxin Demonstration

- *In vivo* tests (Guinea pig inoculation): Subcutaneous and intracutaneous tests
- *In vitro* tests
  - Elek's gel precipitation test
  - Detection of *tox* gene-by PCR
  - Detection of toxin-by ELISA or ICT
  - Cytotoxicity on cell lines.

Laboratory Diagnosis

The diagnosis of diphtheria is based on clinical signs and symptoms plus laboratory confirmation.

- Because of the risk of respiratory obstruction, specific treatment should be instituted immediately on clinical suspicion without waiting for laboratory reports
- Laboratory diagnosis is necessary only for:
  - Confirmation of clinical diagnosis
  - Initiating the control measures
  - Epidemiological purposes.

Laboratory diagnosis consists of isolation of the bacilli and toxin demonstration.

Isolation of Diphtheria Bacilli

Specimen

Useful specimens include: (1) throat swab (one or two) containing fibrinous exudates, (2) a portion of pseudomembrane (3) nose or skin specimens (if infected).

Direct Smear Microscopy

- **Gram-stain:** *C. diphtheriae* appear as irregularly stained club-shaped gram-positive bacilli of 3-6 μm length, typically arranged in **Chinese letter** or **cuneiform** arrangement (V- or L-shaped). It is difficult to differentiate them from other commensal coryneforms found in the respiratory tract (see Fig. 24.1B)
- **Albert's stain:** It is more specific for *C. diphtheriae*, as they appear as green bacilli with bluish black metachromatic granules at the poles (see Fig. 24.1C).

Culture Media

Enriched Medium

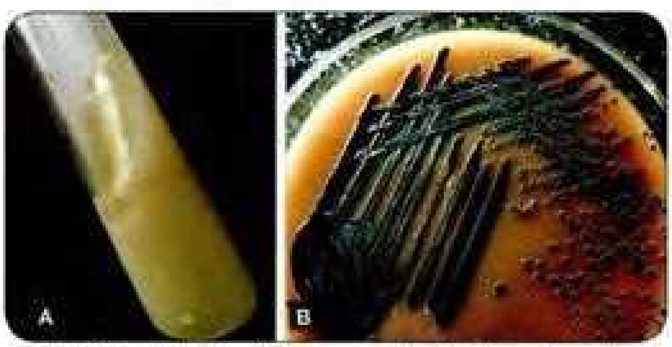
C. diphtheriae is fastidious, aerobe and facultative anaerobe; does not grow on ordinary medium. It grows best in enriched medium such as blood agar, chocolate agar and Loeffler's serum slope. Plates are incubated at 37°C aerobically.

- **Blood agar:** Colonies are small circular, white and sometimes hemolytic (mitis biotype)
- **Loeffler's serum slope:** It is composed of nutrient broth with horse or sheep serum and glucose (Fig. 24.4A)
  - Colonies appear as small, circular, glistening, and white with a yellow tinge in 6–8 hours
  - Advantages: (1) Growth can be detected as early as 6–8 hours. (2) Best medium for metachromatic granules production
  - *Disadvantage*: As it is an enriched medium, if incubated beyond 6–8 hours, it supports growth of other throat commensals also.

Selective Medium

Selective media are best for isolation of *C. diphtheriae* from cases as well as from carriers; as the normal flora will be inhibited. Selective media used are:

- **Hoyle's potassium tellurite agar (PTA):** It contains lysed horse blood agar and potassium tellurite (0.04%) as inhibiting agent
  - *C. diphtheriae* reduces tellurite to metallic tellurium which gets incorporated into the colonies giving them black color (Fig. 24.4B). *C. ulcerans* and *C. pseudotuberculosis* can also grow on PTA producing black colored colonies
  - Advantage: Throat commensals are inhibited
  - Disadvantage: Colonies appear only after 48 hours of incubation.
- **Tinsdale medium:** It is a modified PTA added with cysteine. Black-colored colonies are produced, surrounded by a brown halo, due to breakdown of cysteine by cysteinase enzyme produced by the organism.



Figs 24.4A and B: A. Loeffler's serum slope; **B.** Potassium tellurite agar shows black colonies

Source: A. Department of Microbiology, JIPMER, Puducherry; B. Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (*with permission*).

Biochemical Identification

- **Hiss's serum sugar media:** Diphtheria bacilli ferment glucose and maltose (by all biotypes) and starch (by only gravis biotype), with the production of acid but no gas. As it is fastidious, only enriched sugar media such as Hiss's serum sugar media can be used for testing sugar fermentation test
- **Pyrazinamidase test:** This test is positive by most of the corynebacteria (possessing pyrizinamidase enzyme); however, the exotoxin producing human pathogenic corynebacteria such as, *C. diphtheriae*, *C. ulcerans* and *C. pseudotuberculosis*, give a negative test result as they do not produce this enzyme
- **Urease test:** *C. diphtheriae* does not hydrolyze urea. This property differentiates it from *C. ulcerans* and *C. pseudotuberculosis* which are urease positive
- *Corynebacterium* is catalase positive but oxidase negative and nonmotile.

Toxin Demonstration

As the pathogenesis is due to diphtheria toxin, mere isolation of bacilli does not complete the diagnosis. Toxin demonstration should be done following isolation, which can be of two types—*in vivo* and *in vitro*.

In vivo Tests (animal inoculation)

In vivo toxin demonstration can be done by inoculation of culture broth into **guinea pig**. With the advent of other techniques, this method is rarely followed nowadays.

In Vitro Test

- **Elek's gel precipitation test:** This is a type of immunodiffusion in gel described by Elek (1949)
  - A rectangular strip of filter paper soaked in diphtheria antitoxin (1000 units per mL) is placed on the surface of a 20% horse (or sheep or rabbit) serum agar plate before the medium solidifies
  - When the agar solidifies, the test strain is streaked at right angle to the filter paper strip. The plate is incubated at 37°C for 24–48 hours
  - **Precipitation band:** If the strain is toxigenic, the toxin diffuses in the agar, meets with the antitoxin and produces arrow-shaped precipitation band
  - Nontoxigenic stains will not produce any precipitation line (Fig. 24.5)
  - This test can also be used to know the relatedness between the strains isolated during an outbreak. The precipitate lines would fuse with each other if the toxins produced by the strains are identical (refer Figure 12.4 of Chapter 12)
- **Other *in vitro* tests** include:
  - Detection of *Tox* gene by PCR
  - Detection of diphtheria toxin by ELISA or immunochromatographic test (ICT)
  - Cytotoxicity produced on cell lines.

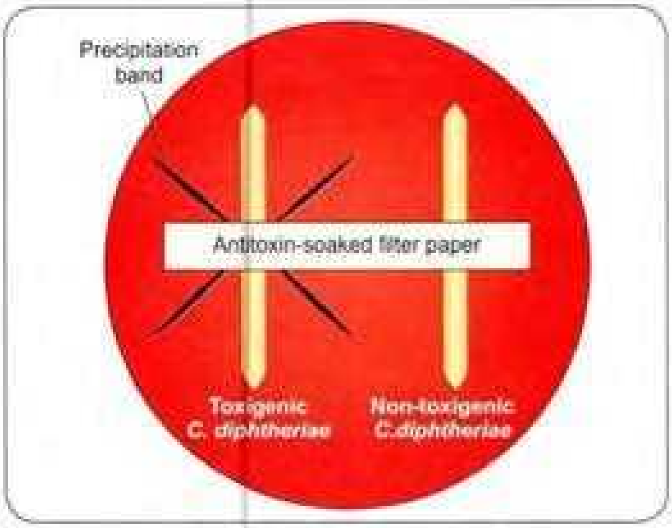


Fig. 24.5: Elek's gel precipitation test

Typing of *C. diphtheriae*

Typing methods are useful for epidemiological studies, to know the relatedness between the isolates. Several methods are available for typing of *C. diphtheriae*.

- **Biotyping (McLeod's classification):** *C. diphtheriae* can be typed into four biotypes such as gravis, intermedius, mitis and belfanti based on various properties (Table 24.1). Biotype belfanti is a nitrate negative variant of mitis biotype
- **Other methods:** such as serotyping, bacteriophage typing, bacteriocin typing and molecular typing (by pulse field gel electrophoresis).

Epidemiology

Worldwide, there is declining trend of diphtheria cases in most countries including India, due to widespread vaccination coverage.

- **Source of infection:** Carriers (95%) are more common source of infection than cases (5%)
- **Carriers:** They may be temporary (persist for a month) or chronic (persist for a year). Nasal carriers are more dangerous due to frequent shedding than throat carriers. Incidence of carrier rate varies from 0.1% to 5%
- **Transmission** is via the aerosol route, or rarely by contact with infected skin lesions
- **Reservoir:** Humans are the only reservoir
- **Age:** Diphtheria is common in children aged 1–5 years. With wide spread immunization, a shift in the age has been observed from preschool to school age. Newborns are usually protected due to maternal antibodies
- **Situation in world:** Due to wide spread immunization, cases were drastically declined by >95% over last 3 decades. Currently around 5000-5500 cases occur per year worldwide (since 2005)

Table 24.1: Biotypes of *Corynebacterium diphtheriae* (McLeod's classification)

	Biotypes		
Properties	**Gravis**	**Intermedius**	**Mitis**
Morphology	Short, no granules	Long barred, poor granules	Long curved, prominent granules
Colonies on PTA	**'Daisy head'** colony	**'Frog's egg'** colony	**'Poached egg'** colony
Colony consistency	Brittle, not emulsifiable	In-between gravis and mitis	Soft, buttery, emulsifiable
Starch fermentation	+ve	-ve	-ve
Toxigenic strains	100%	95–99%	80–85%
Virulence	Severe	Moderate	Mild
Occurrence	Epidemic	Epidemic	Endemic
Complications	Paralytic and hemorrhagic	Hemorrhagic	Obstructive
Hemolysis	Variable	Non-hemolytic	Hemolytic

Abbreviation: PTA, potassium tellurite agar.

- **Russian epidemic:** An epidemic of adult diphtheria occurred in Russia during 1990–97 with > 1.1 Lakh cases and 3000 deaths. It was controlled by improved vaccination of children and adults
- **Situation in India:** India still accounts for the highest burden (60–70%) of diphtheria cases in the world
  - In 2016, nealry 3380 cases have been reported; highest bruden from Uttar Pradesh, Kerala and Karnataka. Recent outrbreaks have been reported from North Kerala and North Karnataka in 2015-16
  - Maximum cases belonged to > 5-10 years; which explains poor vaccination still remains the most important cause; followed by waning immunity leading to adult diphtheria.

TREATMENT ***Corynebacterium diphtheriae***

Treatment should be started immediately on clinical suspicion of diphtheria.

- **Antidiphtheritic serum or ADS (antitoxin):** Passive immunization with antidiphtheritic horse serum is the treatment of choice as it neutralizes the toxin
  - A trial dose should be given to check for hypersensitivity
  - It is given either IM or IV and the dose varies depending on the severity
    - Mild, early pharyngeal cases: 20,000–40,000 units
    - Moderately severe cases: 40,000–60,000 units
    - Severe, extensive or late cases (>3days): 80,000–1,00,000 units.
- **Antibiotics:** Penicillin or erythromycin is the drug of choice. Antibiotic plays a minor role as it is of no use once the toxin is secreted. However, antibiotics are useful:
  - If given early (<6 hrs of infection), before the toxin release
  - Prevent further release of toxin by killing the bacilli
  - Treatment of cutaneous diphtheria
  - Treatment of carriers: Drug of choice is erythromycin.

Prophylaxis

Infection Control Measures

Patient should be kept in isolation room and all the steps of droplet precaution should be followed for the prevention of transmission of *C.diphtheriae* in hospitals (refer chapter 53).

Vaccination

Active immunization is done with diphtheria toxoid as it induces antitoxin production in the body. A protective titer of more than 0.01 Unit/mL of antitoxin can prevent all forms of diphtheria.

However, vaccine is not effective for:
- Prevention of cutaneous diphtheria
- Elimination of carrier state.

Types of Vaccine

- **Single vaccine:** Diphtheria toxoid (alum or formal precipitated)
- **Combined vaccine:** Various vaccines available are:
  - DPT: Contains **D**T (diphtheria toxoid), **P**ertussis (whole cell) and **T**T (tetanus toxoid)
  - DaPT: Contains DT, TT and acellular pertussis (aP)
  - DT: Contains DT and TT
  - dT: Contains TT and adult dose diphtheria toxoid (d).
  - Pentavalent vaccine: DPT can also be given along with hepatitis B and *Haemophilus influenzae* type b.

DPT Vaccine

Among the vaccine preparations available, DPT is the preparation of choice for vaccinating infants, because:
- Infants can be immunized simultaneously against three important childhood diseases—diphtheria, tetanus and pertussis by single injection
- **Pertussis** component acts as **adjuvant** and increases immunogenicity of DT and TT.

Types

Diphtheria toxoid is prepared by two methods:
1. **Plain formol toxoid** (or fluid toxoid): Toxoid is prepared by incubating toxin with formalin.
2. **Adsorbed (alum adsorbed):** Formol toxoid is adsorbed on to alum. Alum (Aluminum phosphate, to less extent Aluminum hydroxide) acts as adjuvant and increases the immunogenicity of toxoid.

Administration of DPT

- **Schedule:** Under National Immunization Schedule (NIS) of India 2018, total five doses are given. Three doses of pentavalent vaccine at 6, 10 and 14 weeks of birth; followed by two booster doses of DPT at 16–24 months and 5 years.
- **Site:** DPT is given deep intramuscularly (IM) at antero-lateral aspect of thigh, (gluteal region is not preferred as fat may inhibit DPT absorption)
- **Thiomersal** (0.01%) is used as preservative
- **Storage:** DPT should be kept at 2–8°C, if accidentally frozen then it has to be discarded
- **Dose:**

  One dose (0.5 mL) of vaccine contains:
  - **Glaxo:** 25 Lf (DT), 5 Lf (TT), 20,000 million (pertussis killed bacilli)
  - **Kasauli:** 30 Lf (DT), 10 Lf (TT), 32,000 million (pertussis killed bacilli).

- **Protective titer:** Following vaccination, an antitoxin titer of ≥ 0.01 unit/mL is said to be protective
- **Td:** It contains TT and adult dose (2 Lf) of diphtheria toxoid. It is recommended after 7 years
- **Tdap:** It is an adult tetanus-diphtheria-acellular pertussis vaccine. This can be given safely to older children, as this form of pertussis vaccine is devoid of neurological complication
- **Adult immunization:** As adult diphtheria cases are increasingly being reported, adult immunization by Td vaccine has been recommended
  - For adults >18 years who have completed their primary vaccination schedule, a booster dose of Td vaccine is indicated once in every 10 years till the age of 65
  - For adults >18 years who have not completed their primary vaccination schedule: 3 doses of Td given at 0, 1 month, and 1 year.

Adverse Reactions following DPT Administration

- **Mild:** Fever and local reaction (swelling and indurations) are observed commonly
- **Severe:** Whole cell killed vaccine of *B. pertussis* is encephalitogenic. It is associated with neurological complications. Hence, DPT is not recommended after 6 years of age
- **Absolute contraindication to DPT:**
  - Hypersensitivity to previous dose
  - Progressive neurological disorder.

Schick Test

It is a toxin-antitoxin neutralization test, was used long back, to test the susceptibility of individual to diphtheria before starting immunization. It is obsolete now.

DIPHTHEROIDS OR NONDIPHTHERIAL CORYNEBACTERIA

Diphtheroids

Diphtheroids or coryneforms are the nondiphtherial corynebacteria, that usually exist as normal commensals in the throat, skin, conjunctiva and other areas. However, they have been associated with invasive disease, particularly in immunocompromized patients. They can be differentiated from *C. diphtheriae* by many features such as:
- Stain more uniformly than *C. diphtheriae*

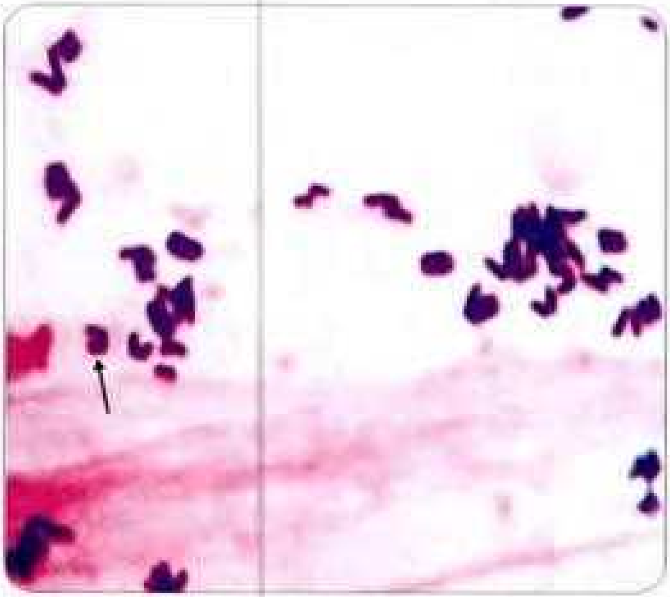


Fig. 24.6: Diphtheroids—Palisade arrangement of gram-positive bacilli

Source: Department of Microbiology, JIPMER, Puducherry (with permission).

- Palisade arrangement: Arranged in parallel rows rather than cuneiform pattern (Fig. 24.6)
- Absence of metachromatic granules (except *C. xerosis*)
- Toxigenicity or virulence test: Negative
- Biochemical reactions may be different from *C. diphtheriae*: e.g. ferments sucrose (*C. xerosis*).

Coryneforms that are rarely pathogenic to man are:

- **Clinically resembling diphtheria:** *C. ulcerans* and *C. pseudotuberculosis* produce diphtheria toxin and cause localized ulcerations in throat, clinically resembling diphtheria. They also produce black colored colony with brown halo on Tinsdale medium similar to *C.diphtheriae*. However, both give a positive urease test, thus can be differentiated from *C.diphtheriae* which is urease negative
  - *C. ulcerans* causes infections in cows. Human infections may occur through cow's milk
  - *C. pseudotuberculosis* (Preisz-Nocard bacillus) causes pseudotuberculosis in sheep and suppurative lymphadenitis in horses. Human infection is very rare.
- ***C. minutissimum:*** It causes a localized infection of skin (axilla and groin), called as '*erythrasma*'. On Wood's lamp examination, erythrasma lesions emit coral red color
- ***C. tenuis:*** It has been associated with *Trichomycosis axillaris*, characterized by the formation of pigmented nodules around axillary and pubic hair shafts
- ***C. jeikeium:*** It is lipophilic species, colonizes skin of hospitalized patients. It can cause bacteremia, endocarditis and meningitis, especially in immunocompromized hosts. It is usually multidrug resistant, responds only to vancomycin
- ***C. urealyticum:*** It is skin commensal, rarely causes urinary tract infection (pyelonephritis) and alkaline encrusted cystitis (struvite stones in alkaline urine) in immunocompromized and renal transplant recipient
- ***C. amycolatum:*** It differs from other corynebacteria as it lacks mycolic acid in the cell wall. It rarely causes human infections such as catheter related infection and surgical site infection
- ***C. pseudodiphtheriticum:*** It is a known commensal in throat. However, in immunocompromized patients, it can cause exudative pharyngitis (may mimic respiratory diphtheria) and endocarditis. It can be easily differentiated biochemically as it is pyrazinamidase test positive, urease positive and does not ferment glucose
- ***C. striatum:*** It is a commensal on human skin and pharynx and also in cattle, rarely infects man
- ***C. parvum*** is frequently used as an immunomodulator
- ***Arcanobacterium haemolyticum*** (formerly placed under *Corynebacterium*): It can cause pharyngitis and skin ulcers. It is β hemolytic, produces a positive reverse CAMP test.

EXPECTED QUESTIONS

I. Essay:

1. A child aged 7 years with high grade fever, toxic, pain in the throat, inability to swallow was brought to the casualty. On examination, a white patch was found on the fauces. No history of immunization is available.
   a. Describe the pathogenesis of the condition.
   b. Write in detail about the laboratory diagnosis of this condition.
   c. Discuss the management of this condition.

II. Write short notes on:

1. DPT vaccines.
2. Diphtheria toxin.

III. Multiple Choice Questions (MCQs):

1. **Production of early metachromatic granules can be seen best in which of the following media:**
   a. Nutrient agar b. Chocolate agar
   c. Loeffler's serum slope
   d. Potassium tellurite agar
2. **Which of the following site is most commonly affected by *C. diphtheriae*?**
   a. Skin b. Conjunctiva
   c. Faucial d. Kidney
3. **Metachromatic granules of *Corynebacterium diphtheriae* can be stained by all of the following special stains, *except*:**
   a. Neisser's stain b. Ziehl-Neelsen stain
   c. Albert's stain d. Ponder's stain
4. **Diphtheria toxin is produced by all, *except*:**
   a. *C. diphtheriae* b. *C. ulcerans*
   c. *C. pseudotuberculosis*
   d. *C. xerosis*

Answers

1. c **2.** c **3.** b **4.** d

Bacillus

25
CHAPTER

Chapter Preview

- *Bacillus anthracis*
- Other *Bacillus* species of human importance

Gram-positive spore forming bacilli belong to two genera:

1. *Bacillus:* They are obligate aerobes; having non bulging spores.
2. *Clostridium:* They are obligate anaerobes with bulging spores.

Bacillus species are obligate aerobic gram-positive spore forming rods. *B. anthracis* and *B. cereus* are the only pathogenic species; other members are ubiquitous, present in soil, dust, air and water, and are also frequently isolated as contaminants in bacteriological culture media. *Bacillus* species are generally motile (with peritrichous flagella) and non-capsulated except anthrax bacillus, which is non-motile and capsulated.

BACILLUS ANTHRACIS

Bacillus anthracis is the causative agent of an important zoonotic disease called **anthrax**. It also gained importance recently because of its ability to be used as biological weapon.

They are gram-positive, large rectangular rods (3-10 µm × 1-1.6 µm) arranged in chains, non-motile and capsulated bearing non-bulging oval spores.

Historical Importance

Considerable historical interest is attached to anthrax bacillus due to the following reasons:

- It was the first pathogenic bacterium seen under microscope (by Pollender, 1849)
- Anthrax was the first communicable disease shown to be transmitted by inoculation of infected blood
- It was the first bacterium to be isolated in pure culture by Robert Koch (1876) and the Koch's postulates were made, based on *B. anthracis*
- Anthrax vaccine was the first live attenuated bacterial vaccine prepared (by Louis Pasteur, 1881).

Virulence Factors and Pathogenesis

Pathogenesis of anthrax is due to two important virulence factors—anthrax toxin and capsule.

Anthrax Toxin

It is a tripartite toxin, consisting of three fragments.

1. **Edema factor:** It is the active fragment; acts as adenylyl cyclase and increases host cell cAMP (cyclic adenosine monophosphate). It is responsible for edema and other manifestations seen in anthrax.
2. **Protective factor:** It is the binding fragment that binds to the host cell receptors and facilitates the entry of other fragments into the host cells.
3. **Lethal factor:** It causes cell death; acts by cleaving host cell MAPK (mitogen-activated protein kinases).

These fragments are not toxic individually, but in combination, they produce local edema and generalized shock. Toxin synthesis is controlled by a plasmid (pX01). Loss of plasmid makes the strain avirulent. This was probably the basis of original anthrax vaccine prepared by Pasteur.

Anthrax Capsule

B. anthracis has a polypeptide capsule, made up of polyglutamate (in contrast to the polysaccharide capsule present in most of the other capsulated bacteria).

- Capsule is plasmid (pX02) coded
- It inhibits complement mediated phagocytosis.

Clinical Manifestations

Animal Anthrax

Anthrax is primarily a zoonotic disease. Herbivorous animals such as cattle, sheep and less often horses and pigs are affected more commonly than the carnivorous animals.

Table 25.1: Differences between cutaneous anthrax and pulmonary anthrax

Features	Cutaneous anthrax	Pulmonary anthrax
Other name	**Hide porter's disease** (As it commonly occurs in dock workers carrying loads of hides and skins on their bare backs)	**Wool sorter's disease** (As it is seen in workers of wool factory, acquire infection by inhalation of dust from infected wool)
Transmission	Cutaneous exposure to spores (enter through abraded skin)	Inhalation of spores
Characterized by	**Malignant pustule (Fig. 25.1)** The lesion begins as a papule that evolves into a painless vesicle followed by the development of a coal-black, necrotic **eschar** surrounded by non-pitting indurated **edema** • The name anthrax, which means coal, comes from the black color of the eschar • However, it is a non-malignant condition	**Hemorrhagic pneumonia** Bacilli spread by lymphatics or blood, leading to— • Bacteremia • Hemorrhagic mediastinitis • Hemorrhagic meningitis
Occupational exposures	Dock worker, butcher, abattoir and farmer	Workers of wool factory
Occurrence	Most common (95%)	Rare
Prognosis	Self-limiting, rarely becomes fatal if untreated	Fatal
Bioterrorism	Rarely associated with bioterrorism	Most common form of anthrax to be associated with bioterrorism

- Infection occurs in susceptible animals by ingestion of the spores present in the soil. Direct spread from animal to animal is rare
- Anthrax in animals presents as a fatal septicemia; however, localized cutaneous lesions may be produced rarely
- Infected animals discharge large number of bacilli from the mouth, nose and rectum. These bacilli sporulate in soil and remain as the source of infection for man.

Human Anthrax

Transmission

Human beings acquire infection by:
- Cutaneous mode—by spores entering through the abraded skin; seen in people with occupational exposure to animals (most common mode)
- Inhalation of spores
- Ingestion of carcasses of animals dying of anthrax containing spores (manifested as bloody diarrhea).

Clinical Types

There are mainly three types of human anthrax.
1. Cutaneous anthrax (described in Table 25.1).
2. Pulmonary anthrax (described in Table 25.1).
3. Intestinal anthrax: It is rare; occurs due to ingestion of spores contaminated with meat of animals dying of anthrax. It is highly fatal and manifests as bloody diarrhea.

Agent of Bioterrorism

B. anthracis is one of the most common agent of bioterrorism. It has been widely used in various biological warfare, such as outbreaks in Sverdlovsk in 1979 and in United States in 2001.
- Pulmonary anthrax is the most common form to cause bioterrorism outbreaks
- Transmission occurs via inhalation of anthrax spores from contaminated animal products
- Though it has a high fatality, with prompt initiation of antibiotic therapy, survival is possible.

Epidemiology

Animal anthrax: It is primarily a disease of herbivorous animals. However, due to effective control measures, there has been a progressive global reduction in livestock anthrax cases over the past three decades.
- Both **enzootic** (endemic) and **epizootic** (epidemic) forms of the disease occur in India, especially in Andhra-Tamil Nadu border, foci in Karnataka, and in West Bengal

Fig. 25.1: Malignant pustule

Source: Public Health Image Library, ID# 1934/Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

- Cycle in animals: Anthrax spores from the carcasses of dead animals remain viable in soil for decades. Grazing animals acquire the spores through abraded skin and the cycle continues.

Human anthrax: Incidence of human anthrax is highest in Africa, and Central and Southern Asia. Human anthrax cases may be of two types:

1. Non-industrial cases: These result from agricultural exposure to animals.
2. Industrial cases: These result from infected animal products such as hides, hair, bristles and wools.

LABORATORY DIAGNOSIS *Bacillus anthracis*

- **Specimen:** Pus, sputum, blood, CSF
- **Direct demonstration**
  - Gram staining: Gram-positive, large rectangular bacilli
  - McFadyean's reaction: Shows amorphous purple capsule surrounding blue bacilli (polychrome methylene blue stain)
  - Direct IF: Detects capsular antigen
  - Ascoli's thermo precipitation test.
- **Culture**
  - Nutrient agar: Medusa head appearance colonies
  - Blood agar: Dry wrinkled, nonhemolytic colonies
  - Gelatin stab agar: Inverted fir tree appearance growth
  - Selective media:
    - Solid medium with penicillin: String of pearl appearance in culture smear
    - PLET medium.
- **Culture smear**
  - Gram-staining: Reveals Gram-positive rods with bamboo stick appearance
  - Spore staining (Ashby's method and acid fast staining)
  - Lipid granules staining with Sudan black B.
- Antibodies detection by ELISA
- Molecular diagnosis: PCR using using *BA pX01/02* primers
- Molecular typing: MLVA or AFLP.

Laboratory Diagnosis

There is high risk of laboratory acquired infection of anthrax, hence utmost precautions should be taken and specimens should be processed in appropriate biological safety cabinets.

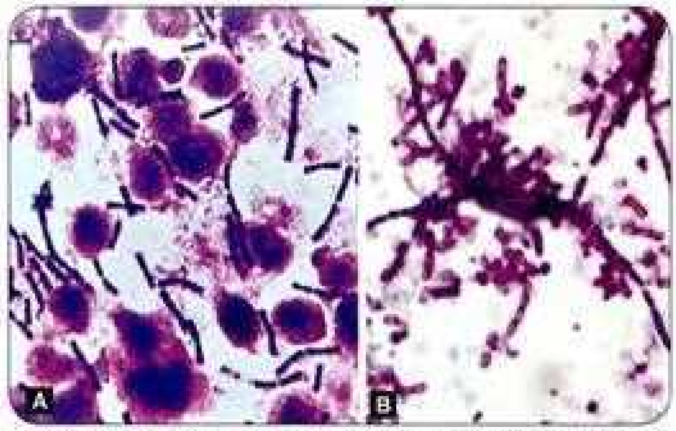
Specimen Collection

The specimens should be collected before starting antibiotic treatment. The useful specimens are:

- Pus or swab from malignant pustule
- Sputum in pulmonary anthrax
- Blood (in septicemia)
- CSF (in hemorrhagic meningitis)
- Gastric aspirate, feces or food (in intestinal anthrax)
- Ear lobes from dead animals.

Direct Demonstration

- **Gram staining:** Reveals gram-positive, large rectangular rods (3-10 μm × 1-1.6 μm). Spores are usually not seen in clinical samples (Fig. 25.2A)



Figs 25.2A and B: Gram-stain of *B. anthracis*: **A.** Direct smear-shows gram-positive, large rectangular bacilli and pus cells; **B.** Culture smear-shows gram-positive bacilli with non-bulging spores (bamboo stick appearance)

Source: A. Public Health Image Library/ID#: 1811, Centers for Disease Control and Prevention (CDC), Atlanta (with permission); B. Department of Microbiology, JIPMER, Puducherry (with permission).

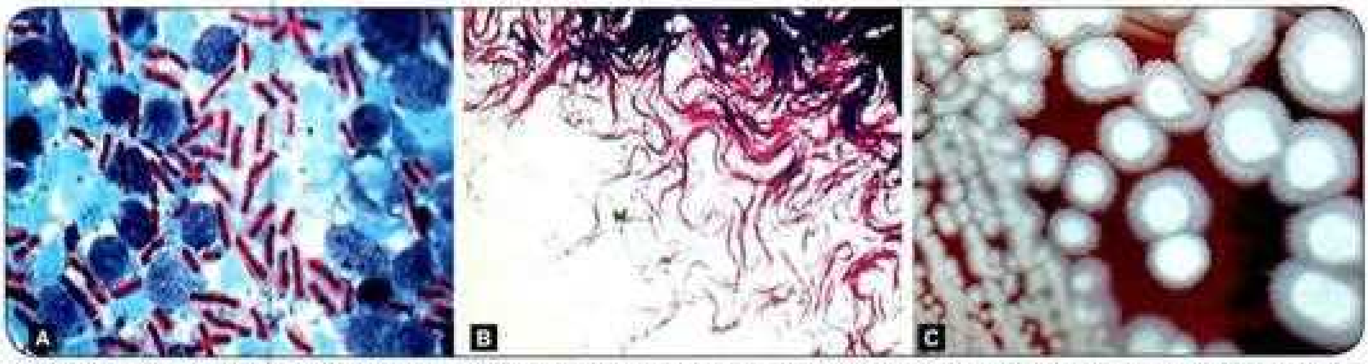
- **McFadyean's reaction:** Polypeptide capsule can be demonstrated by staining with Gurr's polychrome methylene blue stain for 30 seconds. Capsule appears as amorphous purple material surrounding blue bacilli (Fig. 25.3A). This is used for presumptive diagnosis of anthrax in animal
- **Direct immunofluorescence test (direct-IF):** It detects capsular and cell wall polypeptide antigens by using fluorescent tagged monoclonal antibodies. It is used for confirmation of the diagnosis during bioterrorism outbreaks
- **Ascoli's thermoprecipitation test:** It is a ring precipitation test, done when sample is received in putrid form and bacilli are likely to be non-viable. Tissue samples are grounded in saline, boiled and filtered. This antigenic extract is layered over anthrax antiserum on a narrow capillary tube. A ring of precipitate appears at the junction of the two liquids within 5 minutes.

Culture

Bacillus anthracis is aerobic, non-fastidious, grows in ordinary media and has a wide temperature range (12-45°C) of growth. Sporulation is promoted at 25-30°C and in presence of unfavorable conditions such as distilled water, 2% NaCl, oxalate and oxygen.

Colony morphology of *B. anthracis* after 24 hours incubation of plates is as follows:

- **Nutrient agar:** Colonies are 2-3 mm in size, irregular, round, opaque, grayish white with a frosted glass appearance.
  Medusa head appearance: When colonies are viewed under low power microscope, the edge of the colony which is composed of long interlacing chains of bacilli, appears as locks of matted hair (Fig. 25.3B)
- **Blood agar:** It produces dry wrinkled, non-hemolytic colonies (Fig. 25.3C)



Figs 25.3A to C: A. McFadyean's reaction—amorphous purple capsule surrounding blue bacilli (polychrome methylene blue stain); **B.** Medusa head colonies of *Bacillus anthracis* on nutrient agar (10x magnification); **C.** Non-hemolytic dry wrinkled colonies of *Bacillus anthracis* on blood agar

Source: A. Department of Pathobiology, University of Guelph, Canada; B. Dr J Glenn Songer, Iowa State University, USA; C. Public Health Image Library/ID#: 1897/Dr Larry Stauffer, Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

- **Gelatin stab agar:** Growth occurs as **inverted fir tree appearance** (due to liquefaction of gelatin which occurs maximum at the surface, and then slows down towards the bottom)
- **Selective media:**
  - Solid medium with penicillin: Colony smear shows bacilli arranged in string of pearl appearance. (due to cells becoming larger and spherical because of their weaker cell walls under the action of penicillin, and cells tend to arrange in chain)
  - PLET medium: It consists of polymyxin, lysozyme, EDTA and thallous acetate added in heart infusion agar. It has been devised to isolate *B. anthracis* from mixtures of other spore bearing bacilli.

Culture Smear

- **Gram-staining:** Reveals **bamboo stick appearance**, i.e. long chain of gram-positive bacilli with non-bulging spores (appear as empty space) (Fig. 25.2B)
- **Spores:** They can be demonstrated by using special stains, such as hot malachite green (Ashby's method) or 0.25% sulfuric acid (spores are acid fast)
- **Lipid granules:** They can be demonstrated by staining with sudan black B (Burdon's method).

Serology

Antibodies appear in convalescent sera and can be detected by ELISA or immunodiffusion in gel method.

Molecular Diagnosis

PCR with specific primers can be used for further confirmation.

- PCR using *BA pX01* primer targeting gene coding for protective antigen
- PCR *BA pX02* primers targeting capsular gene.

Molecular typing: It is useful for epidemiological studies to trace the source of infection. Various methods available are as follows:

- MLVA (Multiple locus variable number of tandem repeat analysis)
- AFLP (Amplified fragment length polymorphisms).

Guidelines for Diagnosis of Anthrax during Bioterrorism Attacks (CDC, 2001)

In the wake of bioterrorism experience in USA (2001), the Centers for Disease Control and Prevention (CDC) has prepared guidelines for identification of *B. anthracis* during bioterrorism attacks:

- For presumptive identification of anthrax—any large gram-positive bacillus with morphology and cultural properties similar to anthrax bacillus
- For initial confirmation, the tests done are:
  - Lysis by gamma phage
  - Direct immunofluorescence test (direct-IF).
- Further confirmation is done by PCR.

TREATMENT ***Bacillus anthracis***

Anthrax can be successfully treated if the disease is promptly recognized and appropriate therapy is initiated early.

- **Antibiotic regimen for treatment** consists of ciprofloxacin or doxycycline, plus clindamycin, and/or rifampin, for 60 days
- **Antibiotics for post-exposure prophylaxis:**
  - Ciprofloxacin for 60 days ***plus***
  - Doxycycline for 60 days or Amoxicillin for 60 days (given if strain is penicillin sensitive).
- **Raxibacumab:** It is a monoclonal antibody that neutralizes anthrax toxin (protective antigen). It is intended for the prophylaxis and treatment of inhalational anthrax.

Prevention

The general control measures include:

- Disposal of animal carcasses by burning or by deep burial in lime pits

- Decontamination (usually by autoclaving) of animal products
- Protective clothing and gloves for handling potentially infectious materials.

Live Attenuated, Non-capsulated Spore Vaccine (Stern Vaccine)

It is extensively used in animals, remains protective for 1 year following single injection. However, it is not safe for human use.

Adsorbed (Alum Precipitated) Toxoid Vaccine

It is prepared from the protective antigen. Antibodies formed against protective antigen neutralize it and thus prevent attachment of toxin to the host cell. It is safe and effective for human use (e.g. BioThrax). It is indicated for pre-exposure and post-exposure prophylaxis (Table 25.2).

OTHER BACILLUS SPECIES OF HUMAN IMPORTANCE

Anthracoid Bacilli

Bacillus species other than the anthrax bacillus, are collectively called **anthracoid bacilli**.

- Except *B. cereus*, most of them are non-pathogenic and are common contaminants in laboratory cultures
- They have a general resemblance to anthrax bacilli such as producing dry wrinkled colonies and in smear they appear as chains of spore-bearing gram-positive bacilli
- However, they differ from anthrax bacilli in many ways (Table 25.3).

Bacillus cereus

It is a normal habitant of soil, also widely isolated from food items such as vegetables, milk, cereals, spices, meat and poultry. It is an important agent of food poisoning in man.

Clinical Manifestations

- **Food poisoning:** It produces two types of toxins—diarrheal toxin (causes diarrheal type of food poisoning) and emetic toxin (causes emetic type of food poisoning) (Table 25.4)
  - **Emetic toxin:** It is a heat stable preformed toxin, resembling *S. aureus* enterotoxin. It acts immediately on intestine so that the incubation period of food poisoning is short (1–6 hours)
  - **Diarrheal toxin:** Organism secretes this toxin only after entering into the intestine, hence the incubation period is longer (8–16 hours).
- **Ocular disease:** It causes severe keratitis and panophthalmitis following trauma to the eye that may lead to loss of vision
- **Other conditions:** It rarely causes systemic infections, including endocarditis, meningitis, osteomyelitis,

Table 25.2: Indication and schedule of adsorbed anthrax vaccine (BioThrax)

Indication	Route	Dosing schedule
Pre-exposure prophylaxis for persons at high risk of exposure	Intramuscular (0.5 mL/dose)	Primary series: 0, 1, and 6 months Boosters: at 6 and 12 month after primary series and then yearly
Post-exposure prophylaxis following exposure to suspected or confirmed case	Subcutaneous (0.5 mL/dose)	0, 2, and 4 weeks post-exposure combined with antimicrobial therapy

Table 25.3: Differences between *Bacillus anthracis* and anthracoid bacilli

Features	*Bacillus anthracis*	Anthracoid bacilli
Motility	Non-motile	Motile
Capsule	Present	Absent
Bacilli	In long chain	In short chain
Under low power microscope	Medusa head colony seen	Not seen
Blood agar	No hemolysis	Hemolytic colony
Broth	Turbidity absent	Usually turbid
Salicin	Not fermented	Fermented
Gamma phage	Susceptible	Resistant
Gelatin stab agar	• Inverted fir tree appearance seen • Gelatin liquefaction slow	• Inverted fir tree-not seen • Rapid gelatin liquefaction
Solid medium with penicillin	String of pearls appearance	No growth
At 45°C	No growth	Usually grows
Virulence	Pathogenic	Mostly non-pathogenic

Table 25.4: Differences between diarrheal type and emetic type of food poisoning of *Bacillus cereus*

Bacillus cereus	Diarrheal type	Emetic type
Incubation period	8–16 hours	1–5 hours
Toxin	Secreted in intestine (Similar to *C. perfringens* enterotoxin)	Preformed toxin (formed in diet, similar to *S. aureus* enterotoxin)
Heat	Heat labile	Heat stable
Food items contaminated	Meat, vegetables, dried beans, cereals	Rice (Chinese fried rice)
Clinical feature	Diarrhea, fever, abdominal cramps	Vomiting, abdominal cramps
Serotypes involved	2, 6, 8, 9, 10, 12	1, 3, 5

and pneumonia. The presence of a medical device or intravenous drug use predisposes to these infections.

Laboratory Diagnosis

- *Bacillus cereus* can be isolated from feces by using selective media such as:
  - MYPA (mannitol, egg yolk, polymyxin B, phenol red and agar)
  - PEMBA (polymyxin B, egg yolk, mannitol, bromothymol blue and agar).
- It is motile, non-capsulated and not susceptible to gamma phage.

TREATMENT ***Bacillus cereus***

Bacillus cereus is susceptible to clindamycin, erythromycin, vancomycin, aminoglycosides and tetracycline. It is resistant to penicillin (by producing β-lactamase) and trimethoprim.

Bacillus thuringiensis

It is closely related to *B. cereus* and may occasionally produce food poisoning. It is also used as larvicidal agent for mosquito control.

Bacillus (spores) Used as Sterilization Control

Bacillus species are used as biological indicators to check the efficacy of sterilization process. For example;

- *Geobacillus stearothermophilus* (formerly *Bacillus stearothermophilus*) is used for autoclave, hydrogen peroxide gas plasma sterilizer and liquid acetic acid sterilizer
- *Bacillus atrophaeus* is used for ethylene oxide sterilizer and dry heat sterlizer.

Industrial Use of *Bacillus subtilis*

B. subtilis is used in industries for various purposes.

- Used as cleaning agent (detergent)
- In paper and textile industries: It produces amylase that breaks down starch
- Used for pollution treatment: By breaking down pollutants (bioremediation)
- In pesticide industry: It is used for protecting crops against fungi
- In food industry: Helps in fermentation.

EXPECTED QUESTIONS

I. Essay:

1. Alisha, a 30-year-old woman, was admitted into hospital after a prolonged fever, with chills and night sweats, chest discomfort with blood stained sputum, nausea, headache and a sore throat. After being hospitalized, the doctors performed a Gram-stain for sputum specimen and found gram-positive rod-shaped bacteria arranged in chains.
   a. What is the clinical diagnosis and causative agent?
   b. Describe pathogenesis and various forms of clinical presentation of this infection.
   c. Mention the laboratory investigations to confirm the diagnosis.

II. Write short notes on:

1. Malignant pustule.
2. *B. cereus* food poisoning.

III Multiple Choice Questions (MCQs):

1. **Gram-stain morphology of *Bacillus anthracis* is:**
   a. Tennis racket appearance
   b. Drum stick appearance
   c. Bamboo stick appearance
   d. Spectacle glass appearance
2. **"Malignant pustule" is a term used for:**
   a. An infected malignant melanoma
   b. A carbuncle
   c. A rapidly spreading rodent ulcer
   d. Anthrax of the skin
3. **Incubation period for *B. cereus* food poisoning following consumption of contaminated fried rice:**
   a. 1–6 hours b. 8–16 hours
   c. 24 hours d. >24 hours
4. **Adsorbed toxoid vaccine of anthrax contains**
   a. Edema factor b. Protective factor
   c. Lethal factor d. Capsular antigen
5. ***Bacillus anthracis* is differentiated from anthracoid bacilli by all, *except*:**
   a. Medusa head appearance
   b. String of pearl appearance
   c. Hemolytic colonies on blood agar
   d. McFadyean reaction
6. **Selective medium for *B. cereus*?**
   a. PLET medium
   b. Loeffler's serum slope
   c. Potassium tellurite agar
   d. MYPA medium
7. **Emetic toxin of *B. cereus*—all are true, *except*:**
   a. Produces food poisoning similar to *S. aureus*
   b. Chinese fried rice is a common source
   c. Serotype associated 2, 6, 8
   d. Incubation period 1-5 hours
8. **Metachromatic granules are best demonstrated in:**
   a. Tinsdale medium b. Hoyle's medium
   c. Loeffler's serum slope
   d. Potassium tellurite agar
9. **DPT vaccine- all are true, *except*:**
   a. Administered in deep IM
   b. Total five doses are given
   c. Pertussis component acts as adjuvant
   d. Neurological complication is due to diphtheria toxoid component

Answers

1. c 2. d 3. a 4. a 5. c 6. d 7. c 8. c 9. d

Anaerobes (Clostridium and Non-sporing Anaerobes)

26 CHAPTER

Chapter Preview

- *Clostridium*
  - *Clostridium perfringens*
  - *Clostridium tetani*
  - *Clostridium botulinum*
  - *Clostridioides difficile*
- Non-sporing anaerobes

Anaerobic bacteria do not have cytochrome system for oxygen metabolism and hence are unable to neutralize toxic oxygen metabolites. They can be classified as follows:

- **Obligate anaerobes:** They cannot grow in presence of oxygen as they completely lack superoxide dismutase and catalase enzymes and hence are susceptible to the lethal effects of oxygen
- **Aerotolerant anaerobes:** They do not utilize oxygen for growth, but tolerate its presence. This is because they possess small amounts of superoxide dismutase and peroxidase (but lack catalase), which may neutralize the toxic oxygen radicals. Examples include some clostridia (*C. histolyticum*) as well as few non-sporing anaerobes, such as *Bacteroides*.

Anaerobes need special requirements to grow in culture such as:

- **Anaerobic condition:** This can be achieved by various methods such as:
  - McIntosh and Filde's anaerobic jar
  - GasPak system
  - Anoxomat system
  - Anaerobic glove box workstation
  - Pre-reduced anaerobically sterilized (PRAS) media.
- **Medium with low redox potential:** This can be achieved by adding to the media with reducing substances such as unsaturated fatty acid, ascorbic acid, glutathione, cysteine, glucose, sulfites and metallic iron.

Obligate anaerobes can be grouped into spore bearing (e.g. *Clostridium*) and non-sporing anaerobes (described later in this Chapter).

CLOSTRIDIUM

Clostridia are gram-positive bacilli, having bulging spores (in contrast to the genus *Bacillus* which has non bulging spores), and encompass more than 60 species.

- Clostridia are saprophytes found in soil, fresh water, marine water, decaying vegetation, animal matter and sewage; thus play a major role in recycling of organic matter
- They are also harbored in intestine of vertebrates and invertebrates including human beings
- However, few members may cause a variety of infections in humans such as:
  - *C. perfringens*: Causes gas gangrene
  - *C. tetani*: Causes tetanus
  - *C. botulinum*: Causes botulism (food, wound and infant botulism)
  - *C. difficile*: Causes pseudomembranous colitis.
- **Industrial importance:** Some clostridia such as *C. acetobutylicum* and *C. butyricum* are used to prepare chemicals such as acetone and butanol
- They are motile (exhibit stately motility) except *C. perfringens*, *C. ramosum* and *C. tetani* serotype VI
- They are noncapsulated except *C. perfringens* and *C. butyricum*.

Spore

In clostridia, the spores are wider than the vegetative bacteria giving rise to swollen or spindle-shaped appearance (*Clostridium* is named from the word 'Kloster' meaning spindle). Spore formation occurs in unfavorable conditions. Most of the clostridia bear a subterminal spores except (Fig. 26.1):

Fig. 26.1: Types of spores of various *Clostridia* species

- *C. bifermentans:* Produces central and oval spore
- *C. perfringens:* Produces subterminal and oval spore
- *C. tetani:* Produces spherical and terminal spore (drum stick appearance)
- *C. tertium:* Produces oval and terminal spore (tennis racket appearance).

Cultivation

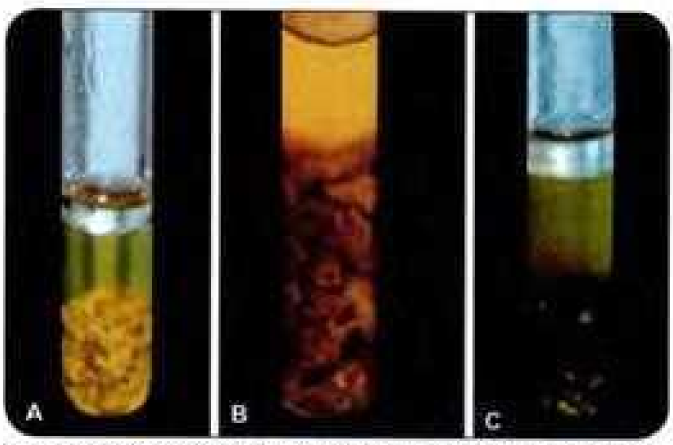
Clostridia grow well in various anaerobic media, such as Robertson's cooked meat (RCM) broth or thioglycollate broth.

Robertson's cooked meat (RCM) broth: It contains chopped meat particles (beef heart), which provide glutathione (a sulfhydryl group containing reducing substance) and unsaturated fatty acids, which take up oxygen and create lower redox potential and thus permit the growth of obligate anaerobes. Growth appears as turbidity in the medium which may be of two types (Figs 26.2A to C):

1. Proteolytic clostridia turn the meat black and produce foul odor, e.g. *C. tetani, C. botulinum* A, B and F.
2. Saccharolytic species turn the meat pink, e.g. *C. perfringens, C. difficile* and *C. botulinum* C, D and E.

CLOSTRIDIUM PERFRINGENS

C. perfringens (previously, *C. welchii*) is a commensal in the large intestine of human beings and animals. It is also found as saprophyte in soil, dust and air.



Figs 26.2A to C: Robertson cooked meat broth: **A.** Uninoculated; **B.** Pink and turbid (*C. perfringens*); **C.** Black and turbid (*C. tetani*)
Source: Department of Microbiology, JIPMER, Puducherry (*with permission*).

- It is capsulated, non-motile, gram-positive bacillus
- It bears subterminal bulging spores; but does not produce spores in tissues or in culture media (especially the gas gangrene strains)
- It is invasive as well as toxigenic.

Virulence Factors

The virulence factors produced by *C. perfringens* can be grouped into different types (Table 26.1).

- *Four major toxins:* Alpha (α), beta (β), epsilon (ε) and iota (ι)
- Eight minor toxins: Gamma (γ), delta (δ), lambda (λ), kappa (κ), theta (θ), eta (η), mu (μ) and nu (υ)
- They also produce heat labile enterotoxin
- Soluble substances are produced such as neuraminidase, histamine, bursting factor (produce muscle lesions) and circulating factor (inhibit phagocytosis).

Toxins of *C. perfringens* show varied biological activity (Table 26.1). Based on production of four major toxins *C. perfringens* can be classified into five types A to E as shown in Table 26.2.

Clinical Manifestations

C. perfringens infections are mostly polymicrobial involving other clostridia species. Various manifestations include:

Clostridial Wound Infection

MacLennan has classified them as follows:

- **Simple wound contamination:** It involves the wound surface contamination, without invasion of underlying tissue, as occurs in absence of devitalized tissue
- **Anaerobic cellulitis:** It involves the fascial plane with minimal toxin release, without muscle invasion
- **Anaerobic myositis (gas gangrene):** Muscle invasion occurs, which leads to gas in the muscle compartment with abundant toxin release (described later).

Clostridial Enteric Infection

- **Food poisoning:** It is caused by *C. perfringens* type A enterotoxin (coded by *cpe* gene)
  - It occurs following consumption of improperly cooked contaminated meat. Spores being heat resistant survive and germinate later when the food is cooled

Table 26.1: *Biological activity of the toxins of Clostridium perfringens*

Toxin	Biological activity
Major toxins	
Alpha (α)	Lethal, lecithinase (phospholipase C) Hemolytic Requires Ca^{+2} ion
Beta (β)	Lethal, necrotizing, trypsin labile
Epsilon (ε)	Lethal, permease, trypsin activatable
Iota (ι)	• Lethal, dermonecrotic • *Binary, has 2 fragments:* ➢ Fragment A-ADP ribosylating ➢ Fragment B-Binding
Minor toxins	
Gamma (γ)	Mechanism of action not defined
Delta (δ)	Hemolysin
Lamda (λ)	Protease
Kappa (κ)	Collagenase and gelatinase
Theta (θ)	Hemolysin (O_2 labile) and cytolysin
Eta (η)	Mechanism of action not defined
Mu (μ)	Hyaluronidase
Nu (υ)	Deoxyribonuclease
Other toxins	
Neuraminidase	It makes RBCs panagglutinable, resulting in increase of blood viscosity and promoting capillary thrombosis
Enterotoxin	Enterotoxic and cytotoxic

Table 26.2: *Classification of Clostridium perfringens*

Types	Major toxin produced	Disease
A	Alpha	Gas gangrene, food poisoning
B	Alpha, beta and epsilon	Lamb dysentery
C	Alpha and beta	Enteritis necroticans in humans
D	Alpha and epsilon	Enterotoxemia and pulpy kidney disease in sheep
E	Alpha and iota	Possible pathogen of sheep and cattle

- Infective dose: About 10^8 viable vegetative bacilli producing enterotoxin are required to initiate the infection
- Enterotoxin acts by forming pores in the intestinal mucosal membrane
- Diagnosis: By detection of enterotoxin in feces by enzyme immunoassay.

❖ **Enteritis necroticans** (gas gangrene of the bowel): It is a life-threatening condition characterized by ischemic necrosis of the jejunum and gas in the tissue plane
- It is also known as **pigbel** in Papua New Guinea and **darmbrand** in Germany
- It is caused by *C. perfringens* type C strains, producing β toxin.

❖ **Necrotizing enterocolitis:** It resembles enteritis necroticans but is associated with *C. perfringens* type A and has been found in North America

❖ **Gangrenous appendicitis.**

Other Clostridial Infections

❖ **Bacteremia:** *C. perfringens* followed by *C. tertium* and *C. septicum* are commonly associated with bacteremia

❖ **Skin and soft-tissue infections**: *C. perfringens, C. histolyticum, C. septicum, C. novyi*, and *C. sordellii* cause necrotizing infections of the skin and soft tissues

❖ Infection of the endometrium leading to toxic shock syndrome—can be associated with *C. sordellii*

❖ Meningitis and brain abscess

❖ Panophthalmitis (due to *C. sordellii* or *C. perfringens*).

Gas Gangrene

Definition (Oakley, 1954)

Gas gangrene is defined as a rapidly spreading, edematous myonecrosis, occurring in association with severely crushed wounds contaminated with pathogenic clostridia, particularly with *C. perfringens*. Previously, the disease was called **malignant edema** or **clostridial myonecrosis**.

Etiological agents

Gas gangrene is always polymicrobial and is caused by many clostridial species.

❖ **Established agents:** *C. perfringens* (most common, 60% of the total cases) and *C. novyi* and *C. septicum* (20–40%)

❖ **Probable agents:** They are less commonly implicated; e.g.: *C. histolyticum, C. sporogenes, C. fallax, C. bifermentans, C. sordellii, C. aerofoetidum* and *C. tertium.*

Pathogenesis

The development of gas gangrene requires:

❖ **Anaerobic environment:** Crushing injuries of muscles such as road traffic accidents (causing laceration of large or medium-sized arteries), open fractures of long bones or foreign bodies (bullet injuries) or devitalized tissues lead to interruption in the blood supply and tissue ischemia. Anoxic muscles start utilizing pyruvate anaerobically to produce lactic acid

❖ **Contamination of wound** with clostridial spores present in the soil (during war or road traffic accident) or clothes

❖ Rarely, spontaneous **non-traumatic gas gangrene** occurs via hematogenous seeding of normal muscle with bowel clostridia, as occurs in people with gastrointestinal pathologies (e.g. colonic malignancy).

Virulence Factors Mediating Gas Gangrene

Toxins produced by *C. perfringens*

Once introduced *C. perfringens* proliferates locally and elaborates exotoxins, chiefly α toxin and θ toxin.

❑ α **toxin** is the principle virulence factor. It has both phospholipase C and sphingomyelinase activities
- ➢ It activates the platelet adhesion molecule ***gpIIbIIIa*** and neutrophil receptors ***CD11b/CD18***, leading to formation of aggregates of platelets and neutrophils in the blood vessels causing occlusion

Contd...

Contd...

- ➢ α toxin directly suppresses myocardial contractility leading to reduction in the cardiac output and results in profound hypotension.
- ❑ θ **toxin** causes marked vasodilation by activating mediators (e.g. prostacyclin, platelet-activating factor).

Toxins Produced by other Clostridia

- ❑ *C. septicum* produces four main toxins: α toxin (lethal, hemolytic, necrotizing activity), β toxin (deoxyribonuclease (DNase)), γ toxin (hyaluronidase), and septicolysin, protease and neuraminidase
- ❑ *C. novyi* has four subtypes A–D. Type-A produces bacteriophage coded alpha-toxin, which commonly causes gas gangrene.

Clinical Manifestation of Gas Gangrene

The **incubation period** is variable. Depending upon the nature of injury, the amount of wound contamination and the type of clostridial species involved, the incubation period varies. For example:

- 10–48 hours for *C. perfringens*
- 2–3 days for *C. septicum*
- 5–6 days for *C. novyi.*

Various manifestations include:

- Sudden onset of excruciating pain at the affected site
- Rapid development of a foul-smelling thin serosanguineous discharge
- Gas bubbles **(crepitus)** in the muscle planes (Fig. 26.3)
- Brawny edema and induration
- Such gangrenous tissues later may become liquefied and sloughed off
- Shock and organ failure develop later
- Associated with higher mortality rate (50%).

LABORATORY DIAGNOSIS — **Gas gangrene**

- ❑ **Specimen:** Necrotic tissues, muscle fragments and exudates from deeper parts of the wound
- ❑ **Direct microscopy:** Thick, stubby, boxcar-shaped gram-positive bacilli without spore are suggestive of *C. perfringens*
- ❑ **Culture:**
  - ➢ Media: Robertson cooked meat broth, egg yolk agar, etc.
  - ➢ Incubation: Anaerobically (by GasPak or Anoxomat, etc.)
- ❑ **Identification of *C. perfringens***
  - ➢ Target hemolysis (double zone hemolysis)
  - ➢ Nagler's reaction: Opalescence surrounding the streak line on egg yolk agar
  - ➢ Reverse CAMP test: Positive
  - ➢ Heat tolerance test: Positive
  - ➢ In litmus milk: Produces stormy clot reaction.

Laboratory Diagnosis of Gas Gangrene

Based on the clinical diagnosis of gas gangrene, treatment should be started as early as possible. Laboratory diagnosis has role only for (1) confirmation of the clinical diagnosis, (2) species identification.

Fig. 26.3: Gas gangrene of the right leg showing swelling and discoloration of the right thigh with bullae, and palpable crepitus

Source: Wikipedia/Cases/Engelbert Schröpfer, Stephan Rauthe and Thomas Meyer (with permission).

Specimen

- Ideal specimens are necrotic tissues, muscle fragments and exudates from deeper part of the wound, where the infection appears to be more active
- Blood culture may be positive for *C. perfringens* and *C. septicum*. However, *C. perfringens* bacteremia can occur even in the absence of gas gangrene
- Swabs rubbed over the wound surface or soaked in exudates are not satisfactory
- Specimens should be put into Robertson's cooked meat broth and transported immediately to the laboratory.

Direct Microscopy

Gram stained films provide clues about the species of clostridia present. Absence of neutrophils in the infected tissues is a characteristic feature.

- Thick, stubby, boxcar-shaped, gram-positive bacilli without spore—suggestive of *C. perfringens* (Fig. 26.4)
- Spore bearing gram-positive bacilli suggest other clostridia species
  - Citron bodies (boat or leaf-shaped pleomorphic irregularly stained bacilli with spores)—suggest *C. septicum*
  - Large rods with oval sub-terminal spores—suggest *C. novyi.*

Identification

C. perfringens can be further identified by the following properties. Culture plates should be incubated anaerobically at 37°C for 2 days.

- **Target hemolysis** (double zone hemolysis, Fig. 26.5A): On blood agar, *C. perfringens* produce an inner narrow zone of complete hemolysis (due to θ toxin), surrounded by a much wider zone of incomplete hemolysis (due to the alpha toxin)

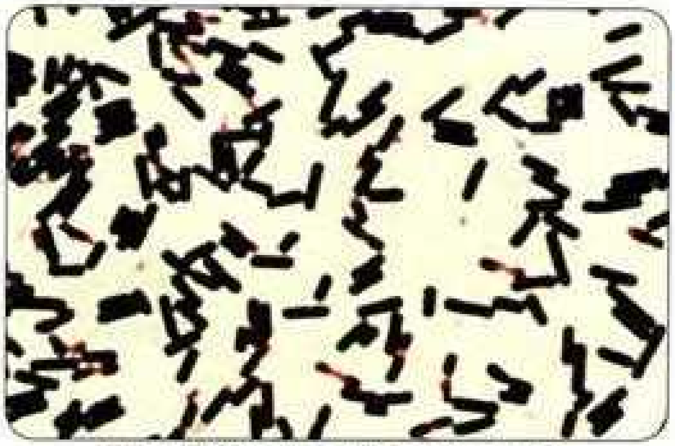


Fig. 26.4: *Clostridium perfringens* (Gram-stain)

Source: Public Health Image Library/ID# 11196, Don Stalons/Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*).

- **Nagler's reaction:** *C. perfringens* produces an opalescence surrounding the streak line on egg yolk agar or media containing 20% human serum (due to lecithinase activity of α toxin). Opalescence can be inhibited by incorporating anti-α toxin to the medium (Fig. 26.5B). The test is also positive for *C. bifermentans, C. baratti* and *C. sordellii* (all produce α toxin)
- **Reverse CAMP test:** *C. perfringens* is streaked over the center of blood agar plate and *Streptococcus agalactiae* is streaked perpendicular to it. Presence of enhanced zone of hemolysis (arrow-shaped) pointing towards *C. perfringens* indicates the test is positive (Fig. 26.5C)
- **Heat tolerance:** *C. perfringens* can grow when RCM broth is incubated at 45°C for 4–6 hours. This differentiates it from other organisms in the specimen
- In litmus milk, *C. perfringens* produces "stormy clot reaction" due to fermentation of lactose producing acid and vigorous gas
- Other clostridia species can be identified by various biochemical tests.

TREATMENT **Gas gangrene**

- **Early surgical debridement** is the most crucial step in the management of gas gangrene. All devitalized tissues should be widely resected so as to remove conditions that produce anaerobic environment. Closure of wounds should be delayed for 5–6 days until the sites are free from infection
- **Antibiotics:** Combination of penicillin and clindamycin is recommended for 10–14 days
- **Hyperbaric oxygen:** It may kill the obligate anaerobic clostridia such as *C. perfringens*; however, it has no effect on aerotolerant clostridia (*C. septicum*)
- **Passive immunization** with anti-α-toxin antiserum.

Prevention

Vaccination against α toxin is protective in experimental animals against gas gangrene, but has not been investigated in humans.

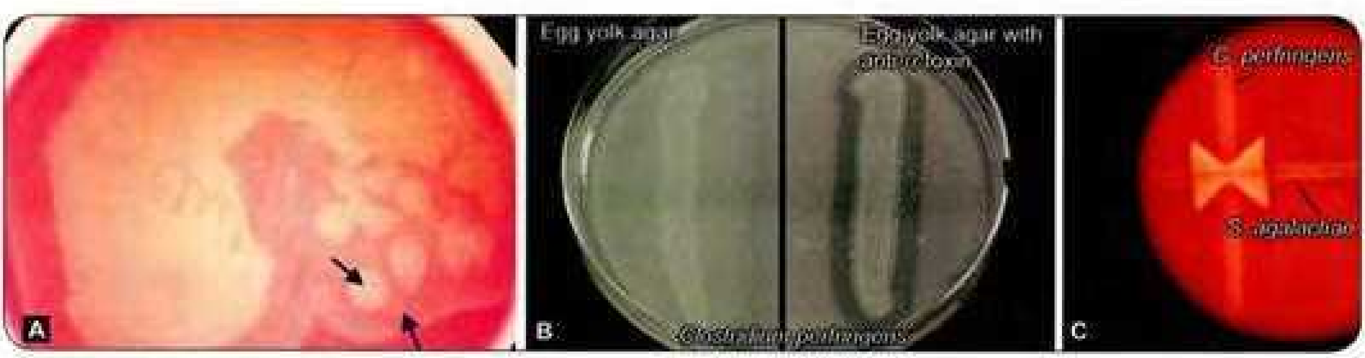
CLOSTRIDIUM TETANI

C. tetani is an obligate anaerobic, gram-positive *bacillus* with terminal round spore (drum stick appearance).

- It is the causative agent of 'tetanus'—an acute disease, manifested by skeletal muscle spasm and autonomic nervous system disturbance
- Tetanus has been known since ancient time; however, its causative organism was isolated later by Kitasato (1889)
- *C. tetani* is ubiquitous in nature, widely distributed in soil, hospital environment and intestine of man and animals.

Virulence Factors

C. tetani produces two exotoxins—tetanolysin and tetanospasmin.



Figs 26.5A to C: A. Target hemolysis of *C. perfringens*-zone of incomplete hemolysis (blue arrow) and zone of complete hemolysis (black arrow); **B.** Nagler's reaction; **C.** Reverse CAMP test

Source: A. Department of Microbiology, JIPMER, Puducherry; C. Dr. Padmaja A Shenoy, Associate Professor, Department of Microbiology, Kasturba Medical College, Manipal, Karnataka (*with permission*).

1. **Tetanolysin:** It is a heat labile, oxygen labile hemolysin antigenically related to the oxygen labile hemolysins produced by *C. perfringens*, *S. pyogenes* and *S. pneumoniae*. It plays no role in the pathogenesis of tetanus.
2. **Tetanospasmin (or tetanus toxin):** It is a neurotoxin responsible for disease manifestations.
   - It is oxygen stable but heat labile
   - Toxin is produced as a single 150 kDa polypeptide chain that is cleaved to produce heavy (100 kDa) and light (50 kDa) chains joined by a disulfide bond
   - It is antigenic and is specifically neutralized by its antitoxin
   - It gets toxoided spontaneously or by formaldehyde. The toxoid form is antigenic, but looses its virulence property, hence, it is used for vaccine preparation
   - Tetanus toxin is plasmid coded, its mechanism of action is given below.

Mechanism of Action of Tetanus Toxin

Tetanus toxin binds to receptors (polysialogangliosides) present on motor nerve terminals which results in toxin internalization.

- Following internalization, tetanus toxin gets transported in retrograde way to the gamma-aminobutyric acid (GABA) and glycine producing inhibitory neuron terminals
- The toxin prevents the presynaptic release of inhibitory neurotransmitters glycine and GABA, which leads to spastic muscle contraction.

Mode of Transmission

Tetanus bacilli enter through:

- Injury (superficial abrasions, punctured wounds, road traffic accidents)
- Surgery done without proper asepsis
- **Neonates:** Following abortion/delivery, due to unhygienic practices
- Otitis media (otogenic tetanus)
- **It is noninfectious:** There is no person-to-person spread.

Clinical Manifestations

Incubation period is about 6–10 days. Shorter the incubation period, graver is the prognosis. Muscles of the face and jaw are often affected first (due to shorter distances for the toxin to reach the presynaptic terminals).

- **First symptom:** Increase in the masseter tone leading to **trismus** or **lock jaw**, followed by muscle pain and stiffness, back pain, and difficulty in swallowing
- In neonates, difficulty in feeding is the usual presentation
- **As the disease progresses, painful muscle spasm develops which may be:**
  - **Localized:** Involves the affected limb
  - Generalized painful muscle spasm → leads to descending spastic paralysis.
- Hands, feet are spared and mentation is unimpaired. Deep tendon reflexes are exaggerated
- Autonomic disturbance is maximal during the second week of severe tetanus-characterized by low or high blood pressure, tachycardia, intestinal stasis, sweating, increased tracheal secretions and acute renal failure.

Complications

Eventually, the following complications may be developed.

- **Risus sardonicus:** It is characterized by an abnormal, sustained spasm of the facial muscles that appears to produce grinning (Fig. 26.6A)
- **Opisthotonos position:** It is an abnormal posture of the body, occurs due to generalized spastic contraction of the extensor muscles (Fig. 26.6B)
- Respiratory muscles spasm: May cause airway obstruction.

Tetanus is more common in developing countries including India due to:

- Warm climate
- Rural area with fertile soil
- Unhygienic surgeries or deliveries.

However, the incidence has been reduced to a large extent due to widespread immunization of infants and pregnant mothers.

Laboratory Diagnosis

Treatment should be started immediately based on clinical diagnosis. Laboratory diagnosis provides supportive evidence for confirmation.

Specimen

Excised tissue bits from the necrotic depths of wounds are more reliable than wound swabs.

Gram Staining

- Gram staining reveals gram-positive bacilli with terminal and round spores (drum stick appearance) (Fig. 26.6C)
- However, microscopy alone is unreliable as it cannot distinguish *C. tetani* from morphologically similar non-pathogenic clostridia like *C. tetanomorphum* and *C. sphenoides*.

Culture

Culture is more reliable than microscopy.

- **Robertson cooked meat broth:** *C. tetani*, being proteolytic turns the meat particles black and produces foul odor
- **Blood agar with polymyxin B:** These plates are incubated at 37°C for 24–48 hours under anaerobic condition. *C. tetani* produces characteristic **swarming growth**.

Figs 26.6A to C: A. Lockjaw and the facial spasms (risus sardonicus); **B.** Patient with opisthotonos seen in tetanus; **C.** Gram-stained smear of *Clostridium tetani* showing round terminal spore bearing gram-positive *bacilli*

Source: A. Wikia/Hoidkempuhtust, B. Public Health Image Library, ID# 6373, C. ID# 12056/Dr Holdeman/Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*).

Toxigenicity Test

As pathogenesis of tetanus is toxin mediated, the association of the isolated organism can only be established when its toxin production is demonstrated. Toxigenicity can be detected by (i) *in vitro* hemolysis inhibition test and (ii) *in vivo* mouse inoculation test.

TREATMENT — Tetanus

Passive immunization (tetanus immunoglobulin).

It is the treatment of choice for tetanus.

- **Two preparations are available:**
  1. HTIG (Human tetanus immunoglobulin), prepared in Serum Institute of India, Pune.
  2. ATS (Antitetanus serum, equine derived).
- **Dosage:** 250 IU of HTIG or 1500 IU of ATS is given as a single IM dose. Intrathecal route is more effective
- **Duration of protection:** Effect of HTIG and ATS last for 30 days and 7–10 days respectively
- HTIG is preferred over ATS as the latter is associated with side effects such as serum sickness and anaphylactoid reactions.

Combined Immunization

(Both active and passive immunization):

In nonvaccinated person, it is ideal to immunize with first dose of tetanus toxoid (TT) vaccine in one arm along with administration of ATS or HTIG in another arm, followed by a complete course of TT vaccine, as per the schedule described later.

Antibiotics:

Antibiotics play only a minor role as they cannot neutralize the toxins which are already released.

- However, they are useful:
  - In early infection, before expression of the toxin (<6 hours)
  - To prevent further release of toxin.
- Metronidazole is the drug of choice. It is given 400 mg rectally or 500 mg IV every 6 hourly for 7 days
- Penicillin can be given alternatively.

Contd...

Contd...

TREATMENT — Tetanus

Other measures:

- Symptomatic treatment: Antispasmodic (benzodiazepines) can be given
- Entry wound should be identified, cleaned and debrided of necrotic material, so as to remove the anaerobic foci of infection
- Patient should be isolated in a separate room as any noxious stimulus can aggravate the spasm.

Prevention

Active Immunization (Vaccine)

It is the most effective method of prophylaxis.

- **Tetanus toxoid (TT)** is commonly used for active immunization. It is available either as:
  - Monovalent vaccine:
    - Plain formal toxoid (or fluid toxoid): Toxoid is prepared by incubating toxin with formalin
    - Adsorbed: Formol toxoid is adsorbed on to alum.
  - Combined vaccine: DPT (consists of diphtheria toxoid, pertussis whole cell killed preparation and tetanus toxoid) (refer Chapter 24 for detail).
- **Primary immunization of children:** Tetanus toxoid is given under National Immunization Schedule of India. Total 'seven doses' are given; three doses of pentavalent vaccine (DPT, hepatitis B and Hib) at 6, 10 and 14 weeks of birth, followed by two booster doses of DPT at 16–24 weeks and 5 years followed by two additional doses of TT at 10 years and 16 years
- **Adult immunization:** If primary immunization is not administered in childhood, then adults can be immunized with tetanus toxoid. Four doses of TT is given:

2 doses of TT at 1 month interval followed by 2 booster doses at 1 year and 6 years

- **Site:** TT is given by deep intramuscular route at anterolateral aspect of thigh (children) and in deltoid (adults)
- **Protective titer:** Persons are said to be protected if tetanus antitoxin titre is ≥0.01 unit/mL.

Prevention of Tetanus after Injury

All types of wounds need surgical toilet followed by immunization which depends on the wound type and immunization status of the individual (Table 26.3).

Prevention of Neonatal Tetanus

Neonatal tetanus is defined by WHO as 'an illness occurring in a child who loses ability to suck and cry between day 3 and 28 of life and becomes rigid and has spasms'. It is also known as **"8th day disease"** as the symptoms usually start after 1 week of birth (Fig. 26.7).

- **Most common reason:** Unhygienic practices during deliveries such as infected umbilical stumps due to application of cow dung, rarely by circumcision or by ear piercing
- **Seasonal:** Neonatal tetanus is seasonal—more common in July, August and September months
- **Neonatal tetanus can be prevented by:**
  - Discouraging home deliveries and promoting hospital or attended deliveries
  - Following aseptic **clean practices** are followed during deliveries—clean hand, clean surface, clean blade for cutting cord, clean cord tie, clean cord stump, clean towel and clean water
  - TT (2 doses) are given to all pregnant women during 2nd trimester at 1 month gap.
- **Neonatal tetanus elimination is based on:**
  - Neonatal tetanus rate: <1/1000 live births in every districts of country
  - TT coverage to pregnant women >90%
  - Attended deliveries >75%.
- **Situation in India:** India has achieved the elimination status for neonatal tetanus; as declared by WHO in 2016.

CLOSTRIDIUM BOTULINUM

Clostridium botulinum produces botulinum toxin and causes **botulism**, a rare disease manifested as various clinical syndromes ranging from food poisoning, wound infection to infant botulism.

- The term is derived from Latin word *botulus*, meaning sausage; as poorly cooked sausages were formerly associated with food poisoning
- *C. botulinum* is anaerobic Gram-positive *Bacillus* with subterminal spore
- It is ubiquitous in nature, widely distributed as saprophyte in soil, animal manure, vegetables and sea mud.

Table 26.3: Recommendation for prevention of tetanus after injury

Immunity category	Simple wound*	Other wounds
Category A	Nothing required	Nothing required
Category B	Toxoid 1 dose	Toxoid 1 dose
Category C	Toxoid 1 dose	Toxoid 1 dose + HTIG
Category D	Toxoid complete dose	Toxoid complete dose + HTIG

Category A: Taken complete course of TT/booster within the past 5 years.
Category B: Taken complete course of TT/booster within the past >5 years to <10 years.
Category C: Taken complete course of TT/booster within the past >10 years.
Category D: Not taken complete course of TT/booster or immunity status is unknown.
*<6 hours, clean, non-penetrating, no/negligible tissue damage.

Fig. 26.7: Neonatal tetanus (neonate displaying a bodily rigidity)
Source: Public Health Image Library, ID#6374/Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*).

Pathogenesis

C. botulinum is non-invasive. Its pathogenesis is due to production of powerful neurotoxin 'botulinum toxin' (BT), probably the most toxic substance known to be lethal to mankind.

- BT is a 150 kDa zinc dependent protein consisting of a 100 kDa heavy chain and a 50 kDa light chain
- **Serotype:** The light chain is serotype specific and can be typed into eight serotypes—A, B, C1, C2, D, E, F and G.
  - Serotypes A, B, E commonly cause human disease; most severe being serotype A
  - All serotypes produce neurotoxin; except C2 which produces an enterotoxin
  - BT types C and D are bacteriophage coded.
- BT differs from other exotoxins, as it is produced intracellularly, not secreted and appears outside only after autolysis of bacterial cell
- Toxin is synthesized initially as a nontoxic protoxin. It requires trypsin or other proteolytic enzymes to convert it into active form
- **Mechanism of action of BT:** It produces flaccid paralysis

Mechanism of Action of Botulinum Toxin (BT)

After entry (either ingested, inhaled, or produced in a wound), botulinum toxin is transported via blood to peripheral cholinergic nerve terminals.

- The most common nerve terminal sites are neuromuscular junctions, postganglionic parasympathetic nerve endings, and peripheral ganglia. It does not affect the CNS
- BT binds to acetylcholine receptors on the nerve terminals at neuromuscular junction, which results in blockage of release of the acetylcholine, leading to flaccid paralysis.

- **Therapeutic uses:** As BT produces flaccid paralysis, it can be used therapeutically for the treatment of spasmodic conditions such as strabismus, blepharospasm and myoclonus
- Botulinum toxin is also produced by other clostridia such as *C. butyricum*, *C. baratti* and *C. argentinense*
- **Recovery:** Blocking of acetylcholine release is permanent, but the action is short lasting as the recovery occurs in 2–4 months, once the new terminal axons sprout
- Spores do not produce toxins. Toxin production, therefore, requires spore germination, which occurs in anaerobic atmosphere. Spores do not normally germinate in adult intestine, however may germinate in the intestine of infants.

Clinical Manifestations

The manifestations of botulism are due to decreased acetylcholine in cranial nerve and parasympathetic nerve terminals. Common symptoms include:

- Diplopia, dysphasia, dysarthria
- Descending symmetric **flaccid paralysis** of voluntary muscles
- ↓Deep tendon reflexes
- Constipation
- There is no sensory or cognitive deficits
- Respiratory muscle paralysis, may lead to death.

Types of Botulism

- **Food-borne botulism:** It results from consumption of foods contaminated with preformed botulinum toxin
  - Most common source: Homemade canned food
  - Most cases are sporadic; outbreaks are rare.
- **Wound botulism:** It results from contamination of wounds with *C. botulinum* spores. It presents like food-borne botulism except for absence of gastrointestinal features
- **Infant botulism:** It results from ingestion of contaminated food (usually **honey**) with spores of *C. botulinum* in children ≤1 year of age. Spores germinate releasing the toxin
  - Manifestations include inability to suck and swallow, weakened voice, ptosis, floppy neck, and extreme weakness (hence called **floppy child syndrome**)
  - It is self-limiting, managed by supportive care and assisted feeding
  - Rarely, it progresses to generalized flaccidity, respiratory failure and sudden death.
- **Adult intestinal botulism:** Rarely, in patients with suppressed normal flora, the colonized clostridial spores may germinate producing toxin
- **Iatrogenic botulism:** It results from injection of overdose of the toxin while used for therapeutic purpose.

Laboratory Diagnosis

Diagnosis of botulism includes isolation of the bacilli and demonstration of the toxin.

Isolation of the Bacilli

- Gram staining of smears made from suspected food or feces-reveals gram-positive, non-capsulated bacilli with subterminal, oval, bulging spores
- It is motile by peritrichate flagella
- **Isolation:** Culture is done on blood agar or Robertson's cooked meat (RCM) broth.
  In RCM broth: Turbidity occurs with meat particles turning:
  - Black and production of foul odor: *C. botulinum* A, B, F (proteolytic)
  - Pink: *C. botulinum* C, D, E (saccharolytic).

  In blood agar: Colonies are large, irregular, semi-transparent, hemolytic with fimbriated border.
- Growth on culture media may be confirmed by Gram staining and biochemical tests
- Mere presence of bacilli in food or feces is of less significance. Toxin demonstration is more meaningful
- Serotyping is done with type specific antisera.

Toxin Demonstration (Mouse Bioassay)

Toxins can be detected in the specimens (serum, stool, sterile water or saline enema, gastric aspirates, wound material) or in samples of ingested foods.

- Specimens are injected into mouse, that develops paralysis in 48 hours; which can be inhibited by prior administration of specific antitoxin
- The sensitivity of the mouse bioassay varies inversely with the time elapsed between onset of symptoms and sample collection.

TREATMENT ***Clostridium botulinum***

- Meticulous intensive care support is needed (such as mechanical ventilation, if respiratory paralysis develops)
- **Botulinum antitoxin:** It should be administered immediately on clinical suspicion, without waiting for laboratory confirmation. Earlier the administration, better is the cure rate because antitoxin can neutralize the unbound free toxin molecules. However, once toxin binds to nerve endings, antitoxin has no role
- In wound botulism, suspected wounds and abscesses should be cleaned, debrided, and drained promptly
- Antibiotics: Though *C. botulinum* is susceptible to penicillin; the role of antibiotics has not been established.

CLOSTRIDIOIDES DIFFICILE

Clostridium difficile is an obligate anaerobic, gram-positive, spore-forming *Bacillus*, responsible for a unique colonic disease—**pseudomembranous colitis** which occurs almost exclusively in association with prolonged antimicrobial use. It was so named due to unusual difficulties involved in the isolation of *C. difficile*. Taxonomically, it is recently placed into a separate genera, *Clostridioides difficile*.

Pathogenesis

Clostridioides difficile is a major cause of hospital-acquired infection; mainly in the Western world. In India, the disease is reported less commonly. It is associated with the following risk factors.

- **Prolonged hospital stay:** Spores are found widely in nature, particularly in the hospitals and get colonized in colon of patients
- **Prolonged antimicrobial use:** This can result in disruption of the normal colonic flora, which enhances the susceptibility to *C. difficile* infection
  - Cephalosporins (e.g. ceftriaxone) are frequently responsible for this condition
  - Other antibiotics, such as clindamycin, ampicillin and fluoroquinolones (ciprofloxacin) are also implicated in hospital outbreaks
  - However, all antibiotics, including vancomycin and metronidazole (which are the drugs of choice in *C. difficile* infection) have been found to carry a risk of infection, if given for prolonged duration.
- **Toxin production:** Pathogenesis is toxin mediated. *C. difficile* may be harbored as a commensal in the intestine; however, only the toxigenic strains can cause pseudomembranous colitis
  - It produces two powerful exotoxins—toxin A (enterotoxin) and toxin B (cytotoxin)
  - Both toxins A and B are secreted in the intestine → glycosylate the GTP binding proteins that regulate the cellular actin cytoskeleton → disruption of the cytoskeleton results in loss of cell shape, adherence, and disruption of epithelial cell barrier → leading to diarrhea, and pseudomembrane formation
  - Infants do not develop symptomatic infection because they lack suitable mucosal toxin receptors which usually develop later in life.
- **Host immune response may determine the outcome of the infection**
  - Persons developing strong IgG response to toxin A—become asymptomatic carriers
  - Persons with inadequate IgG response to toxin A—develop disease.
- **Other risk factors:**
  - Suppression of normal flora (normal flora helps, converting primary bile salts to secondary bile salts, which inturn resist the germination of spores)
  - Advanced age (>65 years)
  - Immunosuppression and malignancy
  - Gastric acid suppressant medications
  - Use of electronic rectal thermometer.
- **Hypervirulent epidemic strain:** Recently, BI/NAP1/027 hypervirulent strain has been described. It produces higher levels of toxins and causes severe infection. Fidaxomicin is the drug of choice.

Clinical Manifestations

- **Diarrhea** is the most common manifestation caused by *C. difficile*. Other manifestations include fever, abdominal pain and leukocytosis. Blood in stool is uncommon
- **Pseudomembrane:** It is composed of necrotic leukocytes, fibrin, mucus, and cellular debris. It attaches to the underlying mucosa
  - It appears as whitish-yellow plaque of size ranging from 1–2 mm to large enough to spread over the entire colonic mucosa (Fig. 26.8A)
  - Relapse after treatment is common and seen in 15–30% of cases.

Laboratory Diagnosis

Laboratory diagnosis of *C. difficile* infection depends on isolation of the bacilli followed by toxigenicity testing.

- **Stool culture:** It is done under anaerobic condition at 37°C for 24–48 hours by using *C. difficile* specific selective media such as **CCFA** (cefoxitin cycloserine fructose agar) or **CCYA** (cefoxitin cycloserine egg yolk agar). Stool culture is highly sensitive and specific. However, since *C. difficile* can colonize the GIT, only isolation is not enough to establish the infection. Toxin demonstration is more meaningful
- **Cell culture cytotoxin neutralization assay:** It is highly specific but not as sensitive as stool culture and also has a long turnaround time

Figs 26.8A and B: A. Endoscopic image of pseudomembranous colitis, with yellow pseudomembranes seen on the wall of the sigmoid colon; **B.** Histopathology (H and E stain) of colonic pseudomembrane in *C. difficile* colitis

Source: A. Wikimedia Commons/Samir; B. Wikimedia Commons/Nephron (with permission).

- **Toxin detection:** Toxin A and B antigens can be detected in stool by various methods such as enzyme immunoassay or rapid tests
- **Toxin and GDH detection:** Glutamate dehydrogenase (GDH) is called as *C. difficile* common antigen; it is present in both toxigenic and non-toxigenic strains. Various methods such as enzyme immunoassay or rapid tests are available for simultaneous detection of toxin A, toxin B and GDH antigens from stool. The result is interpreted as follows (Figs 26.9A to C):
  - Positive for toxin A/B and GDH: Confirms *C. difficile* expressing either toxin A or toxin B
  - Positive for GDH only: Confirms presence of non-toxigenic strain of *C. difficile*
  - All negative: Rules out presence of *C. difficile* in stool.
- **Molecular methods:** Such as PCR, real time PCR, gene Xpert are available targeting genes coding for *C. difficile*

Figs 26.9A to C: C. DIFF QUIK CHEK test: (A) Only control band positive indicate test is negative; (B) Control band and glutamate dehydrogenase (GDH) band positive indicates nontoxigenic *C. difficile* present in stool (commensal); (C) Control band,Tox A/B and and GDH band positive indicates toxigenic *C. difficile* present in stool.

Source: Dr Padmaja A Shenoy, Associate Professor, Department of Microbiology, Kasturba Medical College, Manipal, Karnataka (with permission).

such as *tcd* A (toxin A), *tcd* B (toxin B) and *tpi* (triose phosphate isomerase) in stool. They are highly sensitive and specific with fast turnaround time

- **Colonoscopy:** It is highly specific if pseudomembranes are seen; however, the sensitivity is low, when compared with other tests (Fig. 26.8A)
- **Histopathology:** The histopathology of colonic pseudomembrane (obtained by colonoscopy) can be done by hematoxylin and eosin stain (Fig. 26.8B). It is highly specific; but the sensitivity is very low.

TREATMENT ***C. difficile***

Antimicrobial therapy

- **Initial episode, mild to moderate cases:** Oral metronidazole is the drug of choice (500 mg thrice a day for 10–14 days)
- **Recurrent episodes or severe cases:** Vancomycin is the drug of choice (500 mg, four times a day for 10–14 days)
- **Severe complicated or fulminant infection:** The combination of vancomycin (given via nasogastric tube and by retention enema) plus IV metronidazole has been recommended.

Other modalities of treatment

- **Intravenous immunoglobulin:** Passively provide antibodies to neutralize the *C. difficile* toxins, primarily toxin A
- **Fecal transplant:** It involves replenishing of the gut flora with donated feces from a screened healthy donor
- **Fidaxomicin:** It is a macrolide antibiotic, can be used in cases of relapse and also against hypervirulent strains.

Prevention (Infection Control Measures)

Broad spectrum antimicrobials should be stopped at the earliest. Infection control measures of contact precaution (see Chapter 53) should be followed such as:

- Strict hand hygiene with chlorhexidine 4% hand wash
- Isolation: Patient should be placed at isolation room and transfer should be restricted
- Ensure proper disinfection of floor, surfaces, toilets and other soiled areas using 1% freshly prepared hypochlorite solution.

NON-SPORING ANAEROBES

Medically important non-sporing anaerobes can be classified into gram-positive and gram-negative groups. For the sake of convenience, even the anaerobic cocci and anaerobic gram-negative bacilli are discussed in this Chapter (Table 26.4).

Beside the list, there are several other anaerobes that occur in soil and water and may be of industrial and agricultural importance.

Non-sporing anaerobes are often a part of normal flora of mouth, GIT and genital tract of man (Table 26.5) and animals. Many of these bacteria have also been recognized as important cause of human infections.

Table 26.4: Classification of non-sporing anaerobes

Gram-positive cocci	**Gram-negative cocci**
• *Peptostreptococcus*	• *Veillonella*
• *Peptococcus*	**Gram-negative bacilli**
Gram-positive bacilli	• *Bacteroides*
• *Bifidobacterium*	• *Prevotella*
• *Eubacterium*	• *Porphyromonas*
• *Propionibacterium*	• *Fusobacterium*
• *Lactobacillus*	• *Leptotrichia*
• *Actinomyces*	**Spirochete**
• *Mobiluncus*	• *Treponema, Borrelia*

Beneficial Role of Commensal Non-sporing Anaerobes

Being a part of normal flora, they modulate various physiological functions of human beings.

- They compete with the pathogenic bacteria through depletion of nutrients and production of enzymes and toxic metabolites
- They modulate host's intestinal innate immune response
- *Bacteroides* ferments carbohydrates and produce volatile fatty acids that are used by the host as an energy source
- They are responsible for the production of vitamin K and bile acids in the intestine
- Polysaccharide A of *Bacteroides fragilis* influences the normal development and function of immune system and protects against inflammatory bowel disease
- Lactobacilli maintain the vaginal acidic pH which prevents colonization of pathogens.

Non-sporing Anaerobes Causing Disease

- Anaerobic infections occur when the harmonious relationship between the host and the bacteria is disrupted
- Disruption of anatomical barrier (skin and mucosal barrier) by surgery, trauma, tumor, ischemia, or necrosis (all of which can reduce local tissue redox potentials) allow the penetration of many anaerobes, resulting in mixed infection.

INFECTIONS PRODUCED BY NON-SPORING ANAEROBES

Anaerobic bacteria can cause a wide variety of infections (Table 26.6).

Anaerobic Cocci

The anaerobic cocci occur as normal flora of skin, mouth, intestine and vagina.

- ***Peptococcus:*** It has one species, *P. niger*, which occurs as gram-positive cocci distributed singly or in pair or in clusters (but never in chain). It produces black colored colonies on blood agar and also produces H_2S
- ***Peptostreptococcus:*** It occurs as gram-positive cocci in pair or chains. It has many species which can be differentiated by various biochemical tests, susceptibility to antibiotics, such as SPS (sodium polyanethol sulfonate) and kanamycin and by fermentation of various sugars. Both *Peptococcus* and *Peptostreptococcus* are normal flora of skin, mouth, intestine and vagina. However, they are recovered from various clinical infections such as puerperal sepsis, skin and soft tissue infections and brain abscess (Table 26.6)
- **Veillonellae** are small gram-negative cocci, occurring in pairs or short chains. They are usually nonpathogenic.

Table 26.5: Anaerobes as a part of normal flora

Anatomical site	Bacteria/ g or mL	Anaerobic/ Aerobic ratio	Common anaerobic normal flora
Mouth			
Saliva	10^8–10^9	1:1	Anaerobic cocci
Tooth surface	10^{10}–10^{11}	1:1	*Actinomyces* *Fusobacterium* *Bifidobacterium*
Gingiva	10^{11}–10^{12}	10^3:1	*Prevotella* Spirochetes
Gastrointestinal tract (GIT)			
Stomach	0–10^5	1:1	*Lactobacillus*
Jejunum/ileum	10^4–10^7	1:1	Anaerobic cocci *Bacteroides fragilis*
Terminal ileum and colon	10^{11}–10^{12}	10^3:1	*Fusobacterium* *Bifidobacterium* *Prevotella*
Female genital tract			
Vagina	10^7–10^9	10:1	Anaerobic cocci *Lactobacillus* *Prevotella* *Bifidobacterium*
Skin			
Skin		3:2	*Propionibacterium*

Gram-positive Non-sporing Anaerobic Bacilli

- ***Bifidobacterium* species:** They are non-motile, pleomorphic bacilli that frequently exhibit branching. The name is derived from their typical appearance (bifid Y-shaped cells). They occur as normal flora in the mouth and gut and are mostly nonpathogenic
- ***Eubacterium* species:** They are commensals in mouth and intestine. They are rarely pathogenic (periodontitis)
- ***Propionibacterium* species:** They are related to corynebacteria and are usually labelled as anaerobic diphtheroids. They are skin commensals
  - *P. acnes* is the most common species, which is a common contaminant in blood and CSF. Taxonomically, it is re-classfied as *Cutibacterium acnes*
  - Its pathogenic role is uncertain.
- ***Lactobacillus* species:** They are non-motile gram-positive bacilli that frequently show bipolar and barred staining
  - They are widely distributed as saprophytes and ferment materials such as milk and cheese

Table 26.6: Anaerobic infections—an overview

Infections in various organs	Common anaerobe(s)
Mouth, head, and neck infections	
Dental caries (supra-gingival plaque)	*Lactobacilli*
Periodontal disease • Gingivitis and • Periodontitis	*Prevotella intermedia* *Porphyromonas gingivalis* *Treponema denticola*
Dental root canal infections	*Porphyromonas endodontalis*
Necrotizing ulcerative gingivitis • Vincent's angina • Trench mouth	*Leptotrichia buccalis*
Parapharyngeal space infections: • Peritonsillar abscess (quinsy) • Submandibular space infection (Ludwig's angina)	Mixed flora containing anaerobes and aerobes
Cervicofacial actinomycosis	*Actinomyces*
Sinusitis	*Prevotella, fusobacterium, Peptostreptococcus*
Chronic suppurative otitis media	*Bacteroides fragilis*
Central nervous system infections	
Brain abscess, epidural abscess, and subdural empyema	*Peptostreptococcus, Fusobacterium, Prevotella, Propionibacterium, Eubacterium, Veillonella, Actinomyces*
Pleuropulmonary infections	
Aspiration pneumonitis Necrotizing pneumonitis Anaerobic lung abscesses Empyema	Non-pigmented *Prevotella* *Peptostreptococcus* *Bacteroides* *Fusobacterium*
Abdominal infections	
Peritonitis and abscesses (following a breach in the intestinal mucosa)	Mixed colonic flora Most common—*Bacteroides fragilis*
Diarrhea	Enterotoxigenic *Bacteroides fragilis*
Pelvic and genitourinary infections	
Bartholin gland abscess, salpingitis, tubo-ovarian abscess, septic abortion, pyometra, endometritis and postoperative wound infection	*Bacteroides fragilis, Prevotella* (pigmented), Anaerobic cocci, *Clostridium* species
Puerperal sepsis	Anaerobic cocci
Bacterial vaginosis (also by *Gardnerella vaginalis*)	*Mobiluncus, Prevotella, Peptostreptococcus*
Skin and soft tissue infections	
Crepitant cellulitis, skin abscess, foot ulcers of diabetic patients	*Bacteroides, Peptostreptococcus, Clostridium*
Anaerobic bacterial synergistic gangrene (Meleney's gangrene), a rare infection of superficial fascia (also due to *Staphylococcus aureus*)	*Peptostreptococcus*
Necrotizing fasciitis (also due to *Streptococcus pyogenes*)	*Peptostreptococcus, Bacteroides*
Fournier gangrene (consists of cellulitis involving the scrotum, perineum and abdominal wall)	Mixed anaerobic organisms
Bone and joint infections	
Anaerobic infections of bone	*Actinomyces, Peptostreptococcus, Bacteroides, Fusobacterium*
Anaerobic septic arthritis	*Fusobacterium*
Cervical venous thrombophlebitis (Lemierre's syndrome)	*Fusobacterium necrophorum*
Bloodstream infection	
Bacteremia	*Bacteroides fragilis*
Endocarditis and pericarditis	*Bacteroides fragilis, Peptostreptococcus*

- They produce lactic acid from carbohydrates and grow best at pH of <5.

They are part of normal flora of mouth, gut and vagina.

- **In stomach:** Lactobacilli in the stomach (e.g. *L. acidophilus*) synthesise vitamins, such as biotin, vitamin B12 and vitamin K, which are useful to man
- **In the oral cavity:** It is postulated that lactobacilli may have a role in the pathogenesis of dental caries. Lactobacilli form acid by the fermentation of sucrose and other dietary carbohydrates which dissolve the mineral components of enamel and dentine

- **In vagina:** *Lactobacillus* species in adult vagina (known as Doderlein's bacilli) produce lactic acid that maintains the acidic pH of the adult vagina protecting from various infections. In prepubertal and postmenopausal vagina, lactobacilli are scanty which predispose to many infections.

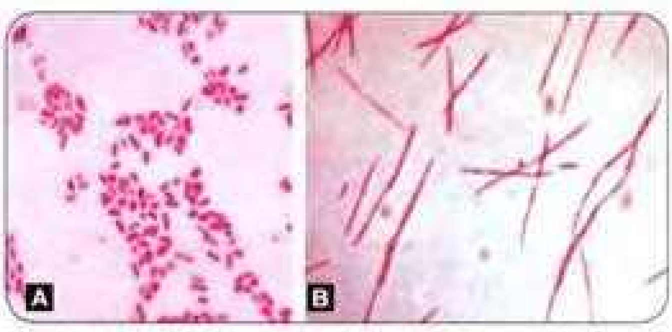
❖ ***Actinomyces:*** They are branching filamentous anaerobic gram-positive bacilli. It is described in Chapter 28

❖ ***Mobiluncus* species:** They are motile, curved, anaerobic gram-variable bacilli, isolated from the vagina in cases of *bacterial vaginosis*, along with *Gardnerella vaginalis*.

Gram-negative Non-sporing Anaerobic Bacilli

❖ ***Bacteroides fragilis:*** It is the most common commensal in the human intestine. At the same time, it is also probably the most frequent anaerobe isolated from the clinical specimens
- They are non-sporing, non-motile, obligate anaerobes, very pleomorphic, appearing as slender rods or coccobacillary forms (Fig. 26.10A)
- Virulence factors include:
  - Capsular polysaccharide
  - Lipopolysaccharide
  - Enterotoxin.
- It causes peritonitis following bowel injury and pelvic inflammatory disease (PID). It is also implicated in abdominal infections, brain abscesses and in empyema producing foul smelling pus. Enterotoxigenic strains can cause diarrhea.

❖ ***Prevotella:*** Previously classified under *Bacteroides*, it differs from the former in being moderately saccharolytic. It has many species which can be grouped into:
- Pigmented (e.g. *P. melaninogenica*):
  - Produces hemin derived black or brown colored colonies
  - Colonies produce characteristic red fluorescence when exposed to ultraviolet light
  - It has been isolated from lung or liver abscess, mastoiditis, and lesions of intestine and mouth.



Figs 26.10A and B: Gram-stained smear of. **A.** *Bacteroides fragilis*; **B.** *Fusobacterium* species

Source: A. Public Health Image Library, ID# 3084/Dr VR Dowell, Jr./Centers for Disease Control and Prevention (CDC), Atlanta. B. Microbes Wiki/Jhonan (with permission).

- Nonpigmented: For example *P. denticola* and *P. buccalis*.

❖ ***Porphyromonas:*** It differs from *Bacteroides* in being asaccharolytic and pigmented
- *P. gingivalis* is responsible for periodontal disease
- *P. endodontalis* causes dental root canal infections.

❖ ***Fusobacterium* species:** They are long, thin spindle-shaped bacilli with pointed ends (Fig. 26.10B)
- *F. nucleatum* is a normal inhabitant of the mouth and is found in oral infection and pleuropulmonary sepsis
- *F. necrophorum* is agent of Lemierre's syndrome (a form of thrombophlebitis).

❖ ***Leptotrichia buccalis*** (formerly called *Fusobacterium fusiforme*): They are long, thin spindle-shaped bacilli with pointed ends
- They are part of the normal oral flora
- They are implicated in an acute necrotizing gingivostomatitis known as **Vincent's angina;** characterized by inflamed pharyngeal mucosa covered by a grayish membrane which peels easily, resembling diphtheria.

CLINICAL PRESENTATION OF ANAEROBIC INFECTIONS

Anaerobic infections are associated with various clinical clues, such as:

❖ Infections adjacent to mucosal surfaces that bear anaerobic flora

❖ Predisposing factors such as ischemia, tumor, penetrating trauma, foreign body, or perforated viscus

❖ Spreading gangrene involving skin, subcutaneous tissue, fascia, and muscle

❖ Foul smelling putrid pus

❖ Abscess formation

❖ Septic thrombophlebitis

❖ Toxemia and fever not marked

❖ Failure to respond to antibiotics that do not have significant anaerobic activity

❖ Organisms are seen under Gram stain, but fail to grow in routine aerobic culture

❖ **Special features like:**
- Gas in specimen (gas gangrene)
- Black pigment that fluoresce (*P. melaninogenica*)
- Sulfur granules (*Actinomyces*).

LABORATORY DIAGNOSIS OF ANAEROBIC INFECTIONS

Specimens

All clinical specimens must be handled meticulously as brief exposure to oxygen may kill obligate anaerobes and result in failure to isolate them in the laboratory.

- Accepted specimens: Tissue bits, necrotic materials, aspirated body fluids or pus in syringes
- Unacceptable specimens: All swabs, sputum or voided urine
- Specimens should be immediately put into RCM broth or other anaerobic transport media and brought to the laboratory as soon as possible.

Microscopy

All clinical specimens from suspected anaerobic infections should be Gram stained and examined for characteristic morphology.

Cultural Identification

- **Anaerobiosis:** Samples should be processed immediately under anaerobic condition which can be created by various methods as described earlier
- **Culture:** Various culture media can be used for isolation of anaerobes, such as:
  - Anaerobic blood agar
  - Neomycin blood agar
  - Egg yolk agar
  - Phenylethyl agar (PEA)
  - BHIS agar: Brain-heart infusion agar added with supplements, such as vitamin K and hemin
  - *Bacteroides* bile esculin agar (BBE agar).
- **Identification of anaerobes** is based on:
  - Biochemical tests
  - Susceptibility to antibiotic disks
  - Gas liquid chromatography.

TREATMENT **Anaerobic infections**

Common antibiotics given for anaerobic infections are:
- Metronidazole
- Carbapenems (imipenem)
- β-lactam/β-lactamase inhibitor combination (ampicillin/sulbactam)
- Chloramphenicol.

Choice of antibiotics depends on the site of infection, type of anaerobe involved and susceptibility to antibiotics. Antimicrobial resistance in anaerobic bacteria is an increasing problem.

EXPECTED QUESTIONS

I. Essays:

1. Rajesh, a 23-year-old male was admitted 5 days after a crush injury to his right leg following a road traffic accident. He had been treated by a local village quack. On examination, the wound which was bandaged with a soiled gauze, appeared to be heavily contaminated with soil, the local muscles appeared to have been crushed, there was edema and pain at the site and crepitus was felt on palpation.
   a. What is the clinical condition? List the etiological agents responsible for this condition.
   b. Describe in detail the pathogenesis of this condition.
   c. Describe in detail the laboratory diagnosis of this condition.
2. 3-5 days following a bullet injury, a person developed trismus followed by muscle pain and stiffness, back pain, and difficulty in swallowing. As the disease progressed, painful muscle spasms developed which became generalized leading to a descending type of spastic paralysis. In the later stage, the patient assumed typical opisthotonus position of the body. Excised tissue bits from the necrotic depths of the wound revealed gram-positive bacilli with terminal and spherical spores.
   a. What is the diagnosis of this clinical condition?
   b. Describe in detail the pathogenesis and clinical manifestations of this condition.
   c. Describe in detail the laboratory diagnosis for confirming the etiological agent.
   d. Add a note on vaccination to prevent this condition.

II. Write short notes on:

1. Botulism
2. *Clostridioides difficile*
3. Non-sporing anaerobes

III. Multiple Choice Questions (MCQs):

1. **Characteristic of anaerobic bacteria is:**
   a. Foul smelling discharge
   b. Fail to grow in aerobic media
   c. Gas in tissue d. All of the above
2. **Principle toxin responsible for gas gangrene is:**
   a. Alpha toxin b. Theta toxin
   c. Beta toxin d. Delta toxin
3. **Site of action of tetanus toxin:**
   a. Presynaptic terminal of spinal cord
   b. Postsynaptic terminal of spinal cord
   c. Neuromuscular junction
   d. Muscle fibers
4. **The most effective way of preventing tetanus:**
   a. Hyperbaric oxygen b. Antibiotics
   c. Tetanus toxoid
   d. Surgical debridement and toilet
5. **Pseudomembranous colitis is caused by?**
   a. *Clostridium perfringens*
   b. *Clostridioides difficile*
   c. *Clostridium tetani*
   d. *Clostridium botulinum*
6. **Spore with drum stick appearance is produced by-**
   a. *C. bifermentans* b. *C. perfringens*
   c. *C. tetani* d. *C. tertium*
7. **Which of the following toxin of Clostridium perfringens has lecithinase activity?**
   a. Alpha b. Beta
   c. Epsilon d. Iota

Answers

1. d **2.** a **3.** a **4.** c **5.** b **6.** c **7.** a

Mycobacteria

27
CHAPTER

Chapter Preview

- *Mycobacterium tuberculosis* complex
- Nontuberculous mycobacteria
- *Mycobacterium leprae*

INTRODUCTION

Mycobacterium belongs to the family Mycobacteriaceae, order Actinomycetales.

All the species under the genus *Mycobacterium* should have the following minimum properties:

- **Acid fastness:** They resist decolorization by dilute mineral acids. Acid fastness is due to—(1) presence of high content of mycolic acids in the cell wall, and (2) integrity of the cell wall
- **Guanine plus cytosine** (G+C) content of DNA of *Mycobacterium* is 61–71 mol %, the only exception being *M. leprae* with a G+C content of 54 to 57 mol %.

Mycobacteria are non-motile, non-sporing, non-capsulated, weakly gram-positive, straight or slightly curved rod-shaped bacteria, which are obligate aerobes (or microaerophilic). They sometimes show branching filamentous forms resembling fungal mycelium (*myces* meaning fungus, reflecting the mould-like pellicle formation on liquid media).

History

- Lepra bacillus was discovered by Armauer Hansen (1874), hence it is called **Hansen's bacilli**
- Robert Koch (1882) isolated the tubercle bacillus and proved its causative role in tuberculosis as it satisfies the Koch's postulates.

Classification

Mycobacteria can be classified into:

- ***M. tuberculosis* complex:** It is responsible for tuberculosis in man
- ***M. leprae*** (Hansen's bacilli): It causes leprosy
- **Nontuberculous mycobacteria (NTM):** These are diverse group of mycobacteria. They are either saprophytic in nature; isolated in soil, water and other environmental sources, e.g. *M. phlei* (from grass) and *M. gordonae* (from tap water); or may be found as commensal in humans or animals (*M. smegmatis* in urine). Some of them can occasionally cause opportunistic human infection, e.g. *M. kansasii.*

M. leprae and NTM have been described in detail later in this Chapter.

MYCOBACTERIUM TUBERCULOSIS COMPLEX

M. tuberculosis complex causes tuberculosis, which is one of the oldest disease of mankind, and is a major cause of death worldwide. It usually affects the lungs, although other organs are also involved.

***M. tuberculosis* complex includes:**

- *M. tuberculosis* (human tubercle bacillus)
- *M. bovis* (bovine tubercle bacillus)
- *M. caprae* (closely related to *M. bovis*)
- *M. africanum* (isolated from a few West African cases)
- *M. microti* ('vole' bacillus, rare and less virulent)
- *M. pinnipedii* (infects seals in the Southern hemisphere and recently isolated from humans)
- *M. canetti* (a rare isolate from East African cases, that produces unusual smooth colonies on solid media)
- **Others:** *M. orygis, M. mungi* and the recently described dassie bacillus and chimpanzee bacillus.

These species are so closely related to each other by antigenic and molecular analysis that, they are regarded by many authors as variants of a single species. However, they can be distinguished from each other by certain properties.

Among all, *Mycobacterium tuberculosis* (MTB) is the most common cause of tuberculosis in man. The subsequent discussion about *M. tuberculosis* complex in this Chapter will be restricted to *M. tuberculosis.*

Antigenic Structure

Antigens of *M. tuberculosis* are mainly of two types:

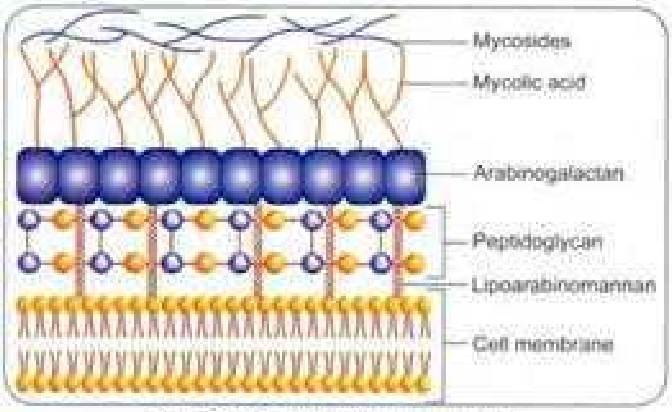


Fig. 27.1: Cell wall of *M. tuberculosis*

1. **Cell wall (insoluble) antigens:** The cell wall consists of several distinct layers (Fig. 27.1):
   - *Peptidoglycan layer:* It maintains the shape and rigidity of the cell
   - *Arabinogalactan layer:* It facilitates the survival of *M. tuberculosis* within the macrophages
   - *Mycolic acid layer:* It is the principal constituent, made up of long chain fatty acids attached to arabinogalactan. It confers very low permeability to the cell wall and is responsible for **acid fastness** and also reduces the entry of most antibiotics
   - *Outermost layer:* It consists of lipids (mycocerosates and acylglycerols), glycolipids and mycosides (phenolic glycolipids)
   - *Proteins* (e.g. porins, transport proteins): They are found throughout the various layers
   - *Plasma membrane:* This layer is present beneath the cell wall, into which various proteins, phosphatidylinositol mannosides, and **lipoarabinomannan (LAM)** are inserted. LAM is an important antigen, helps in attachment to host cell and is also a target antigen used for diagnosis.
2. **Cytoplasmic (soluble) antigens:** These include antigen 5, antigen 6, antigen 60; and are used in serodiagnosis of tuberculosis.

Pathogenesis

Source of Infection

The source of infection of *M. tuberculosis* may be—(1) human (e.g. cases of pulmonary tuberculosis), (2) bovine source (e.g. consumption of unpasteurized infected milk).

Mode of Transmission

Inhalational mode: *M. tuberculosis* is mainly transmitted by inhalation of droplet nuclei, generated while coughing, sneezing, or speaking of infected patients. There may be as many as 3000 infectious nuclei per cough. The tiny dry droplets (<5–10 µm size) may remain suspended in the air for several hours and are easily inhaled.

Other modes of transmission are rare, such as:
- **Inoculation:** Transmission of infection through direct skin contact with an infected person is uncommon
- **Ingestion:** Swallowing of sputum (in infants) or consumption of unpasteurized (infected) milk.

Risk Factors

The risk factors favoring the transmission of infection include:
- Sputum positive patients (sputum showing acid fast tubercle bacilli in microscopy) transmit more efficiently than sputum negative patients
- **Bacillary load:** At least 10^4 bacilli/mL in sputum is required for an effective transmission
- Adult patients with cavitary lesions in lung have more bacillary load in sputum and transmit more efficiently.
- Overcrowding in poorly ventilated rooms.

Following infection, not all, but only a minor proportion of people develop progressing disease. They usually have the following endogenous risk factors such as:
- **Low cell-mediated immunity:** For example, HIV-infected people
- Other comorbid conditions such as: Post-silicosis, post-transplantation (renal, cardiac), jejunoileal bypass, gastrectomy, chronic renal failure/hemodialysis, diabetes, IV drug abuse, smoking, etc.
- **Age:** Late adolescence and early adulthood periods are more prone
- **Sex:** Risk is higher in women at 25–34 years of age, while at older ages, men have greater risk.

Sequence of Pathogenic Events

The sequence of pathogenic events that take place are as follows:
- **Droplet nuclei** containing tubercle bacilli from infectious patients are inhaled. Majority are trapped in the upper airways and expelled out by the ciliary action of the mucosal cells; only a fraction (usually <10%) of small droplets reach the alveoli
- **Adhesion to macrophages:** Mycobacterial surface lipoarabinomannan (LAM) binds to complement receptors and mannose receptors present on the surface of macrophages. This leads to internalization of bacilli
- **Phagocytosis by macrophages:** It is enhanced by complement (C3b) mediated opsonization of bacilli
- **Survival inside the macrophages:** This is due to bacterial cell wall LAM which **impairs phagosome-lysosome fusion** by inhibiting increase in intracellular Ca^{2+} and phosphatidylinositol 3-phosphate
- If the bacilli are successful in arresting phagolysosome fusion, then they happily replicate inside the macrophage. The macrophage eventually ruptures and releases its bacillary contents which infect other phagocytes and the cycle continues.

Host Immune Response

Cell-mediated Immune Response

Host's cell-mediated immune response to tubercle bacilli is critical to contain the infection.

- Macrophages present the mycobacterial antigens to T_H (T helper) cells and activate them into T_H1and T_H2 subsets. T_H1 cells release cytokines such as IL-2 and IFN-γ, which activate monocytes and macrophages
- Thus, activation of T_H1 cells leads to development of two host responses: A macrophage-activating response and a tissue-damaging response. The balance between the two determines the outcome of the infection, as follows:

1. **Macrophage-activating response:**
   Majority of individuals show resistance to infection and are able to contain the bacilli.
   - IFN-γ activates the resting alveolar macrophages into activated macrophages which are capable of killing and digesting the tubercle bacilli
   - These activated macrophages aggregate around the center of the lesion and form characteristic granuloma called tubercles.

   Tubercles: Tubercles are the essential pathological findings in tuberculosis. Formation of tubercle is a favorable sign. They are primarily of two types:
   i. **Hard tubercles:** Tubercles are initially hard, composed of a central zone containing activated macrophages (epithelioid and giant cells) and a peripheral zone of lymphocytes and fibroblasts.
   ii. **Soft tubercles:** Later, the central part of the lesion undergoes caseous necrosis, and it contains necrotic material resembling soft cheese.
   Growth of *M. tuberculosis* is inhibited within this necrotic environment because of low oxygen tension and low pH. Eventually, the lesion heals and calcifies. The viable bacilli may remain dormant within the macrophages or within the necrotic material for many years without causing further tissue destruction.

2. **Tissue-damaging response:**
   In a minority of cases, especially those associated with risk factors (as mentioned above), the macrophage-activating response is weak and the bacilli are more virulent.
   - Here the mycobacterial growth can be inhibited only by an intensified delayed hypersensitivity reaction (DTH) which leads to lung tissue destruction
   - The caseous necrosis becomes liquefied, containing large numbers of bacilli which further spread by three ways:
     1. Direct draining into the airways, and then get discharged into the environment (while coughing and talking).
     2. Lymphatic spread and thereby reseeding into the same or opposite lung → then disseminate to other organs.
     3. Hematogenous spread to various organs.

Humoral Immune Response

T_H2 cells derived cytokines such as IL-4, IL-5 activate B-cells to produce antibodies.

Contd...

Contd...

- *M. tuberculosis* being obligate intracellular organism, humoral immunity plays a minor role
- However, the anti-LAM antibodies play a role in preventing dissemination of tuberculosis in children.

Clinical Manifestations

Tuberculosis (TB) is classified as pulmonary and extra-pulmonary forms.

Pulmonary Tuberculosis (PTB)

Pulmonary tuberculosis (PTB) accounts for 80% of all cases of tuberculosis (TB). It can be further categorized into primary or postprimary (secondary) types (Table 27.1).

Extrapulmonary Tuberculosis (EPTB)

EPTB results from hematogenous dissemination of tubercle bacilli to various organs. Though EPTB constitutes about 15-20% of all cases of TB, in HIV-positive patients, the frequency is much higher accounting for 20-50% of all cases of tuberculosis.

Though, virtually all organ systems may be affected however, the sites commonly involved (in order of frequency) are:

- **Tuberculous lymphadenitis:** It is the most common form, accounting for 35% of all EPTB cases. The most common sites are posterior cervical and supraclavicular lymph nodes. It presents as painless swelling in the neck region without warmth or color change
- **Pleural tuberculosis:** It accounts for 20% of all EPTB cases. It presents as pleural effusion
- **Tuberculosis of the upper airways**: Involving larynx, pharynx, and epiglottis
- **Genitourinary tuberculosis:**
  - Renal tuberculosis
  - Genital tuberculosis: In female patients, fallopian tubes and the endometrium are commonly involved causing infertility. In males, epididymis is the most common site.
- **Skeletal tuberculosis:** Weight-bearing joints, such as spine (Pott's disease or tuberculous spondylitis is most common), hips and knees are commonly affected. With advanced disease, collapse of vertebral bodies results in kyphosis (gibbus) and a paravertebral 'cold' abscess may also form
- **Tuberculosis of CNS:** It occurs commonly in children. Tuberculous meningitis and tuberculoma are the common forms
- **Gastrointestinal tuberculosis:** Terminal ileum and cecum are the most common sites involved. The route of spread may be due to swallowing of sputum with

Table 27.1: Comparison of primary and secondary pulmonary tuberculosis

Features	Primary pulmonary tuberculosis	Postprimary/secondary pulmonary tuberculosis
Results due to	Initial exogenous infection with tubercle bacilli	• Exogenous reinfection • Endogenous—reactivation of the latent primary lesion
Age group affected	Children	Adults
Parts of the lungs commonly affected	Subpleural lesion affecting, upper part of the lower lobe and lower part of the upper lobe	Apical and posterior segments of the upper lobes (areas of high oxygen tension)
Lesions formed at the initial sites	Fibrotic nodular lesions are formed **(Ghon focus)**	Calcified nodules are formed (**Assmann** focus) Hematogenous seedling in the apex of lungs called **Simon's focus**
Lymph node	Ghon focus with associated hilar lymphadenopathy is common (called **primary complex**)	Lymph node involvement is unusual
Clinical feature	It may be asymptomatic or may present with fever, productive cough (with or without hemoptysis) and occasionally chest pain, night sweating, weight loss	Lesions undergoing necrosis and tissue destruction, leading to cavity formation Symptoms are similar, but more pronounced
Fate	In the majority of cases: • Lesions heal spontaneously. • Primary complex becomes calcified **(Ranke complex)** Rarely, in children with impaired immunity, progressive primary TB (develops and spreads by local invasion and by lymphatics)	*In majority of cases:* The necrotic material breaks into the airways, leading to: • Bronchogenic spread to the same or opposite lung forms satellite lesions, which coalesce producing caseating pneumonia • Expectoration of bacteria-laden sputum • Hematogenous spread leading to seedling of bacilli in various parts of the body and granuloma formation. Rarely heals spontaneously

direct seeding, hematogenous spread, or ingestion of cow's milk contaminated with *M. bovis* (in developing countries)

- **Tuberculous pericarditis:** It occurs as direct extension from adjacent lymph nodes or following hematogenous spread. It occurs in elderly people, in countries with low TB prevalence
- **Tuberculous skin lesions:**
  - ***Scrofuloderma:*** It is a skin condition caused by tuberculous involvement of the skin by direct extension, usually from underlying tuberculous lymphadenitis
  - ***Lupus vulgaris:*** Apple jelly nodules are formed over the face in females.
- **Miliary or disseminated tuberculosis:** Hematogenous spread of tubercle bacilli results in the formation of yellowish 1-2 mm size granulomatous lesions resembling **millet seeds** (thus termed as miliary) in various organs. It is more common in HIV-infected people.

HIV-associated Tuberculosis

Tuberculosis is one of the most common opportunistic diseases among HIV-infected persons due to low CMI. Worldwide, TB occurs in 70-80% of HIV-infected individuals, EPTB being more common than PTB.

Epidemiology

About one-third of the current world population is infected asymptomatically with *M. tuberculosis*, of which 5-10% develop clinical disease during their lifetime.

- WHO (World Health Organization) has estimated that 10.4 million new cases of TB occurred in 2016 with 1.7 million deaths (including 0.4 million deaths among people co-infected with HIV). Over 95% of TB deaths occur in low and middle-income countries
- Seven countries account for 64% of the total TB burden, with India leading the count (25% of total TB cases and 33% of total TB deaths), followed by Indonesia, China, Philippines, Pakistan, Nigeria, and South Africa. Patients with infectious pulmonary TB can infect 10-15 people in a year
- TB is the ninth leading cause of death worldwide and the leading cause among infectious diseases, ranking above HIV/AIDS.

LABORATORY DIAGNOSIS ***Mycobacterium tuberculosis***

Diagnosis of Active Tuberculosis

Specimen collection
- In pulmonary TB: Sputum (2 specimens—spot and early morning), gastric aspirate (in children)
- In EPTB: Specimens vary depending on the site involved.

Digestion, decontamination and concentration of specimen:
- Modified Petroff's method (4% NaOH)
- NALC (N-acetyl-L-cysteine) + 2% NaOH.

Direct microscopy by acid-fast staining:
- Ziehl-Neelsen (ZN) technique—long slender, beaded, less uniformly stained red color acid fast bacilli
- Kinyoun's cold acid fast staining
- Fluorescent (auramine) staining—it is more sensitive and smears can be screened more rapidly than ZN stain.

Contd...

Contd...

LABORATORY DIAGNOSIS	*Mycobacterium tuberculosis*

Conventional culture media—take 6–8 weeks

- Solid media, e.g. Lowenstein Jensen (LJ) medium—shows rough, tough and buff colored colonies in 6–8 weeks
- Liquid media—Kirchner's medium and Middlebrook 7H9 medium.

Automated culture methods—take 3–4 weeks

- **MGIT system:** Detects growth and resistance to antitubercular drugs (ATDs); with a turnaround time of 2–3 weeks
- BacT/ALERT: Detects growth
- VersaTek system: Detects growth and resistance to ATDs.

Culture identification

- MPT 64 antigen detection—by ICT
- Biochemical identification—Niacin test (obsolete).

Molecular methods

- PCR detecting IS6110 gene
- **CBNAAT (GeneXpert)**—for identification and detection of resistance to rifampicin; has a turnaround time 2 hours
- **Line probe assay (e.g. Genotype TB)**—for identification and detection of resistance to 1st and 2nd line ATDs; has a turnaround time 2–3 days.

Diagnosis of latent tuberculosis:

By tuberculin test (e.g. Mantoux test) and interferon gamma release assay (IGRA).

Laboratory Diagnosis

Laboratory diagnosis of active tuberculosis can be established by various methods described below.

The diagnosis of latent tuberculosis is explained later in the description.

Specimen Collection

In PTB, *two sputum* samples are recommended—**spot sample** (collected on the same day under supervision) and **early morning sample** (collected on the next day). Alternatively 2 spot samples at least one hour apart can be collected. Sputum should be at least 2–5 mL in quantity and preferably mucopurulent; without blood tinged. The extrapulmonary specimens vary depending on the site involved, which can be divided into two categories (Table 27.2).

Laboratory diagnosis of PTB is described below. The differences between the laboratory diagnosis of EPTB from that of PTB is followed thereafter.

Table 27.2: Extrapulmonary specimens

Sterile site specimens collected aseptically	
Optimum specimens	CSF, spinal, pericardial, synovial, ascitic, blood and bone marrow, pleural biopsy, tissues (collected in sterile saline)
Suboptimal specimens (organism load is less)	Pleural fluid (20–50 mL is collected and centrifuged) Blood (indicated only for disseminated TB and co-infected with HIV)
Specimens containing normal flora	
Swabs	Considered suboptimal specimen. The only recommended swabs are: • *Laryngeal swabs:* Collected early morning in empty stomach or • Swab from discharging sinus
Urine	Three early morning specimens collected (500 mL/specimen, centrifuged) on different days as TB bacilli in urine are shed intermittently
Stool	For disseminated TB in HIV infected patients and infants
Other respiratory specimens	Bronchial secretions (2–5 mL) Bronchoalveolar lavage (20–50 mL) Transbronchial and other biopsies
Gastric lavage	Recommended for children (tend to swallow sputum), or ICU patients (aspiration) Early morning lavage should be collected and processed early (<4 hours)

Note: Samples for culture should never be collected in formalin.
If histopathological examination is required, two samples should be collected.

Digestion, Decontamination and Concentration

Sputum and specimens from non-sterile sites subjected to smear microscopy and culture need prior treatment for digestion (to liquefy the thick pus cells and homogenization), decontamination (to inhibit the normal flora) and concentration (to increase the yield). However, this step is not required for molecular methods and also for processing of extrapulmonary specimens collected aseptically from sterile sites. Commonly used methods are:

- **Modified Petroff's method (4% NaOH):** Sputum is thoroughly mixed with equal volumes of 4% sodium hydroxide, centrifuged and the sediment is neutralized with phosphate buffer saline of pH 6.8. This method is recommended for LJ culture
- **NALC (N-acetyl-L-cysteine) + 2% NaOH:** This is superior to Petroff's method for isolation. NALC liquefies the sputum and NaOH kills the normal flora. This method is more compatible with automated culture systems.

Direct Microscopy by Acid Fast Staining

Ziehl-Neelsen (ZN) technique (hot method)

Smears are prepared from thick mucopurulent part of sputum or with the sediment obtained after concentration. Optimum thickness of the smear can be assessed by placing the smear on printed matter. The print should be just readable through the smear. Then the smear is stained by acid fast stain (for procedure, refer 'acid fast staining' in Chapter 2).

- **Interpretation**
  - *Negative result:* At least 100 oil immersion fields should be examined for 10-15 minutes before giving a negative report
  - *Positive result: M. tuberculosis* appears as long slender, beaded, less uniformly stained red colored acid fast bacilli (AFB) (**Fig. 27.2A**).
- **Presumptive diagnosis:** Microscopy provides only presumptive diagnosis. If typical beaded appearance is seen, then it should be reported as *'acid fast bacilli resembling M. tuberculosis are seen by smear microscopy by ZN stain'*
- **Advantages:** Smear microscopy is rapid, easy to perform at peripheral laboratories and is cheaper
- **Disadvantages:** (i) Smear microscopy is less sensitive than culture. (ii) Low sensitivity with a detection limit of 10,000 bacilli/mL of sputum. (iii) It cannot determine the viability of bacilli
  - It is difficult to differentiate *M. tuberculosis* from saprophytic mycobacteria present in tap water or even as commensal in clinical samples such as gastric aspirate, and urine
  - *Acid alcohol* (3% hydrochloric acid+ 95% ethyl alcohol) can be used to differentiate *M. tuberculosis* (acid and alcohol fast) from *M. smegmatis* (only acid fast but not alcohol fast) in urine sample.

RNTCP guidelines for grading of sputum smear

Revised National Tuberculosis Control Program (RNTCP) of India has given guidelines for grading of **ZN stained sputum smears** (Table 27.3).

RNTCP grading is useful for:

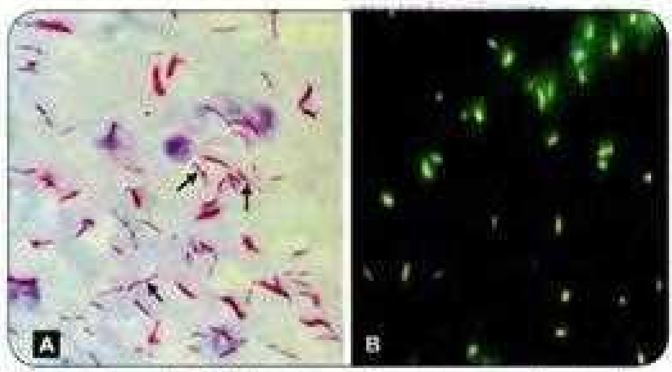
- Monitoring the treatment response of the patients
- Assessing the severity of disease
- Assessing the infectiousness of the patient: Higher the grade more is the infectiousness. Smear negative patients (<10,000 bacilli/mL of sputum) are less infectious.

However, grading of the sputum smear depends upon the quality of sputum collected.

Table 27.3: RNTCP guidelines for grading of ZN stained sputum smears

No. of AFB seen	OIF to be screened	Grading	Result
No AFB in 100 OIF	100	0	Negative
1–9/ 100 OIF	100	Scanty*	Positive
10–99/100 OIF	100	1+	Positive
1–10/ OIF	50	2+	Positive
>10/ OIF	20	3+	Positive

Abbreviations: AFB, acid fast bacilli; OIF, Oil immersion fields; ZN, Ziehl-Neelsen.
*Record the actual no. of bacilli seen in 100 fields e.g. "Scanty 8".



Figs 27.2A and B: A. ZN staining of sputum smear showing long, slender and beaded red colored acid-fast bacilli; **B.** Auramine phenol staining of sputum smear—Tubercle bacilli appear bright brilliant green against dark background

Source: A and B. Department of Microbiology, JIPMER, Puducherry (with permission).

Kinyoun's Cold Acid Fast Staining

It differs from ZN stain in that—(i) Heating is not required (ii) Phenol concentration in carbol fuchsin is increased and (iii) Duration of carbol fuchsin staining is more.

Fluorescence Staining

It is a fluorescent staining technique, uses 0.3% auramine solution (for 20 min) as primary stain, 0.5% acid alcohol (for 3 min) as decolorizer and 0.1% potassium permanganate (for 1 min) as counter stain. Then the slide is examined under fluorescent LED (light-emitting diode) microscope.

- The bacilli appear brilliant yellow against dark background (Fig. 27.2B)
- Smears are screened by using 20X or 25X objective, hence can be screened faster (2 min for 100 fields)
- It is more sensitive than ZN staining and has been the recommended screening method by RNTCP
- However, artifacts may confound with the interpretation. Hence the reading should be taken by an expert.

Culture Methods

Culture is traditionally considered as gold standard method of diagnosis of TB. It offers several advantages:

- It is more sensitive than microscopy with detection limit of 10-100 viable bacilli
- Indicates viability: TB bacilli growing on culture indicates that they are viable
- Drug susceptibility testing can be performed.

RNTCP recommended culture media include both conventional solid media (Lowenstein Jensen medium) and automated liquid culture, such as Mycobacteria Growth Indicator Tube (MGIT).

Conventional Solid Media (Lowenstein Jensen Medium)

Lowenstein Jensen (LJ) medium has been the most widely used and recommended by RNTCP.

- It is composed of coagulated hen's eggs, mineral salt solution, asparagine and malachite green (as a selective agent)
- Inoculated media are incubated for prolonged duration of 6–8 weeks. This is because of the slow growing nature of tubercle bacilli (long generation time of 10–15 hours)
- *Colonies: M. tuberculosis* produces typical rough, tough and buff colored colonies (Fig. 27.3A). In contrast, *M. bovis* produces smooth, moist and white colored colonies which break up easily when touched.

Conventional liquid media are Kirchner's medium and Middlebrook 7H9 medium. They are not routinely used.

Automated Liquid Culture

Automated culture systems monitor the growth continuously and offer a faster turnaround time compared to conventional culture.

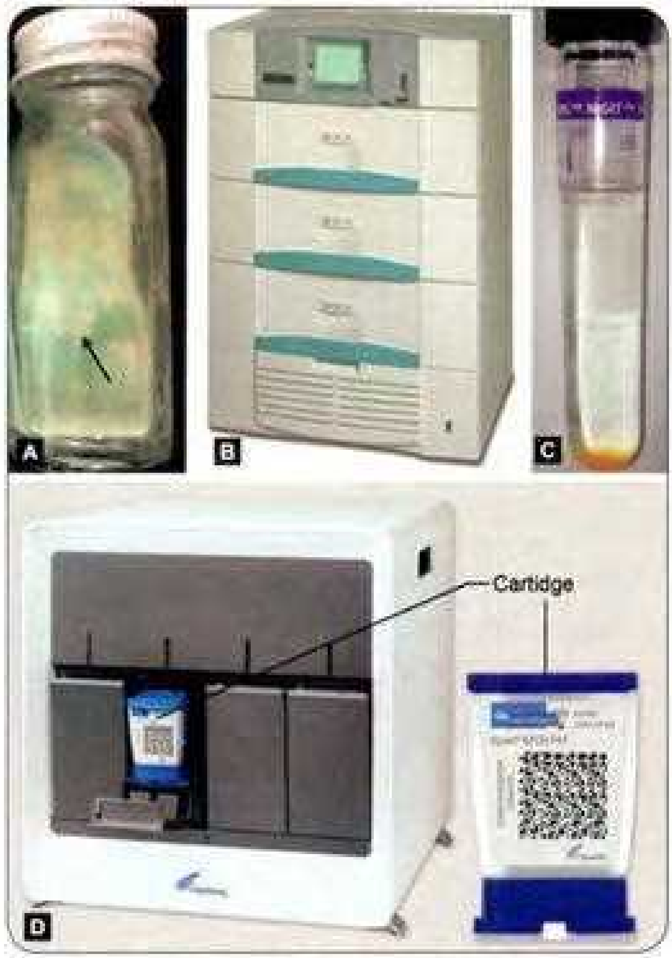
- Positive growth (99%) gets detected within 3–4 weeks. However, the negative result is reported after 6 weeks of incubation
- They use liquid broth such as Middlebrook 7H9 medium supplemented with:
  - OADC enrichment growth media (oleic acid, albumin, dextrose and catalase): to promote the growth *M. tuberculosis* and
  - PANTA antibiotic mixture (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin): To inhibit other organisms present in specimen.

Various automated systems available are:

- **BACTEC MGIT** (**M**ycobacteria **g**rowth **i**ndicator **t**ube): This is the automated system endorsed by WHO and RNTCP (Figs 27.3B and C)
  - Uses: (i) It detects growth of mycobacteria and (ii) also performs the drug susceptibility testing against first line and second line antitubercular drugs
  - Principle: It uses an oxygen sensitive fluorescent compound, dissolved in the broth. Initially, the large amount of dissolved oxygen in the medium quenches emissions from the fluorescent compound. Later, actively respiring microorganisms consume the oxygen; the quenching effect is lost which allows the fluorescence to be detected.
- Other automated systems include VersaTek and BacT/ALERT systems.

Culture Identification

The colonies grown on LJ media and the broth from a positively flagged automated culture bottle are first subjected to acid fast stain. If found AFB positive, then further tests are done for species identification.



Figs 27.3A to D: Culture media/culture systems for *M. tuberculosis*. **A.** Lowenstein-Jensen medium (arrow showing rough, tough and buff colored colonies); **B.** BACTEC MGIT **C.** MGIT liquid culture medium **D.** GeneXpert system with cartridge

Source: Department of Microbiology, JIPMER, Puducherry (with permission).

- **MPT 64 antigen** detection by rapid immunochromatographic test: MPT64, a 28 Da antigen is specific for *M. tuberculosis* complex (*M. tuberculosis, M.bovis* and *M. africanum*) and negative for NTM (nontuberculous mycobacteria)
- **Biochemical tests** such as niacin test, nitrate reduction and pyrazinamidase test: *M. tuberculosis* gives a positive reaction; *M. bovis* is negative. These tests are almost obsolete now
- **Rabbit pathogenicity test:** It was previously followed; now obsolete. *M. bovis* is pathogenic to rabbit, whereas *M. tuberculosis* is not pathogenic.

Serology

Serological methods (both antigen and antibody detection methods) are not recommended because of low sensitivity; cross reactivity with other mycobacteria and variable antibody response against different epitopes. WHO has banned the use of serological tests.

Molecular Methods

Molecular methods are extremely useful as:
- They take less time than culture
- They are more sensitive than culture. This is very much useful for extrapulmonary samples that are usually paucibacillary
- They can also detect the genes coding for drug resistance
- Used for epidemiological typing of strains.

There are several molecular methods available as described below.

Polymerase Chain Reaction (PCR)

Nested PCR targeting IS6110 gene was the most common molecular test used earlier. Other genes which were targeted by using PCR: MPT64 gene, 65 KDa and 38 KDa genes.

Cartridge-based nucleic acid amplification test (CBNAAT)

With the advent of automated real time PCR systems such as CBNAAT; the diagnosis of TB has been completely revolutionized. It is the real game changer.

GeneXpert

GeneXpert (Cepheid's) is the CBNAAT system endorsed by WHO and is used in India under RNTCP (see Fig. 27.3D).
- **Rapid:** It has the lowest turnaround time (2 hours) among all the diagnostic methods currently available for TB
- **Principle:** It is based on by real-time PCR technique; simultaneously detects: (i) MTB complex DNA and (ii) rifampicin resistance (mutations of the *rpoB* gene). It uses five probes targeting various sequences of *rpoB* gene
- **No contamination:** It employs single-use disposable cartridges containing PCR reagents. Because the cartridges are self-contained, cross-contamination between samples is eliminated (see Fig 27.3D)
- **Procedure:** Specimen is added to a buffer solution (to liquefy the sample) and then inoculated into cartridge. The entire process is fully automated (2 hours), starting from sample processing, nucleic acid extraction, amplification, and reporting of the result
- **EPTB:** WHO recommends GeneXpert as the initial test for diagnosis of EPTB; especially for CSF, lymph nodes and other tissues specimens. As the bacilli are more in number in the pleural wall; the ideal specimen for pleural TB is pleural biopsy; not pleural fluid
- **Diagnostic utility:** The detection limit of GeneXpert is about 131 bacilli/mL of specimen. Compared to culture, the sensitivity and specificity are as follows:
  - For detection of TB bacilli: It is 88% sensitive and 99% specific
  - For detection of rifampicin resistance: It is 95% sensitive and 98% specific.
- **Disadvantages:** (i) very expensive, (ii) cannot further speciate MTB complex.

Line Probe Assay (LPA)

Line probe assay involves probe-based detection of amplified DNA in the specimen.
- **Use of LPA in TB diagnostics:**
  - The basic LPA kits are used for (i) identification of MTB complex and (iii) detection of resistance to anti-tubercular drugs such as rifampicin and isoniazid
  - Newer kits of LPA are available which can also be used for: (i) speciation of MTB complex and NTM; and (ii) detection of resistance to second line drugs such as fluoroquinolones, aminoglycosides, and ethambutol.
- **Limitation:** LPA can be performed only on positive cultures or smear positive clinical specimens. It is not recommended for smear negative specimens as the sensitivity is low
- **Kits and systems:**
  - The commercial kits available to perform LPA are GenoType and INNO-LiPA
  - There are two systems available to perform the assay by using the commercial kits: (i) TwinCubator (manual system) and GT-Blot (automated system).
- **Principle:** The whole process takes 2-3 days of turnaround time and is divided into three steps:
  i. *DNA extraction* from clinical specimens (pulmonary, decontaminated) or culture (solid/liquid medium).
  ii. *Multiplex PCR amplification* with biotinylated primers.
  iii. *Reverse hybridization:* This involves chemical denaturation of amplicons into single stranded amplicons. Then the membrane strips coated with specific probes are added which bind to the complementary sites of the amplified nucleic acids (hybridization). Then the streptavidin-conjugated alkaline phosphatase is added which detects the hybrids by biotin-streptavidin moieties.
- **Diagnostic utility:** The sensitivity and specificity of LPA:
  - For the detection of TB bacilli: 81.5% and 87.5% respectively
  - For detection of rifampicin resistance: 97% and 99% respectively
  - For detection of INH resistance: 90% and 99% respectively.
- LPA is useful particularly in isoniazid mono-resistant cases of TB, which are not diagnosed by GeneXpert.

Upcoming Methods for TB Diagnosis

Chip Based Real Time PCR (TrueNat)

TrueNat (Molbio's) is a chip-based real-time PCR system, validated recently by Indian Council of Medical Research (ICMR), in 2017; yet to be endorsed by WHO.

- **Advantage:** It is an automated battery operated device; can be used at level of primary health center where GeneXpert cannot be used as it needs uninterrupted power supply and air conditioning
- **Disadvantages:** (i) very expensive, (ii) cannot further speciate MTB complex and (iii) it tests one sample at a time; in contrast to GeneXpert which can test single or multiple samples.

Other Newer Methods

- **TB-LAMP** (Loop-mediated isothermal amplification): It is endorsed by WHO to use as an alternative to smear microscopy for identification. However, it does not detect drug resistance
- **Next generation GeneXpert** (Xpert Ultra): It was endorsed by WHO in 2017
  - It uses an improved version of cartridge (called Ultra cartridge) which has a larger chamber for DNA amplification (to accommodate larger amount of sputum) and contains two additional molecular targets to detect TB
  - These changes made Xpert Ultra more sensitive and specific with detection limit of 16 bacilli/mL, compared to 131 bacilli/mL of first generation GeneXpert.
- **Breath biomarkers** such as volatile organic compounds (VOCs) detection in breath
- **LAM** (lipoarabinomannan) antigen detection in urine and sputum
- **IRISA TB** (InterGam Rapid Immuno Suspension Assay): It detects interferon gamma from extrapulmonary sites such as pleural, pericardial and ascitic fluid
- Automated microscopy and image detection
- **Non-commercial culture and drug-susceptibility testing (DST):** Useful for settings with resource limited settings. Promising methods in the pipeline are: (i) microscopic observation of drug susceptibility (MODS), (ii) colorimetric redox indicator (CRI) methods, etc.

RNTCP Guideline 2016 for Diagnostic Algorithm for Tuberculosis

RNTCP has recently modified the guideline for the diagnostic algorithm to be followed for tuberculosis. This algorithm is being followed across India in every laboratory. Before looking into the algorithm, the following terminologies need to be understood first.

- **Presumptive pulmonary TB:** It refers to a person with any of the symptoms or signs suggestive of TB: (i) cough >2 weeks, (ii) fever >2 weeks, (iii) significant weight loss, (iv) hemoptysis, (v) any abnormalities in chest X-ray
- **Presumptive DRTB (drug resistant TB) case:** This includes: (i) TB patients who have failed treatment with first line drugs, (ii) pediatric TB non-responders, (iii) TB patients who are contacts of DRTB cases, (iv) TB patients found positive on any sputum smear examination during treatment with first-line drugs, (v) previously treated TB case, (vi) new TB patients with HIV co-infection
- **RIF status:** The rifampicin susceptibility testing report is expressed as RIF sensitive (if found sensitive), RIF resistant (if found resistant), RIF indeterminate (if result is not clear) and RIF unknown (if not tested).

Diagnostic Algorithm

For pulmonary TB: The diagnostic algorithm for pulmonary TB in adults is explained in Figure 27.4. For detail, refer to RNTCP guideline 2016.

For extrapulmonary TB (EPTB): EPTB specimens are paucibacillary. Hence, CBNAAT is directly performed. If not available, liquid culture (MGIT) is performed.

For pediatric presumptive pulmonary TB: CBNAAT is directly performed on sputum. If MTB is not detected or CBNAAT is not available; then decision is made based on chest X-ray and tuberculin skin test (TST).

- If chest X-ray is highly suggestive (miliary shadows, hilar or mediastinal lymphadenopathy or chronic fibrocavitary shadows) perform CBNAAT on alternate specimens (gastric aspirate or induced sputum)
- If chest X-ray is non-specific (e.g. consolidations): treat for bacterial pneumonia. If symptoms still persists, perform CBNAAT on alternate specimens
- If chest X-ray negative, TST positive—evaluate for EPTB
- If chest X-ray negative, TST negative—look for alternate cause.

Typing of Mycobacteria

Typing of mycobacteria is useful for epidemiological studies in determining the relatedness between various isolates in the communities. Currently the following genotyping methods are used for molecular typing of *M. tuberculosis*.

- PCR-RFLP (Restriction fragment length polymorphism) is used to detect variations in IS6110 gene
- **Spoligotyping:** This is based on detecting the polymorphisms in DR locus (direct repeat) of *M. tuberculosis* genome. It is more useful in strains having no or few copies of IS6110 gene
- Pulse field gel electrophoresis (PFGE)
- DNA sequencing.

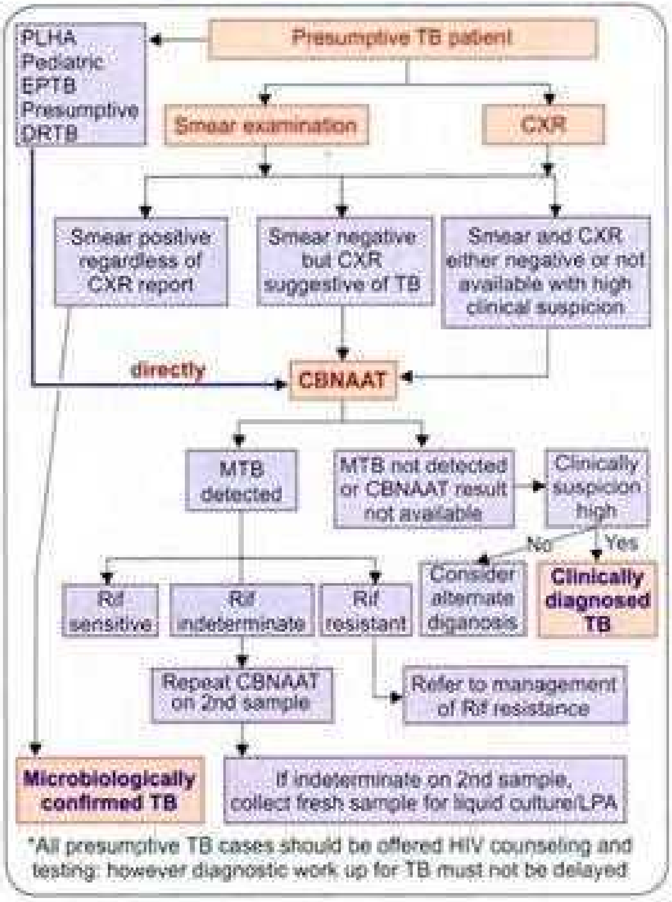


Fig. 27.4: Diagnostic algorithm for pulmonary tuberculosis (*Adapted from* RNCTP guideline 2016)

Abbreviations: Rif, Rifampicin; CBNAAT, Cartridge-based nucleic acid amplification test; PLHA, people living with HIV/AIDS; DRTB, drug resistant tuberculosis; CXR, chest X-ray; LPA, line probe assay.

Diagnosis of Latent Tuberculosis

Latent tuberculosis is diagnosed by demonstration of delayed or type IV hypersensitivity reaction against the tubercle bacilli antigens. Two methods are available, (1) tuberculin test, (2) IFNγ release assay.

Tuberculin Test

Traditionally, the tuberculin test has been in use for diagnosis of latent TB for >100 years. It was discovered by Von Pirquet in 1907.

- **Antigens used in tuberculin test:**
  - **OT** (Old tuberculin antigen): It is a crude preparation of tubercle bacilli. It was described by Robert Koch, now rarely used
  - **PPD** (Purified protein derivative antigen): It is a purified preparation of the active tuberculoprotein, prepared by Seibert in 1941(PPD-S) by growing *M. tuberculosis* in a semisynthetic medium
    - WHO recommends a preparation of PPD, known as PPD-RT-23 with Tween 80
    - PPD preparation from atypical mycobacteria such as PPD-B (Battey mycobacteria), PPD-Y (from *M. kansasii*) and scrofulin (from *M. scrofulaceum*) are also available.
- **Dosage:** It is expressed in tuberculin unit (TU). One TU is equal to 0.01 mL of OT or 0.00002 mg of PPD
- **Procedure:**
  - Mantoux test: It is the most commonly employed method. 0.1 mL of PPD containing 1 TU is injected intradermally into flexor surface of forearm
  - Heaf and Tine multiple puncture tests: Both the techniques are not in use as they are less precise in measurement of tuberculin reactivity.
- **Reading:** It is taken after 48–72 hours. At the site of inoculation, an induration surrounded by erythema is produced. If the width of the induration is:
  - ≥10 mm: Positive (tuberculin reactors)
  - 6–9 mm: Equivocal/doubtful reaction
  - <5 mm: Negative reaction.
- **Interpretation of result:**
  - Adults: Positive tuberculin test in adults only indicates present or past exposure with tubercle bacilli but does not confirm the presence of active stage of the disease. Hence, it is only used as an epidemiological marker
    - Prevalence of tuberculosis is calculated by counting all tuberculin reactors in a community
    - Incidence of tuberculosis is calculated by counting new converters to tuberculin test in a community.
  - Children: In children, positive test indicates active infection and used as **diagnostic marker**.
- **False-positive:** The test becomes positive after
  - BCG vaccination (after 8–14 weeks)
  - Nontuberculous mycobacteria infection.
- **False-negative:** The test may become negative in various conditions such as—early or advanced TB, miliary TB, decreased immunity (HIV-infected people)
- **Two-step testing:** In adults, tuberculin reactivity slowly wanes with time and it may become negative after some years. In such a case, a repeat test 1–2 weeks after the first test exerts a booster effect and gives a strong positive reaction (>20 mm).

Interferon Gamma Release Assay (IGRA)

This uses highly specific *M. tuberculosis* antigens such as CFP10 (culture filtrate protein) and ESAT6 (early secreted antigenic target-6); both coded by RD1 genes.

- **Procedure:** In contrast to tuberculin test, it is an *in vitro* test. Sensitized T lymphocytes collected from suspected individuals, are exposed to ESAT-6/CFP-10 antigens, which lead to release of high level of IFNγ from the T lymphocytes. An ELISA format is available commercially (QuantiFERON-TB Gold assay)

- **Advantage:** It is highly specific; there are no false-positive results.

TREATMENT — Tuberculosis

Anti-tubercular drugs (ATDs) can be classified into:

First line drugs:
- Isoniazid (H)
- Rifampin (R)
- Pyrazinamide (Z)
- Ethambutol (E)
- Streptomycin (S)

Second line drugs:
- Ethionamide and prothionamide
- Quinolones: levofloxacin, moxifloxacin and ofloxacin.
- Aminoglycosides: kanamycin, capreomycin and amikacin
- Cycloserine and para-aminosalicylic acid
- Macrolides: clarithromycin
- Bedaquiline (approved in 2015)
- Agents with unclear efficacy—Clofazimine, linezolid, amoxicillin-clavulanic acid, thiacetazone, meropenem-clavulanic acid, imipenem/cilastatin, and clarithromycin.

Treatment of tuberculosis aims to:
- Interrupt transmission by rendering patients non-infectious.
- Prevent morbidity and death by curing patients.
- Prevent the emergence of drug resistance.
- Prevent relapse.

To achieve the aims, the following strategies are followed:
- **Multidrug therapy:** Combination of more than one drug for rapid and effective killing of tubercle bacilli.
- **Short course chemotherapy** lasting for 6 months (or 8 months in previously treated cases).
- **Two phase chemotherapy:** The short course is divided into—
  - *Intensive phase* (initial phase, 2–3 months): Aims at aggressive treatment with 4–5 first line drugs that rapidly kill the bacilli making the smear negative, followed by:
  - *Continuation phase* (given for 4–5 months, with 2 or 3 first line drugs): Aims at killing the remaining dormant bacilli and prevents relapse.
- **DOTS strategy** (Directly Observed Treatment, Short course) is recommend by RNTCP and WHO. Here, the strategies used are:
  - The entire treatment course is supervised to improve the patient's compliance.
  - Treatment response is also monitored by sputum smear microscopy at the end of each phase.
- **Treatment regimens:** There are two treatment regimens, category I and II; both have different indications (Table 27.4).
- **Dosage:** All drugs must be given in fixed dose combination as per appropriate weight bands and **given daily**.

DST (Drug Susceptibity Testing)

Several methods of DST (drug susceptibity testing) are available which can be grouped into:

Phenotypic Methods

- **MGIT** (used for 1st and 2nd line drugs): Resistance is determined by growth of TB bacilli in drug containing tube as compared to control tube (drug free) within 4–21 days of incubation. The instrument interprets results at the time when the growth unit (GU) in control tube reaches 400. At this point, the GU values of the drug vial are interpreted as below
  - Susceptible if the GU of the drug tube is less than 100
  - Resistant if the GU of the drug tube is 100 or more.
- **Proportion method** (used for 1st and 2nd line drugs): An isolate is considered resistant to a given drug when growth of 1% or more is observed in the drug containing LJ medium compared to the control LJ medium without drug after 42 days of incubation
- **Other older methods** such as resistance ratio and absolute concentration methods are obsolete now.

Genotypic Methods

- **GeneXpert** (used only for rifampicin): Five probes targeting different wild sequences of *rpoB* gene are used. If mutations would be there in any of these wild gene, the corresponding probe will not bind or bind weakly. Low or absence of signal indicates presence of resistance to rifampicin. Turnaround time is <2 hours
- **Line probe assay**: Detects resistance to both 1st and 2nd line drugs in 2–3 days of turnaround time.

Resistance to Antitubercular Drugs

Drug resistance is the most worrisome aspect of management of tuberculosis. Development of drug resistance may occur due to:

- **Primary or pretreatment drug resistance:** It develops in a strain infecting a patient who has not previously been treated. It mostly occurs due to infection of an individual by a drug resistant strain. Primary resistance accounts for minority of cases
- **Acquired resistance** (secondary or post treatment): It develops when the infective strain is initially sensitive, becomes resistant later. It is usually due to inappropriate or inadequate treatment. This is much more common than primary resistance.

Mechanism of Drug Resistance

Mechanism of resistance in tubercle bacilli is due to point mutation in the genome of *M. tuberculosis* which occurs at a rate of once in 10^6 cell divisions (Table 27.5).

Rationale of Using Multidrug Therapy

The most worrisome aspect of chemotherapy is development of drug resistance especially when monotherapy is used.

Table 27.4: Category-wise treatment regimen for tuberculosis		Intensive phase	Continuation phase
Category-I	New patients: received ATDs for <1 month	(2)HRZE	(4)HRE
Category-II	*Previously treated patients*: received ≥1 month ATD • *Treatment after failure*: Microbiologically confirmed* after completion of treatment • *Recurrent TB case*: Previously declared cured; becomes microbiologically confirmed* • *Treatment after loss to follow up*: treatment is interrupted for ≥1 consecutive month(s)	(2)HRZES + (1) HRZE	(5)HRE
Follow up	• **Follow up during treatment:** Clinical follow up should be done at least once a month (improvement of chest symptoms, weight gain, etc.) and microbiological* follow-up at the end of intensive phase and continuation phase • **Long-term follow up after treatment is completed**: Patients should be followed up: at the end of 6,12,18 and 24 months		
For EPTB	The continuation phase in both new and previously treated cases may be extended for 3–6 months in certain extrapulmonary TB cases such as CNS, skeletal, disseminated TB; based on clinical decision		

Note: *Microbiologically confirmed means positive by any method such as smear microscopy, liquid culture or CBNAAT.
In liver disorders: if serum alanine transaminase level is ≥3 times; then regimen should be modified. All the three hepatotoxic drugs (INH, rifampicin and pyrazinamide) should not be given together.
Adapted from Revised National Tuberculosis Control Programme (RNTCP) 2016.
Abbreviation: ATDs, antitubercular drugs.

Table 27.5: Drug resistance genes present in *M. tuberculosis*	
Drugs	**Drug-resistant genes**
Isoniazid	Enoyl ACP reductase (*inhA*) Catalase-peroxidase (*katG*) Alkyl hydroperoxide reductase (*AhpC*)
Rifampicin	RNA polymerase subunit B (*rpoB*)
Pyrazinamide	Pyrazinamidase (*pncA*)
Ethambutol	Ribosomal protein subunit 12 (*rpsL*)
Streptomycin	Ribosomal protein subunit 12 (*rpsL*) 16s ribosomal RNA (*rrs*) Aminoglycoside phosphotransferase gene (*strA*)
Fluoroquinolones	DNA gyrase (*gyr A* and *B*)

- This can be effectively checked by multiple drug therapy
- Incidence of resistance to one drug is independent of that to another. Hence, the probability of a strain to be resistant to two drugs will be the product of the probabilities of resistance to each drug and thus drug resistance is much lower when multidrug regimen is used.

Failure to adhere to the multidrug regimen is the most important reason for development of resistance, which may be due to:
- Prolonged duration of regimen
- Poor compliance of the patient
- Development of toxicity to the drugs
- Improper supervision and follow up
- Irregular supply of drugs
- Incorrect prescription.

Resistance to Antitubercular Drugs (ATDs)

Mono resistance

Defined as resistant to one first-line ATD only.

Poly resistance

Defined as resistant to >1 first-line ATD except other than both INH and rifampicin resistant.

Contd...

Contd...

Rifampicin Resistance (RR)

Defined as rifampicin resistance with or without resistance to other ATDs (excluding isoniazid).

Multidrug-resistant Tuberculosis (MDR-TB)

- **Definition:** MDR-TB is defined as resistance to isoniazid and rifampicin with or without resistance to other first line anti-tubercular drugs
- **Epidemiology:**
  - World: 4.1% of new and 19% of previously treated TB cases are estimated to have RR and/or MDR-TB. In 2016, an estimated 6,00 000 new cases of MDR/RR-TB emerged globally
  - In India, MDR-TB accounts for 2.3% of all new TB cases and 12–17% of retreatment cases
  - The actual number of MDR-TB in India may be even higher as the drug sensitivity testing facility is not available in many centers.
- **Treatment of MDR-TB** requires complex regimen of 2nd line drugs for longer course which are more toxic and expensive
- **DOTS-Plus program** is initiated by RNTCP in the year 2000, to cover the diagnosis and treatment of MDR-TB cases. This standardized RNTCP regimen for MDR-TB is as follows (Table 27.6):
  - 6 drugs are given in the intensive phase for 6–9 months, followed by
  - 4 drugs in continuation phase for 18 months.

The 2nd line ATDs are 100 times more expensive and highly toxic than 1st line ATDs.

Extensively Drug-resistant Tuberculosis (XDR-TB)

- **Definition:** They are MDR-TB cases which are also resistant to:
  - Fluoroquinolones (ofloxacin/levofloxacin) and
  - At least one injectable aminoglycosides (kanamycin, amikacin or capreomycin).
- **Epidemiology:** 6.2% of MDR-TB cases have been found to be XDR. The exact incidence of XDR-TB in India is not known. The MDR-TB treatment failure cases (2–6%) may be presumed to be XDR-TB cases
- **Treatment** of XDR-TB is extremely difficult. XDR TB has a very rapidly progressing clinical course with high mortality.

Bedaquiline

Bedaquiline is a new second line ATD of diarylquinoline class, approved for use as second line ATD since 2015. It is considered as miracle drug for the treatment of XDR-TB.
- It acts by inhibiting mycobacterial ATP synthase
- It is strongly mycobactericidal; has no cross resistance with other ATDs
- Showed significant benefit in improving the time to culture conversion in MDR-TB patients
- **Indication:** It is recommended MDR-TB or XDR-TB cases which are resistance to fluoroquinolones and/or second line injectable drugs
- It is contraindicated in pregnancy and age <18 years
- **Regimen:** Bedaquiline containing regimen should contain four other second line ATDs depending up on the sensitivity.

Global and National TB Programs

Revised National Tuberculosis Control Program (RNTCP)

The Government of India has launched this health program in 1992, in collaboration with WHO and World Bank.

The main strategies of RNTCP are:
- Detecting >70% of estimated cases by quality sputum microscopy
- Cure rate not less than 85%
- Involvement of NGOs (Non-Government Organizations)
- Implementing DOTS (Directly Observed Treatment, Short course): A community-based treatment and care of TB patients under supervision
- Implementing DOTS Plus: For detection and treatment of MDR-TB.

The End TB Strategy

In 2016, the WHO has launched "The End TB Strategy by 2035" in order to end the global tuberculosis epidemic. It has the following objectives:
- About 95% reduction by 2035 in number of TB deaths compared with 2015
- About 90% reduction by 2035 in TB incidence rate compared with 2015
- Zero% TB-affected families facing catastrophic costs due to TB by 2035.

Every year, **March 24th** is observed as 'World Tuberculosis Day' globally.

National Strategic Plan, India (2017–2025)

In parallel to WHO, Government of India has initiated National Strategic Plan (2017-2025) aiming at elimination of TB epidemic with a vision of TB-Free India with zero deaths, disease and poverty due to tuberculosis by 2025.

Table 27.6: Standardized RNTCP regimen for MDR-TB (DOTS Plus guideline)

Intensive phase (for 6–9 months)	Continuation phase (for 18 months)
Kanamycin	Ofloxacin or levofloxacin
Ofloxacin or levofloxacin	Ethionamide
Ethionamide	Cycloserine
Cycloserine	Ethambutol
Pyrazinamide	
Ethambutol	

Note: If rifampicin resistance (RR) with isoniazid sensitive or not known: then isoniazid should be added to the MDR-TB regimen of both the phases.

If isoniazid is found resistant:
- For high level resistance or resistant due to *Kat G* gene mutation—INH should be removed.
- For low level resistance or resistant due to *INH A* gene mutation—High dose INH should be added.

Nikshay: It is a web-based solution for monitoring of TB patients. All new cases should be reported into this web portal.

Prophylaxis Against Tuberculosis

Bacillus Calmette Guerin Vaccine (BCG)

BCG vaccine was developed by Calmette and Guerin (1921). They attenuated the strain by serial subculturing in glycerol bile potato medium for 230 times over a period of 13 years.
- **BCG strain:** Live-attenuated *M. bovis* was the strain originally used by Calmette and Guerin
  - Though the same strain is used currently, due to different methods of maintenance in various vaccine laboratories, many substrains have evolved in the past few decades
  - In India, WHO recommended ***Danish 1331*** strain of BCG is used. It is prepared in Central BCG laboratory, Guindy, Chennai.
- **Types of vaccine:** BCG is available in two forms
  - Liquid (fresh) form: It is less stable
  - Lyophilized form (freeze-dried) form: It is more stable and is recommended for use.
- **Reconstitution of BCG:** Lyophilized form should be reconstituted before administration. This is done by using normal saline as diluent. Distilled water is never used as it is irritant. Once reconstituted; it has to be administered within 1 hour
- **Administration of BCG**
  - Dose and strength: 0.1 mL containing 0.1 mg TU
  - Alcohol should not be used to wipe the skin
  - Site: It is given above the insertion of left deltoid
  - Route: It is administered by intradermal route by using a 26 gauge tuberculin syringe.

- **Phenomena after BCG:** If BCG is properly injected intradermally, then the following phenomena develop at the inoculation site:
  - After 2–3 weeks: Papule develops
  - 5–6 weeks: Shallow ulcer develops, which is covered with crust
  - 6–12 weeks: Permanent tiny round scar (4–8 mm diameter) is formed
  - 8–14 weeks: Mantoux test becomes positive.

  If overdose is given: The lesion or scar becomes larger and irregular in size.
- **Protection**
  - Efficacy: Many trials have shown that BCG has a variable efficacy of 0–80%
  - Duration of immunity lasts only for 15–20 years
  - Though BCG may not protect from the risk of tuberculosis infection, it surely gives protection to infants and young children against development of complications such as tuberculous meningitis and disseminated tuberculosis.
- **Complications following BCG**
  - Most common complications include ulceration at the vaccination site and regional lymphadenitis
  - Rarely, keloid or lupus lesion, and osteomyelitis may develop
  - Very rarely, non-fatal meningitis, progressive tuberculosis and disseminated BCG infection ("BCGitis") are reported in people with low immunity.
- **Indications of BCG**
  - Direct BCG: BCG is directly given to newborn soon after the birth. This strategy is followed by most of the developing countries including India. If not given at birth it can be given later, maximum up to 2 years
  - Indirect BCG: BCG is given after performing tuberculin test.
- **Contraindications to BCG include:**
  - HIV-positive child
  - Child borne to AFB positive mother
  - Child with low immunity
  - Generalized eczema
  - Pregnancy.
- **Other uses of BCG are:**
  - BCG induces non-specific stimulation of the immune system; thus provides some protection against certain diseases such as leprosy and leukemia
  - BCG has been tried as an adjunctive therapy in malignancies, such as bladder carcinoma (Onco TICE strain of BCG)
  - BCG may be superior to PPD for tuberculin test, as reported by some workers.

VPM1002

It is a recombinant BCG vaccine, under phase II trial (2017). It is prepared by replacing the urease C encoding gene from *Listeria monocytogenes* which improves its immunogenicity (promotes phagolysosome fusion).

Chemoprophylaxis

Treatment of selected high risk tuberculin reactors (i.e. people with latent tuberculosis) aims at preventing active disease. Isoniazid or ethambutol for six months have been tried. However, chemoprophylaxis has several shortcomings such as—(1) it is expensive, (2) risk of developing tuberculosis is minimal in tuberculin reactors, and (3) side effects of the drugs.

Hence, INH prophylaxis can be restricted to limited indications such as:
- Adults with HIV who are unlikely to have active TB
- Children with HIV who have no TB symptoms and who are unlikely to have active TB
- All children with HIV who have successfully completed treatment for TB.

NONTUBERCULOUS MYCOBACTERIA

Nontuberculous mycobacteria (NTM) were formerly called atypical mycobacteria or mycobacteria other than tubercle bacilli (MOTT).

NTM are diverse group of mycobacteria that are isolated from birds, animals, and from environmental sources, such as soil and water. They are opportunistic pathogens, occasionally associated with human infection. Man-to-man transmission is not known.

Saprophytic mycobacteria are isolated from soil, water and other environmental sources. They do not cause any disease in humans and are distinct from NTM. Examples include *M. phlei* (from grass), *M. smegmatis* (from smegma, a common contaminant in urine).

Classification of NTM

Nontuberculous mycobacteria (NTM) have been classified (Table 27.7) into four groups by Runyon (1959), based on pigment production and rate of growth.

1. Photochromogens

They produce pigments only when the colonies are exposed to light. This group contains the following pathogens:
- ***M. marinum:*** It grows poorly or not at all at 37°C, but grows well at 33°C. It is acquired from water sources (fish tanks, swimming) and enters through minor trauma. Various lesions produced are:

- It typically causes papules or ulcers known as **swimming pool granuloma** or **fish tank granuloma**
- Tendonitis
- Tender nodules: Spread in a sporotrichoid pattern similar to *Sporothrix schenckii*.

❖ ***M. asiaticum:*** It is rarely associated with pulmonary disease and bursitis

❖ ***M. simiae:*** It was originally isolated from monkeys
- It gives a positive niacin test (so may be confused with *M. tuberculosis*)
- It is principally isolated from pulmonary lesions.

❖ ***M. kansasii:*** It causes chronic pulmonary disease resembling tuberculosis
- *Risk factors:* Old persons with preexisting lung disease and with impaired immune responses
- It has been isolated from soil, water and milk
- It grows well at 37°C on LJ medium and produces a yellow-orange pigment.

❖ ***M. genavense:*** It grows very slowly and rarely causes infection in patients with advanced HIV.

2. Scotochromogens

They produce pigments (yellow, orange or red) even when cultures are incubated in dark, but intensity of color may increase on exposure to light.

❖ ***M. scrofulaceum:*** It causes scrofula (cervical lymphadenitis) in children

❖ ***M. gordonae:*** It is often found as commensal in tap water and is a common contaminant of clinical specimens. It is rarely isolated from pulmonary specimens; however its pathogenic potential is doubtful

❖ ***M. szulgai:*** It behaves as a scotochromogen at 37°C and photochromogen at 25°C. It may occasionally cause pulmonary disease and bursitis

❖ ***M. celatum:*** It is a rare cause of pulmonary infection.

Table 27.7: Runyon's classification of nontuberculous mycobacteria

Runyon group	Property	Species
I. Photochromogens	Produce pigments only in light	*M. marinum, M. asiaticum, M. simiae, M. kansasii, M. genavense*
II. Scotochromogens	Produce pigments both in dark and light	*M. scrofulaceum, M. szulgai, M. gordonae M. celatum, M. flavescens*
III. Non-photochromogens	Do not produce pigments	*M. avium-intracellulare* complex (MAC) *M. xenopi, M. ulcerans, M. paratuberculosis, M. malmoense*
IV. Rapid growers	Grow within one week	*M chelonae, M fortuitum, M. smegmatis, M. abscessus*

3. Nonphotochromogens

They do not produce any pigments. Examples include:

❖ ***M. avium-intracellulare* complex (MAC):** They comprise of two related organisms—*M. avium* (Battey bacillus, isolated from birds) and *M. intracelluare*
- They are opportunistic pathogens, especially in HIV-infected people with low CD4 T-cell count (<50/µL)
- MAC can cause various manifestations: Lymphadenitis, respiratory infection and disseminated disease.

❖ ***M. xenopi:*** It has been isolated from hospital water supplies, and associated with nosocomial outbreaks
- It is found as a commensal, but rarely causes pulmonary disease especially in HIV-infected people
- Though classified as a non-photochromogen, *M. xenopi* may form yellow colored colonies similar to scotochromogens
- It was originally isolated from toads.

❖ ***M. ulcerans:*** It is a waterborne skin pathogen, found mainly in the tropics of Africa, Central South America and South-east Asia
- It is the agent of **Buruli ulcer** (derived from Buruli district of Uganda where a large outbreak had occurred). Lesions are typically painless ulcers and nodules that become necrotic later
- It can also cause osteomyelitis and limb deformities
- *Exotoxin:* It produces mycolactone toxin which may be involved in the pathogenesis of the disease
- It has a narrow temperature range of 31°-34°C. It grows slowly, colonies appear in 4-8 weeks.

❖ ***M. malmoense:*** It can cause pulmonary disease and rarely lymphadenitis

❖ ***M. paratuberculosis*** (Johne's bacillus): It mainly causes disease in cattle. It is associated with the pathogenesis of Crohn's disease, but this link has not been proved yet

❖ Other rarely encountered non-photochromogens are—*M. shimoidei, M. terrae,* and *M. gastri*.

4. Rapid Growers

This group of NTM grow in culture within 1 week of incubation. Examples include:

❖ *M. fortuitum* and *M. chelonae:* They cause post-trauma injection abscess and catheter-related infections

❖ *M. abscessus:* It can cause pulmonary infection.

The following tests are done for rapid growers:

❖ Arylsulfatase test: Positive for all rapid growers

❖ Growth on MacConkey agar: *M. fortuitum* grows on MacConkey agar in presence of 5% NaCl, whereas *M. chelonae* fails to grow.

The clinical manifestations of NTM are tabulated in Table 27.8.

Table 27.8: Clinical manifestations of nontuberculous mycobacteria (NTM)

Disease	Organisms
Pulmonary infection	*M. avium-intracellulare* (MAC) *M. kansasii, M. xenopi, M. malmoense, M. szulgai, M. abscessus*
Lymph node infection	*M. avium-intracellulare* (MAC) *M. scrofulaceum*—causes scrofula *M. malmoense*
Cutaneous infection	*M. marinum*—causes swimming pool or fish tank granuloma *M. ulcerans*—causes Buruli ulcer *M. abscessus* *M. fortuitum* and *M. chelonae*—cause injection abscess *M. avium-intracellulare* (MAC)
Disseminated infection	*M. avium-intracellulare* (MAC) *M. kansasii*

Laboratory Diagnosis

- **Specimens:** Sputum, lymph node aspirate, pus or exudate, biopsy from skin lesions are the usual specimens, depending on the type of infection
- **Microscopy by ZN staining:** Shows red acid fast bacilli which needs to be differentiated from *M. tuberculosis*
- **Culture on LJ media:** Several species of NTM grow well on LJ medium, however a few grow sparsely
- **Pigment production:** LJ media are incubated in dark and light separately for distinguishing between photochromogens and scotochromogens
- **Identification:** Species of NTM can be differentiated from *M. tuberculosis* complex by:
  - Negative for MPT64 antigen by ICT: Suggestive of NTM infection differentiating it from *M. tuberculosis*
  - Positive for following biochemical identification tests (less commonly used): such as (i) catalase test (semi-quantitative and heat-stable catalase tests) and (ii) tween 80 hydrolysis test
  - Newer methods: MALDI-TOF (matrix-assisted laser desorption ionization-time of flight) and molecular methods such as PCR are currently the preferred methods used for species identification of NTM.

TREATMENT **Nontuberculous mycobacterial disease**

Just as in tuberculosis, NTM infections are treated with multidrug therapy and are associated with the emergence of drug resistance and relapse.

- *M. avium-intracellulare* complex (MAC) *M. kansasii* and *M. marinum* infections often require multidrug therapy with macrolide (clarithromycin or azithromycin), ethambutol, and a rifamycin (rifampin or rifabutin)
- NTM are resistant to most of the first and second line anti-tubercular drugs.

MYCOBACTERIUM LEPRAE

History

Mycobacterium leprae is the causative agent of leprosy: a disease of antiquity, having been recognized since long time such as:

- Vedic times in India (described as Kushta Roga in Sushruta Samhita, 600 BC)
- Biblical times in the Middle East
- Hippocrates, 460 BC.

The credit of discovery of lepra bacilli goes to **G. H. Armauer Hansen** (1873) in Norway. Although, *M. leprae* was the first bacterial pathogen of humans to be described, still it remains one of the least understood organisms probably because it is not cultivable. However, **Shepard** (1960) had done a breakthrough by multiplying the lepra bacilli in the footpads of mice kept at a low temperature (20°C).

Social stigma: Leprosy was once believed to be highly contagious disease.

- Due to fear, ignorance, superstitious beliefs and characteristic deformities and disfigurement produced in the patients, leprosy remained as a social stigma over many years
- Patients were considered as 'unclean' and socially out casted
- Today, with early diagnosis and effective treatment, patients can lead productive life in the community and the deformities can largely be prevented.

General Properties of Lepra Bacilli

- **Not cultivable:** *M. leprae* is not cultivable either in artificial culture media or in tissue culture; hence, it does not follow the Koch's postulates.

  ICRC bacillus: In Indian Cancer Research Center (ICRC), Bombay (1962), an acid-fast bacillus was isolated from leprosy patients, employing human fetal spinal ganglion cell culture. It was shown to grow on LJ medium. However, its relation to the lepra bacillus is uncertain
- **Animal models:** Lepra bacilli can be maintained in animals, such as **nine banded armadillo** (*Dasypus novemcinctus*) and **foot pad of mice** (kept at a low temperature, 20°C). It can produce natural disease in armadillo as well as other susceptible animals such as slender loris, Indian pangolin, chimpanzees and West African mangabey monkeys
- **Intracellular:** Lepra bacilli are obligately intracellular and strict aerobe
- **Less acid fast:** Compared to tubercle bacilli, they are less acid fast and can resist up to 5% sulfuric acid
- **Appearance:** In smears made from skin lesions, they appear in groups, called **cigar-like bundles of**

bacilli present inside lipid laden macrophages called **Virchow's lepra cells**

- **Grow in cooler areas:** They have a tendency to grow in cooler areas of the body; hence, the clinical manifestations are largely confined to the skin, peripheral nerves, upper respiratory tract, eyes, and testes
- **Generation time:** Lepra bacillus has a long generation time of **12-13 days** as compared to 14 hours in tubercle bacillus and about 20 minutes for coliform bacilli
- **Genome of *M. leprae*** has undergone reductive evolution over the time. Almost half of genes have become non-functional, many of those used to code for metabolic and respiratory pathways. This made the bacilli obligately intracellular and are dependent on host biochemical support.

Clinical Manifestations

Leprosy is a chronic granulomatous disease of humans, primarily involving cooler parts of the body but are capable of affecting any tissue or organs causing bony deformities and disfigurements in untreated cases.

Incubation period: Leprosy has a long incubation period, an average of 3-5 years (vary between 2 and 40 years)

- This can be attributed to the longer generation time of lepra bacilli
- Lepromatous cases have longer incubation period than tuberculoid cases.

Classification of Leprosy

Leprosy can be classified into various categories based on clinical, bacteriological, immunological and histological status of the patients (Table 27.9). Following initiation of treatment or alteration of host immunity, the leprosy category of patients changes from one type to another type.

Table 27.9: Classifications of leprosy

Ridley-Jopling classification (1966)	Madrid classification (1953)	Indian classification* (1981)
Lepromatous leprosy (LL)	Lepromatous type	Lepromatous type
Borderline Lepromatous leprosy (BL)	Borderline	Borderline
Borderline leprosy (BB)	Indeterminate type	Indeterminate type
Borderline tuberculoid leprosy (BT)	Tuberculoid type	Pure neuritic type
Tuberculoid leprosy (TT)	—	Tuberculoid type

Note: There is another **clinical classification of leprosy** (two types—paucibacillary and multibacillary) available. This classification is used by the leprosy control program for the treatment of patients (described later).

* by Leprosy Association of India.

Leprosy is a bipolar disease. Under any classification scheme, lepromatous and tuberculoid cases are the two extreme poles of the disease (Table 27.10).

Lepromatous Leprosy (LL)

It is seen when the host resistance is low to lepra bacilli.

- **Multibacillary disease:** Large number of acid fast bacilli are found in large clumps (globi) inside the macrophages (lepra cells) (see Fig 27.7)
- **Skin lesions** are many, symmetrical with irregular margin. Lesions appear as superficial nodular lesions (Fig. 27.5A) (lepromata) or plaques or xanthoma-like papules. Lesions on face produce **leonine facies** appearance. Loss of eye brow/lashes may occur late
- **Nerve lesions:** The nerve involvement occurs very late in LL patients
- **Host immunity:**
  - The CMI of the LL patient is grossly impaired. As a result the lepromin test and lymphocyte transformation test become negative
  - In contrast, the humoral immune response is exaggerated in LL patients (described later)

Table 27.10: Differences between lepromatous leprosy and tuberculoid leprosy

Characters	Lepromatous leprosy (LL)	Tuberculoid leprosy (TT)
Bacillary load	Multibacillary	Paucibacillary
Bacteriological index	4–6+	0–1+
Skin lesions	Many, symmetrical Margin is irregular Lesions appear as: • Multiple nodules (lepromata) • Plaques and xanthoma-like papules	One or few, asymmetrical Margin is sharp Lesions appear as: Hypopigmented, annular macules with elevated borders Tendency towards central clearing
Nerve lesion	Nerve lesions appear late Hypoesthesia is a late sign	Early anesthetic skin lesion. Enlarged thickened nerves. Nerve abscess seen (common in BT)
CMI	Low	Normal
Lepromin test	Negative	Positive
Humoral immunity	Exaggerated	Normal
Macrophages	Foamy type (lipid-laden)	Epithelioid type
Langhans giant cells	Not seen	Found

Abbreviations: VDRL, venereal disease research laboratory; BT, borderline tuberculoid leprosy; CMI, cell-mediated immunity.

- **Spread:** Bacilli invade nasal mucosa and are shed in large numbers in nasal secretions. LL patients are most infectious. Later on, reticuloendothelial system, eyes, testes, kidneys and bones are also involved. Eventually, any organ can be involved except CNS and lungs. Bacteremia is common
- **Prognosis:** LL patients have a poor prognosis.

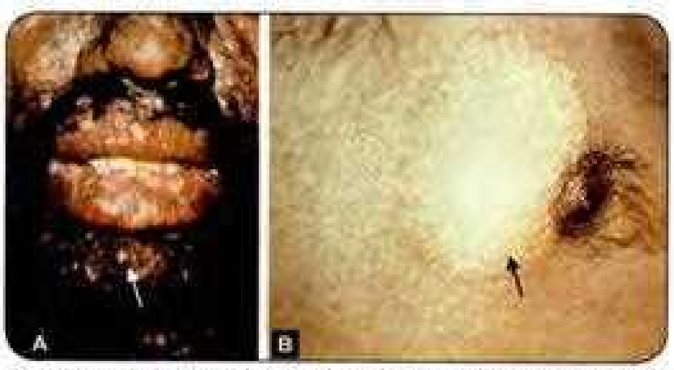
Tuberculoid Leprosy (TT)

It is seen in patients showing a high degree of resistance to lepra bacilli.

- **Paucibacillary disease:** Bacilli are scanty in the lesions
- **Skin lesions** are few, asymmetric and sharply demarcated, consisting of hypopigmented, annular macules (anesthetic patches) with elevated borders (Fig. 27.5B)
- **Nerve lesion:** Nerve involvement occurs early. Nerves are often enlarged and thickened. Nerve abscess may be seen (common in TT and BT). Pronounced nerve damage may lead to deformities, particularly in the hands and feet
  - Most common nerves involved are **ulnar** nerve followed by post-auricular nerve
  - Medial popliteal nerve is never involved.
- **Host immunity:**
  - CMI is adequate and the lepromin test is positive
  - Humoral immune response is also normal
- **Least infectious:** TT patients are least infectious among all types
- **Prognosis:** TT patients have a good prognosis among all the types.

Other Categories of Leprosy

- **Borderline type:** It is seen in patients possessing characteristics in between tuberculoid and lepromatous types. They may shift to either TT or LL type, depending on chemotherapy or alterations in the host resistance



Figs 27.5A and B: A. Nodular lesions of lepromatous leprosy; **B.** Hypopigmented skin lesions of tuberculoid leprosy (arrows showing)

Source: Public Health Image Library, A. ID# 15508/ Dr. Andre J. Lebrun, B. ID# 15503/ Arthur E. Kaye, Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

- **Indeterminate type:** This denotes those early unstable cases with one or two hypopigmented macules and definite sensory impairment. Lesions are bacteriologically negative
- **Pure neuritic type:** These patients develop neural involvement without any skin lesion. Cases are bacteriologically negative.

Immunne Response

Immune respone to the lepra bacilli is the most important factor that determines the outcome of the infection.

- **Innate immunity**: People show a high degree of innate immunity to lepra bacilli so that only a minority of those infected develop clinical disease
- **Humoral antibodies** are produced against various lepra antigens. However *M. leprae* being intracellular, antibodies have a minor role in disease control
- **CMI:** It plays a vital role in the control of the disease. The category of leprosy develops, is determined by the CMI status of the individual (see the box below)
- **Genetic predisposition:** Association has been found between HLA DQ1, HLA MTI with LL type and HLA DR2 with TT type of leprosy.

Role of CMI in the Control of Leprosy

People with low CMI usually develop LL type of lesions:

- **Delayed hypersensitivity (DTH)** to the lepra antigens (which is usually positive in sensitized individual) is absent. Hence, the lepromin test is negative in these patients
- **Virchow's Lepra cells:** The macrophages are able to phagocytose the lepra bacilli but instead of being killed, the bacilli proliferate inside the cells. Macrophages are often lipid laden and called **Virchow's lepra cells**
- **Antigen specific:** The deficiency in CMI appears to be antigen specific as LL patients are not susceptible to any opportunistic infections or any conditions where CMI is important
- **CD4 : CD8 ratio:** It is reversed (1:2) in LL patients. CD8 T-cells predominate in the circulation, as well as in skin granulomas
- **Predominant T_H2 response**: In LL patients, there occurs release of T_H2 specific cytokines such as IL4, IL5, IL6, IL10—which leads to an exaggerated antibody response
- **Humoral immune response** is exaggerated in LL patients. There occurs polyclonal B-cell activation producing high titer of antibodies—both specific against lepra antigens as well as against several other antigens
  - Autoantibodies are common
  - Patients often show biological false positive reaction to VDRL test
  - The albumin: globulin ratio is reversed.

People with intact CMI usually develop TT type lesions:

- **DTH response:** TT patients exhibit a delayed hypersensitivity response to lepra antigen (lepromin test is positive)

Contd...

Contd...

- **CMI is intact and there is predominant T_H1 response:**
  - In TT patients, there occurs release of T_H1 specific cytokines such as IL2, IFN-γ; which in turn activate the macrophages
  - Activated macrophages phagocytose and kill the bacilli.
- **CD4: CD8 ratio** is normal (2:1). CD4 T-cells predominate in the circulation, as well as in skin granulomas
- **TT patients show a normal humoral response.**

Complications

Complications in leprosy patients may be of two types—deformities and allergic response (called **lepra reactions**).

Deformities

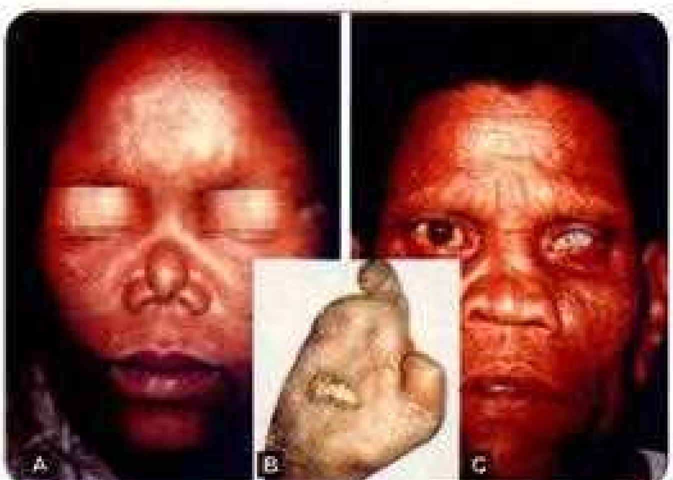
About 25% of untreated cases develop deformities in due course of time which may arise due to—(1) nerve injury leading to muscle weakness or paralysis, or (2) disease process (facial deformities or loss of eyebrow), or (3) infection or injury (ulcers).

Common deformities include (Figs 27.6A to C):

- **Face:** Leonine facies, sagging face, loss of eyebrow/ eye lashes, saddle nose and corneal opacity and ulcers
- **Hands:** Claw hand and wrist drop
- **Feet:** Foot drop, clawing of toes, inversion of foot, and plantar ulcers.

Lepra Reactions

Though leprosy runs as a chronic disease, several allergic type of acute exacerbations occur throughout its course.



Figs 27.6A to C: Deformities seen in untreated lepromatous leprosy: **A.** Saddle nose deformity; **B.** Bony deformity; **C.** Corneal opacity

Source: Public Health Image Library, ID#, A. 15456, B. 15459, C. 15494/ Dr Andre J Lebrun, Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

called lepra reactions (two types, I and II); described in detail in Table 27.11.

Lucio Phenomenon

It is an unusual reaction distinct from lepra reactions; seen in some cases of Mexico and the Caribbean; who have diffuse dermal infiltration without visible skin lesions (termed ***diffuse lepromatosis***).

Epidemiology

- **Source of infection:** Multibacillary (LL and BL) cases are the most important sources of infection. Asymptomatic cases can also have a role in transmission. Tuberculoid leprosy cases do not transmit infection efficiently
- **Mode of transmission:** *M. leprae* has multiple routes of transmission. Portal of entry is either nose or skin
  - Nasal droplet infection (aerosols containing *M. leprae*) is the most common mode. A sneeze from an untreated LL patient may contain $>10^{10}$ lepra bacilli
  - Contact transmission (skin):
    - Direct contact from person to person
    - Indirect contact with infected soil, fomites such as clothes and linens
  - Direct dermal inoculation during tattooing.

Table 27.11: Lepra reactions

Characters	Lepra reaction type I	Lepra reaction type II*
Hypersensitivity	Type IV (delayed hypersensitivity)	Type III (immune complex-mediated)
Seen with	Borderline leprosy	Lepromatous variety (BL, LL)
Manifests as	Inflammation of previous lesions, new skin lesions and neuritis	Crops of painful erythematous papules which become nodular called ENL
Progresses as	If occurs before treatment —progresses towards LL (**down grading reaction**) If occurs after treatment—progresses towards TT (**reversal reaction**)	It usually occurs following the start of chemotherapy
T-helper response	T_H1 predominates leads to ↑ IFN-γ and IL-2	T_H2 predominates-leads to ↑ IL-6, IL-8. TNF-α plays a central role
Other organs	Usually not affected	Eyes, testes and kidney are affected
Treatment	Glucocorticoid	Glucocorticoid, thalidomide, clofazimine and antipyretics

Note: *Type II lepra reaction occurs following the institution of chemotherapy. Antibiotics kill the bacilli releasing the antigens which combine with circulating antibodies and the immune complexes formed are deposited in skin and various parts of the body.

Abbreviations: ENL, Erythema nodosum leprosum; IFN, interferon; IL, interleukin.

- **Communicability:** Leprosy is not highly communicable. Intimate and prolonged contact is necessary for transmission. Only about 5% of spouses living with leprosy patients develop disease. The disease is more likely if contact occurs during childhood
- **Environmental factors** that promote infection include people of rural areas, moist soil, humidity and overcrowding. Males are affected twice common than females.

Geographical Distribution

Once leprosy was worldwide in distribution, but now, it is almost exclusively confined to the developing nations of Asia, Africa, Latin America, and Pacific.

- **World:** In 2016, the new leprosy cases registered globally were 2,16,108 with a prevalence of 0.29/10,000
- **India:** Though the disease burden has declined considerably, still India accounts for maximum number of leprosy cases globally
  - As of 1st April 2017, total of 88,166 cases are on record in India with a prevalence rate of of 0.66 per 10,000 population
  - 34 out of 36 states and UTs achieved elimination status. Chhattisgarh and Dadra and Nagar Haveli are yet to achieve elimination
  - Five more states/UTs wherein elimination was achieved earlier, namely Odisha, Bihar, Chandigarh, Goa and Lakshadweep have reported leprosy cases with prevalence of >1/10,000 population, as on 31st March 2017.

Laboratory Diagnosis

1. Smear Microscopy

Smear microscopy is done to demonstrate the acid fast bacilli in the lesions.

Specimen Collection

Total six samples are collected; four from skin (forehead, cheek, chin and buttock), one from ear lobe and nasal mucosa by nasal blow/scraping.

- **Slit skin smear** is the technique followed to collect the skin and ear lobe specimens
  - The **edge of the lesion** is the preferred site. Lesion is cleaned with spirit, then is pinched up tight to minimize bleeding
  - A 5 mm long incision is made with a scalpel, deep enough to get into the infiltrated layers
  - After wiping off blood or lymph that may have exuded, the scalpel blade is rotated transversely to scrape the sides and base of the incision so as to obtain a tissue pulp from below the epidermis which is smeared uniformly over an area of 8 mm diameter on a slide.
- **Nasal specimens:** (1) **Nasal blow:** Early morning mucus material is collected by blowing the patient's nose on a clean cellophane sheet; or (2) **Nasal scraping:** By using a mucosal scraper to scrape the nasal septum sufficiently so as to remove a piece of mucous membrane, which is transferred onto a slide and teased out into a uniform smear. Nasal scrapings are not recommended as routine
- **Biopsy** from the thickened nerves and nodular lesions may be necessary in some cases.

Appearance

The smears are stained by Ziehl-Neelsen technique by using 5% sulfuric acid for decolorization. Under oil immersion objective, red acid fast bacilli are seen, arranged singly or in groups (cigar like bundles), bound together by lipid-like substance, the glia to form **globi**. The globi are present inside the foamy macrophages called **Virchow's lepra cells or foamy cells** (Fig. 27.7).

- **Live bacilli** will be uniformly stained with parallel sides and round ends and length is five times the width
- **Dead bacilli** are less uniformly stained and have fragmented and granular appearance.

Grading of the Smear

The smears are graded, based on the number of bacilli per oil immersion field (OIF) as follows:

1–10 bacilli in 100 OIF = 1+
1–10 bacilli in 10 OIF = 2+
1–10 bacilli per OIF = 3+
10–100 bacilli per OIF = 4+
100–1000 bacilli per OIF = 5+
>1000 bacilli or bacilli in clumps and globi in each OIF = 6+

Bacteriological index (BI): It is based on the total number of bacilli (live and dead) seen per oil immersion field. The bacteriological index (BI) is calculated by totalling the number of pluses scored in all the smears and divided by the number of smears examined.

Morphological index (MI): It is expressed as the percentage of uniformly stained bacilli out of the total number of bacilli counted. MI is a better marker to monitor the treatment response. Following clinical improvement by treatment, the MI should fall down, whereas the BI may remain the same.

SFG percentage (solid, fragmented granular rod percentage): Since the percentage of solid, fragmented granular rods are recorded separately, this method gives better picture of bacterial morphology and is a more sensitive indicator of monitoring the treatment response than MI.

2. Mouse Foot Pad Cultivation

M. leprae is not cultivable either in artificial culture media or in tissue culture. The only certain way to cultivate *M. leprae* is by inoculating the specimens into foot pad of mice and keeping at 20°C for 6–9 months. Other animals such as nine banded armadillo can also be used.

- **Advantages:** (1) it is 10 times more sensitive than microscopy, (2) useful in detecting drug resistance, (3) evaluating the potency of drugs, (4) detects viability of the bacilli

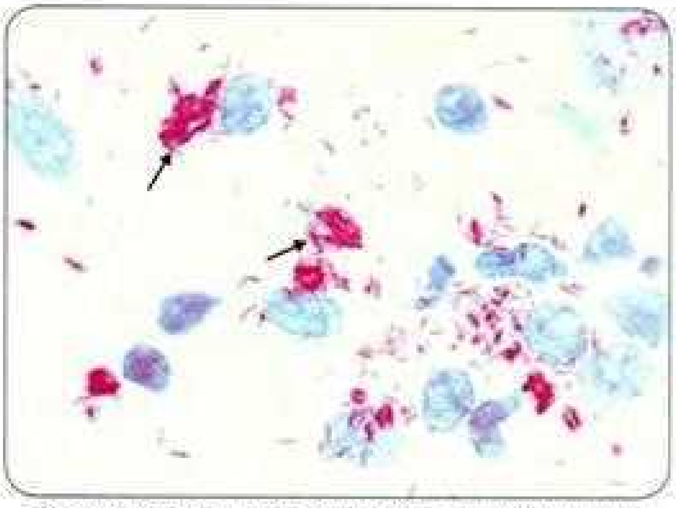


Fig. 27.7: Acid-fast stained slit skin smear showing numerous *Mycobacterium leprae* singly or in globi (arrows)

Source: Dr Isabella Princess, Apollo hospital, Chennai (with permission).

- **Disadvantages:** (1) time-consuming (6-9 months), and (2) ethical issues regarding use of animals.

3. Antibody Detection

- **FLA-ABS** (Fluorescent leprosy antibody absorption test): It is widely used to identify subclinical cases. It detects *M. leprae* specific antibodies irrespective of duration and stage of the disease. It claims to be 92% sensitive and 100% specific
- **ELISA** detecting IgM antibodies to PGL-1 (phenolic glycolipid-1) antigen of *M. leprae* are found in 95% of patients with untreated LL patients and the titre decreases with effective therapy. However, its sensitivity is low (60%) in TT patients.

4. Test for Detecting CMI (Lepromin test)

Lepromin test is discovered by Mitsuda (1919). It demonstrates the delayed hypersensitivity reaction and an intact CMI against the lepra antigen.

- **Procedure:** Standard lepromin antigens prepared from armadillo-derived lepra bacilli (lepromin -A), are increasingly used, replacing the earlier crude antigens. 0.1mL of lepromin antigen is injected intradermally to inner forearm and reading is taken at two occasions; at 48 hours and 21 days
- **Early or Fernandez reaction:** Induration surrounded by erythema (red area) is produced at the site of inoculation within 24–48 hours
  - Early reaction corresponds to that of tuberculin test and indicates a delayed hypersensitivity reaction to soluble constituents of lepra antigen
  - Red area of >10 mm diameter is considered as positive, which indicates past exposure to lepra bacilli. However, it does not indicate active infection.
- **Late or Mitsuda reaction:** Reading is taken after 21 days
  - Positive test is indicated by a nodule formation of >5 mm size at the site of inoculation which ulcerates later on
  - Late reaction is produced against the bacillary component of the lepra antigen and indicates that the patient's CMI is intact.
- **Uses of Lepromin test:** The late reaction is a useful tool to measure the immune status (CMI) of the individual. Lepromin test is said to be positive only when the CMI is intact. Thus, it can be used for:
  - **Classifying lesions of leprosy:** In TT patients with intact CMI, the test is strongly positive. Gradually the positivity becomes weaker as the patient progresses towards lepromatous end. In LL patients; the test is negative indicating a low CMI
  - **Assessing prognosis:** Intact CMI (as in TT patients) indicates good prognosis
  - **Assessing resistance to leprosy in individuals:** Lepromin negative persons are at higher risk of developing multibacillay leprosy than lepromin positive persons.

TREATMENT **Leprosy**

Because of risk of development of drug resistance to single drug, WHO recommends multidrug therapy (MDT) for treatment of leprosy (Table 27.12).

- Recommended drugs: Dapsone, rifampicin and clofazimine
- Alternate drugs: Ethionamide, quinolones (ofloxacin), minocycline and clarithromycin.

WHO recommended treatment regimens are administered based on the clinical type of leprosy (Table 27.12).

Drug Resistance to Anti-leprosy Drugs

Resistance to anti-leprosy drugs is not common. However, for surveillance purpose, WHO has recommended sequencing of the resistant determining regions in the *folP*, *rpoB* and *gyrA* genes for detection of mutations that confer resistance to dapsone, rifampicin and quinolones respectively.

Prevention of Leprosy

Active case finding and effective treatment of cases is the most important measure to control leprosy.

- **BCG vaccine:** There is no effective vaccine available so far. Trials were done using BCG vaccine alone or in combination with killed lepra bacilli, ICRC bacillus
- **MIP vaccine:** A killed leprosy vaccine has been recently developed in India in 2018, using *Mycobacterium indicus pranii* (MIP). Trials have shown that if the vaccine is given to people in close contact, cases can be brought down by 60% in three years
- **Chemoprophylaxis:** Dapsone may be effective when given to high-risk household contacts of tuberculoid

Table 27.12: Clinical classification of leprosy and WHO treatment regimens

Criteria	Paucibacillary	Multibacillary
Skin lesions	1–5	6 or more
Nerve involvement	1	2 or more
Microscopy	Smear negative	Smear positive
Leprosy type	TT, BT and Intermediate	BB, BL, LL
Treatment regimen	• Dapsone (100 mg) given daily, self-administered • Rifampicin (600 mg) given once a month under supervision	• Dapsone (100 mg) given daily • Rifampicin (600 mg) given once a month • Clofazimine—300 mg once a month under supervision, followed by 50 mg daily, self-administered
Duration of treatment	Up to 6 months	Up to 1 year or till smear negative
Follow up	Annually till 2 years	Annually till 5 years

patients, but not for lepromatous patients; hence not recommended

- Hospitalized patients need not be isolated as transmission requires prolonged contact.

Leprosy Elimination

In 1992, the WHO launched a campaign to eliminate leprosy as a public health problem by year 2000. The goal was kept as <1 case per 10,000 population. India has achieved the status by December 2005. However, many other SEAR countries are yet to achieve this level. Though achieved the elimination level, still India accounts for the highest number of leprosy cases worldwide. Hence, the long-term aim is to eradicate leprosy. The National Leprosy Eradication Program (NLEP) in India has been in operation since 1983.

The national institutes for leprosy are:

- **National JALMA** Institute of Leprosy and other mycobacterial diseases, Agra
- **CLTRI** (Central Leprosy Training and Research Institute), Chengalpattu, Tamil Nadu.

EXPECTED QUESTIONS

I. Essay:

1. Rajesh, a 28-year-old male, was admitted to the hospital with complaints of low-grade fever, loss of weight and appetite and chronic cough with expectoration for past 6 months. Sputum examination revealed long, slender and beaded acid fast bacilli.
   a. What is your provisional diagnosis?
   b. Describe the pathogenesis of this condition.
   c. Mention the laboratory diagnosis in detail.
   d. Mention briefly about drug resistance that can occur in this etiological agent.

II. Write short notes on:

1. BCG vaccine.
2. Tuberculin test.
3. Laboratory diagnosis of leprosy.
4. MDR-TB.
5. Nontuberculous mycobacteria.

III. Multiple Choice Questions (MCQs):

1. **Humans become infected by *M. tuberculosis* commonly by:**
   a. Ingestion b. Contact
   c. Inhalation d. Inoculation
2. **Which of the following mycobacteria are micro-aerophilic?**
   a. *M. tuberculosis*
   b. *M. bovis*
   c. *M. leprae*
   d. None of the above
3. **A positive tuberculin test is indicated by an area of induration of:**
   a. <5 mm in diameter
   b. 6–9 mm in diameter
   c. ≥10 mm in diameter
   d. No induration
4. **Which of the following mycobacteria produces Buruli ulcer?**
   a. *M. kansasii* b. *M. tuberculosis*
   c. *M. ulcerans* d. *M. marinum*
5. **Fish tank granuloma is caused by:**
   a. *M. kansasii* b. *M. fortuitum*
   c. *M. marinum* d. *M. ulcerans*
6. **The generation time of lepra bacilli is:**
   a. 20 minutes b. 2 hours
   c. 20 hours d. 12–13 days
7. ***M. tuberculosis* complex includes all, *except*:**
   a. *M. tuberculosis* b. *M. bovis*
   c. *M. caprae* d. *M. smegmatis*
8. **Acid-fastness of *M. tuberculosis* is due to:**
   a. Peptidoglycan layer
   b. Arabinogalactan layer
   c. Mycolic acid layer
   d. Lipoarabinomannan
9. **How much bacillary load in sputum is required for an effective transmission of *M. tuberculosis*?**
   a. 10 bacilli/mL b. 100 bacilli/mL
   c. 1000 bacilli/mL d. 10000 bacilli/mL
10. **Which component of *M. tuberculosis* cell wall helps in attachment to host cell surface?**
    a. Peptidoglycan layer
    b. Arabinogalactan layer
    c. Mycolic acid layer
    d. Lipoarabinomannan

11. Survival of *M. tuberculosis* inside the macrophages is due to:
a. Inhibition of entry into the host cell
b. Inhibition of entry into the phagosome
c. Inhibition of phagosome-lysosome fusion
d. Inhibits degradation by lysosomal enzymes

12. Most common form of extrapulmonary tuberculosis include:
a. Tuberculous lymphadenitis
b. Pleural tuberculosis
c. Genitourinary tuberculosis
d. Tuberculosis of CNS

13. Which type of host immune response against *M. tuberculosis* is responsible for formation of tubercle?
a. Macrophage-activating response
b. Tissue-damaging response
c. Humoral immune response
d. Innate immune response

14. Which of the following is a feature of primary pulmonary tuberculosis?
a. Assmann focus
b. Simon's focus
c. Ranke complex
d. Cavity formation

15. Which is not an ideal specimen for GeneXpert?
a. Sputum b. Pleural fluid
c. Synovial fluid d. CSF

16. MGIT: all are true, *except*:
a. It contains OADC enrichment growth media
b. It contains PANTA antibiotic mixture
c. It detects growth of mycobacteria within 3–4 weeks
d. It cannot be used for drug susceptibility testing

17. MPT 64 antigen is used to detect:
a. *M. tuberculosis* from *M. bovis*
b. *M. tuberculosis* complex from NTM
c. Acid-fast bacilli from non acid-fast bacilli
d. *M. tuberculosis* from *M. leprae*

18. Cartridge-based nucleic acid amplification test (CBNAAT)- all are true, *except*:
a. Works on real-time PCR principle
b. Detects MTB complex DNA
c. Detects rifampicin resistance
d. Detects isoniazid resistance

19. GeneXpert- all are true, *except*:
a. Detection limit 131 bacilli/mL
b. Gives result in 2 hours
c. Can speciate MTB complex
d. Uses five probes targeting various sequences of *rpoB* gene

20. Line probe assay for *M. tuberculosis*—all are true, *except*:
a. Used for identification of MTB complex
b. Detects rifampicin resistance
c. Detects isoniazid resistance
d. Useful for paucibacillary smear-negative specimens

21. Spoligotyping is used for typing of:
a. *Salmonella* Typhi
b. *M. tuberculosis*
c. *S. aureus*
d. *Vibrio cholerae*

22. False-positive tuberculin skin test is seen with:
a. Following BCG vaccination
b. Early or advanced TB
c. Miliary TB
d. HIV-infected people

23. Tuberculin skin test, all are true, *except*:
a. Not useful in infant
b. Marker of prevalence in adults
c. >10 mm is considered as reactive
d. False positive in NTM infection

24. Interferon gamma release assay (IGRA) has the advantage over tuberculin test:
a. Positive indicates active infection
b. Has less false negative result
c. Has no false positive result
d. Can be used as marker of prevalence

25. Which of the following cannot be used for drug susceptibility testing for *M. tuberculosis* for second line antitubercular drugs?
a. Line probe assay b. MGIT
c. GeneXpert d. Proportion method

26. Multidrug-resistant tuberculosis (MDR-TB) is defined as resistant to:
a. All first line ATD
b. Second line ATDs
c. Rifampicin
d. Rifampicin and isoniazid

27. Which of the following NTM is not a photochromogen?
a. *M. marinum* b. *M. asiaticum*
c. *M. kansasii* d. *M. scrofulaceum*

28. Which of the following is the causative agent of swimming pool granuloma?
a. *M. marinum* b. *M. asiaticum*
c. *M. kansasii* d. *M. scrofulaceum*

29. Which NTM produces clinical disease similar to pulmonary TB?
a. *M. marinum* b. *M. asiaticum*
c. *M. kansasii* d. *M. scrofulaceum*

30. Which NTM causes injection site abscesses?
a. *M. marinum*
b. *M. asiaticum*
c. *M. fortuitum*
d. *M. avium-intracellulare* complex

31. The agent of Buruli ulcer?
a. *M. ulcerans* b. *M. asiaticum*
c. *M. fortuitum* d. *M. kansasii*

Answers

1. c **2.** b **3.** c **4.** c **5.** c **6.** d **7.** d **8.** c **9.** d **10.** d **11.** c **12.** a **13.** a **14.** c **15.** b **16.** d
17. b **18.** d **19.** c **20.** d **21.** b **22.** a **23.** a **24.** c **25.** c **26.** d **27.** d **28.** a **29.** c **30.** c **31.** a

Miscellaneous Gram-positive Bacilli

28
CHAPTER

Chapter Preview

- Actinomycetes
  - *Actinomyces* species
  - *Nocardia* species
  - *Actinomadura* species
- *Listeria* species
- *Erysipelothrix* species
- *Tropheryma whipplei*

ACTINOMYCETES

Actinomycetes are diverse group of gram-positive, non-motile, non-sporing, non-capsulated bacilli arranged in chains or branching filaments. Though they are true bacteria, but similar to fungi, they form a mycelial network of branching filaments. They are related to mycobacteria and corynebacteria. Most of them are soil saprophytes or normal human commensals. Important genera include:

- *Actinomyces:* They are anaerobe and non-acid fast; produce a clinical condition called actinomycosis
- *Nocardia:* They are aerobe and acid fast; cause actinomycetoma and pulmonary infection
- *Actinomadura:* They are aerobe and non-acid fast; cause actinomycetoma
- *Streptomyces:* They are aerobe and non-acid fast; rarely cause actinomycetoma in man. They also remain as an important source of antibiotics such as streptomycin
- Thermophilic actinomycetes such as *Micropolyspora* and *Thermoactinomyces* can cause hypersensitivity pneumonitis (farmer's lung and bagassosis).

Actinomyces

Actinomyces are soil saprophytes and commensals of oral cavity. In humans, they cause actinomycosis. *A. israelii* is the most common species infecting man. Others such as *A. naeslundii* and *A. odontolyticus* are rare pathogens.

Pathogenesis

Actinomycosis is a chronic suppurative and granulomatous infection characterized by multiple abscesses with formation of sinuses, discharge containing granules and on later stage; fibrosis and tissue destruction.

- The name refers to ray-like appearance of the organism in the granules (*Actinomyces*, meaning ray fungus)
- **Mode of infection:** As *Actinomyces* are commensals of oral cavity, the infection is mostly **endogenous** and may result from trauma, e.g. dental extraction
- The bacteria bridge the mucosal or epithelial surface of the mouth, grow in an anaerobic niche, induce a mixed inflammatory response, and form painless indurated swelling with sinuses which may drain pus containing granules to the skin surface
- The infection may spread to the neighboring organs including the bones and induce tissue destruction
- Often, the hard indurated swellings are mistaken as malignant tumors.

Clinical Manifestations (Actinomycosis)

Cervicofacial actinomycosis: This is the most common form, usually presents as a painless, slow-growing, hard mass with cutaneous fistulas, a condition commonly known as **lumpy jaw** (Fig. 28.1A).

Other forms are rare such as:

- **Abdominal form:** It occurs due to spillage of intestinal flora secondary to bowel surgery or other conditions of bowel such as appendicitis
- **Pelvic form:** It occurs following intrauterine contraceptive devices (IUCDs) insertion
- Brain abscesses
- Bone destruction and soft tissue infections
- **Disseminated form:** It may occur due to hematogenous spread. Lungs and liver are the common sites; where multiple nodules are formed
- **Dental caries and periodontal diseases:** Mainly caused by *A. naeslundii* and *A. odontolyticus*.

Laboratory Diagnosis

Specimen

Based on the affected site, the specimens collected include discharge from the sinuses or fistula, rarely bronchoalveolar lavage, sputum or tissue sections.

Direct Microscopy

Pus discharge is thoroughly washed in saline in a test tube and the sediment is collected that contains gritty, white or yellowish **sulfur granules**, of <5 mm in size. Granules are crushed between two slides and smears are made.

- **Gram-staining (Brown-Brenn modification):** It shows a central mass of gram-positive filamentous bacilli, radiating peripherally with hyaline, club-shaped ends. Clubs are composed of complexes formed due to interaction of bacteria derived polysaccharide and protein with host cell salts and polypeptides (Fig. 28.1B)
- Granules of actinomycosis are hard and not emulsifiable which differentiates them from granules produced in other conditions
- *Actinomyces* species can also be detected directly from the sample by methods such as:
  - Fluorescent antibody techniques using fluorescent tagged species specific monoclonal antibodies
  - Fluorescent in situ hybridization (FISH) using species specific probes.
- **Histopathological staining** such as hematoxylin-eosin and Gomori's stained tissue sections may reveal granules composed of eosinophilic clubs surrounding basophilic filaments and inflammatory cells such as neutrophils and foamy macrophages (sun-rays appearance) (Fig. 28.1C).

Culture

Pus containing sulfur granules are washed and cultured anaerobically at 37°C on media such as:

- **Thioglycollate broth:** Growth of *A. israelii* resembles **fluffy balls** at the bottom of the tube, this can be differentiated from other species (*A. bovis* produces uniform turbidity)
- **Brain heart infusion (BHI) agar:** It forms small **spidery colonies** at 48 hours which become enlarged and heaped up in 10 days.

Species Identification

It is done when the culture isolate is subjected to:

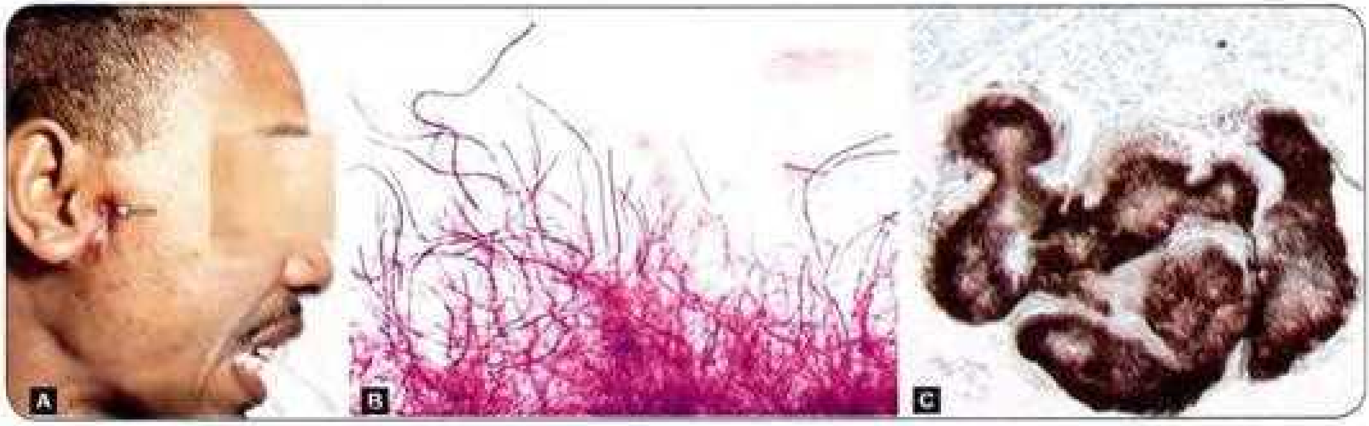
- Biochemical reactions
- Gas-liquid chromatography (GLC) for detection of the products of glucose metabolism
- Molecular methods, such as PCR-RFLP are also available for speciation.

TREATMENT ***Actinomycosis***

- Recommended regimen include IV ampicillin or IV penicillin G for 4–6 weeks followed by oral penicillin V or amoxicillin for 6–12 months. Lesser duration may cause relapse
- Doxycycline, ceftriaxone or clindamycin can be given to people with penicillin allergy
- Surgical removal of the affected tissues may be required for extensive lesions.

Nocardia

Nocardia species (named after Edmond Nocard, 1898) are gram-positive branching filamentous bacilli similar to *Actinomyces*; however, they differ from the later by being aerobic and acid-fast (Table 28.1). They are environmental saprophytes found in soil and vegetations. Though more than 50 species have been identified, only few (nine) species are associated with human disease. *N. asteroides* (so named due to its star-shaped colonies) and *N. brasiliensis* are the most common pathogens.



Figs 28.1A to C: A. Actinomycosis (painless, slow-growing, hard mass with cutaneous fistula) (arrow showing); **B.** Gram-positive filamentous bacilli; **C.** Gomori's stained smear showing sun-ray appearance

Source: A. Public Health Image Library, ID# 2856; B. Dr Isabella Princess, Apollo Hospitals, Chennai; C. ID# 10601/Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*).

Table 28.1: *Differences between Actinomyces and Nocardia*

Features	*Actinomyces*	*Nocardia*
Acid-fastness	Nonacid-fast	Partially acid-fast
Oxygen requirement	Anaerobe	Obligate aerobe
Sugar	Fermenter	Utilizes sugar oxidatively
Habitat	• Found as oral flora • Infections occur endogenously	• Usual habitat is soil • Infections occur exogenously
Risk factors	Disease occurs in immunocompetent host also	Usually affects people with low immunity
Clinical forms	Cervicofacial, abdominal and others	Pulmonary, CNS forms, Actinomycetoma
Granules	**Sulfur granules** are hard and not emulsifiable, consist of branching filamentous bacilli and surrounded by clubs (**sun-ray appearance**)	• Granules are soft and lobulated and also show sun-ray appearance • Commonly found in mycetoma, rare in other conditions
Culture	• **Spidery molar** teeth colony in solid media • **Fluffy ball** at bottom of the liquid medium	Colonies are creamy, wrinkled and pink Isolation is done in: • Selective media • Paraffin bait technique • LJ medium
Drug of choice	Penicillin	Sulfonamide or cotrimoxazole

Abbreviation: LJ, Lowenstein-Jensen medium

Pathology and Pathogenesis

Nocardiosis occurs worldwide, more common among adult males. Soil is the natural habitat of *Nocardia*. Infection is acquired from soil either by:

- **Inhalation of fragmented bacterial filaments:** Leads to development of pulmonary nocardiosis that may disseminate later. It is often associated with various species such as *N. asteroides, N. cyriacigeorgica, N. farcinica* and *N. pseudobrasiliensis*
- **Transcutaneous inoculation of the bacteria:** Leads to various cutaneous and subcutaneous manifestations (e.g. mycetoma). This is often associated with various species such as *N. brasiliensis, N. asteroides* and rarely by *N. otitidiscaviarum* and *N. transvalensis*
- Person-to-person spread is not known.

The characteristic histologic feature seen in nocardiosis is an abscess with extensive neutrophil infiltration and prominent necrosis, surrounded by granulation tissue. Nocardiae survive within the neutrophils by:

- Neutralization of oxidants
- Prevention of phagosome-lysosome fusion
- Prevention of phagosome acidification.

Risk Factors

Cell-mediated immunity plays an important role in controlling the disease. Hence nocardiae act as opportunistic pathogen, tend to occur frequently in immunocompromised conditions including AIDS, corticosteroid treatment, organ transplantation and tuberculosis.

Clinical Manifestations

Pulmonary Nocardiosis

Lobar pneumonia is the most common form, characterized by subacute onset of cough with thick, purulent sputum. It may rarely spread directly to adjacent tissues, leading to pericarditis, mediastinitis, laryngitis, tracheitis and bronchitis.

Extrapulmonary (Disseminated) Nocardiosis

In half of the pulmonary nocardiosis cases, dissemination occurs via blood. It typically presents as subacute abscess. Brain is the most common site followed by skin, kidneys, bone and muscle. Brain abscesses are usually supratentorial, often multiloculated, and may be single or multiple. Meningitis is uncommon.

Actinomycetoma

Mycetoma is a chronic granulomatous condition affecting subcutaneous tissues of the feet and hands, characterized by:

- Subcutaneous nodular swelling
- Multiple sinuses
- Discharge containing granules
- Tendency of spreading to adjacent bones (bony deformities).

Mycetoma usually affects people residing in tropical countries. The organism enters through skin on exposure to contaminated soil. Broadly, mycetoma is classified into two types:

1. **Eumycetoma:** It is caused by fungi such as *Madurella* (Refer Chapter 52 for detail).
2. **Actinomycetoma:** It is caused by filamentous bacteria such as *Nocardia, Actinomadura* and *Streptomyces somaliensis*.

Laboratory Diagnosis

Specimen

Depending on the site affected, various specimens collected such as sputum, pus from abscess and granules.

Granules present in discharge are collected in sterile gauze or loop by pressing the sinuses from the periphery to express them out (as in case of actinomycetoma).

Direct Microscopy

- **Gram staining (Brown-Brenn modification):** Reveals gram-positive branching and filamentous bacilli of

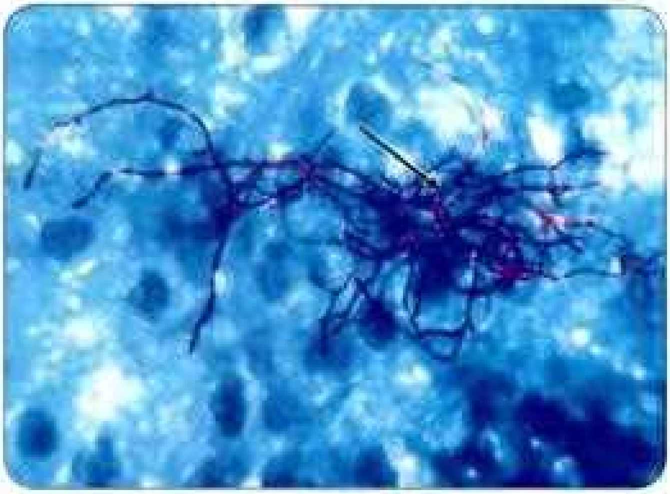


Fig. 28.2: Acid-fast filamentous branching bacilli of *Nocardia* (modified acid-fast stain)

Source: Dr Suchitra Mishra, Assistant Professor, Department of Microbiology, HiTech Medical College, Rourkela, Odisha (*with permission*).

width 0.5–1 µm. They stain irregularly as their filaments are beaded. Sputum examination may show numerous lymphocytes and macrophages, some of which contain branching bacilli

- **Modified acid-fast staining** using 1% sulfuric acid as decolorizer (Kinyoun method): Nocardiae are partially acid fast and appear as branching and filamentous red colored acid-fast bacilli (Fig. 28.2)
- Granules are washed several times in saline, crushed between two slides and observed under microscope. Granules are 0.5-2 mm sized microcolonies composed of branching filamentous bacilli
- **Histopathology (H and E stain) of the granules:** Shows multilobulated with sun-ray appearance.

Culture

Nocardiae are obligate aerobes that grow on various media such as brain heart infusion agar and Sabouraud dextrose agar (SDA) when incubated at 37°C for 2 days to 2 weeks. Colonies are creamy, wrinkled, pigmented (orange or pink colored due to carotenoid-like pigments) and adhere firmly to the medium. Some colonies possess abundant aerial growth and have a cotton wool ball appearance.

Recovery of *Nocardia* from the samples containing *Actinomadura* and *Streptomyces* can be done by:

- **Using selective media:**
  - Buffered yeast extract containing polymyxin and vancomycin
  - Sabouraud dextrose agar with chloramphenicol.
- **Paraffin bait technique:** Media using paraffin as the sole carbon source have been shown to be effective for isolation of nocardiae from soil and clinical samples
- **Lowenstein-Jensen medium:** Produces moist glabrous colonies (differentiates from mycobacteria).

Biochemical Identification

Nocardia species are non-motile, catalase positive and utilize a number of sugars oxidatively. Various biochemical tests are done for species identification such as:

- Decomposition of casein, hypoxanthine, tyrosine
- Growth in lysozyme
- Acetamide utilization
- Growth at 45°C for 3 days
- Acid from rhamnose.

TREATMENT ***Nocardia***

- **Sulfonamide or cotrimoxazole** are the drug of choice for all forms of nocardiosis
- For severe disease such as brain abscess or pneumonia, cotrimoxazole plus imipenem is recommended
- **Duration of treatment**
  - For severe disease: 6–12 months (for intact host defense) or 1 year (for deficient host defense)
  - For lymphadenitis, skin abscess: 2 months
  - For actinomycetoma: 6–12 months after cure
  - For keratitis: 2 months after cure.
- Aspiration or drainage of the abscesses should be carried out to limit the spread of infection.

Actinomadura

Actinomadura is the most frequent cause of actinomycetoma, significantly out numbering the cases caused by *Nocardia*.

- *Actinomadura madurae* and *A. pellettieri* are important species
- Granules are usually white to yellow except in case of *A. pellettieri* that produces red colored granules
- Microscopy of the specimens containing granules reveals branching filamentous bacilli
- Colonies have a **molar tooth** appearance after 48 hours in culture with sparse aerial growth
- Speciation is on the basis of biochemical tests
- Most isolates are susceptible to amikacin and imipenem.

LISTERIA MONOCYTOGENES

Listeria monocytogenes is a food-borne pathogen that can cause serious infections, particularly in neonates, pregnant women and elderly people.

Epidemiology

L. monocytogenes is a ubiquitous saprophyte. It has been isolated from birds causing epizootic disease and also found as asymptomatic carriage. It causes abortion and circling disease, a form of basilar meningitis in sheep and cattle.

Human Infection

- **Mode of transmission:** It is transmitted most commonly through contaminated food followed by vertical transmission (mother to fetus)

- **Age:** Listeriosis is common among extremes of age (neonate and old age)
- Use of proton pump inhibitors increases the risk by reducing the gastric acid mediated killing of *Listeria*
- **Other risk factors:** Pregnant women and immunocompromised individuals are at higher risk
- Due to its ability to survive refrigeration (4°C), it is **commonly found in stored foods** especially aged soft cheeses, packaged meats, milk and cold salads
- Listeriosis is most often sporadic, although outbreaks do occur.

Pathogenesis

Important steps involved in pathogenesis are entry into cells, intracellular growth and cell-to-cell spread.

- **Entry:** Entry into intestinal epithelium is mediated by host surface protein **internalins**
- **Intracellular survival:** It is a facultative intracellular organism. Survival inside the host cells is mainly due to inhibition and lysis of phagosome by forming pores (mediated by listeriolysin O)
- Surprisingly, it is observed that there is an inverse relationship between toxicity and virulence, i.e. more cytotoxic strains are less virulent. This is because, as an intracellular pathogen, *L. monocytogenes* benefits from leaving its host cell unharmed
- **Direct cell-to-cell spread:** *Listeria* escapes into the host cytoplasm, expresses a surface protein, *Act A*, that mediates the nucleation of host actin filaments which in turn helps the bacterium to reach cell membrane and migrate to the adjacent epithelial cells/macrophages (mediated by listeriopods).

Clinical Manifestations

Clinical manifestation depends on the age of the patient and other risk factors, such as immunosuppression. Most of the human infections are caused due to serotypes 1/2a, 1/2b, and 4.

- **Neonatal listeriosis:** Two clinical presentations are recognized—early-onset and late-onset neonatal disease (Table 28.2)
- **In pregnant women:** It affects both mother and the fetus. (1) Fetal complications, such as abortion, preterm delivery lead to early onset disease; (2) Maternal complications, such as flu-like symptoms, bacteremia and rarely meningitis
- **Adults:**
  - It produces manifestations such as bacteremia and meningitis
  - Common risk factor is immunosuppression (steroid therapy, HIV, diabetes, malignancy)

Table 28.2: Differences between early and late onset neonatal listeriosis

Early onset neonatal disease	Late onset neonatal disease
Occurs <5 days of birth (Mean age 1.5 days)	Occurs >5 days of birth (Mean age 14.2 days)
Acquired from maternal genital flora	Acquired from environment
Associated with obstetrical complications like premature delivery and low birth weight	Not associated
Most common form is neonatal sepsis	Most common form is neonatal meningitis
Granulomatosis infantiseptica: Occurs rarely, characterized by miliary microabscesses and granulomas, mostly in skin, liver and spleen	Not seen
Mortality rate is >30%	Mortality rate is <10%
Does not cause nosocomial outbreaks	Nosocomial outbreaks are seen

  - *Listeria* can cause meningitis in kidney transplanted patients after 1 month
  - It also causes gastroenteritis following consumption of contaminated milk, meat and salads.

Laboratory Diagnosis

- **Specimens** collected are CSF, blood and amniotic fluid
- **Gram stain:** Gram-positive short coccobacilli, often confused with diphtheroids
- **Motility:** It shows **tumbling type of motility** at 25°C but non-motile at 37°C (called **differential motility,** which is due to temperature dependent flagella expression)
- **Culture:** It grows on blood agar (β-hemolytic colonies), chocolate agar and selective media, such as **PALCAM agar** (polymyxin, acriflavine, lithium chloride, ceftazidime, aesculin and mannitol)
- Growth improves on refrigeration at 4°C (called **cold enrichment**) in trypticase soy broth followed by subculture on to plates and incubated at 37°C
- **Biochemical reaction:** It is catalase positive, can grow in presence of 10% salt and low pH. *L. monocytogenes* is differentiated from other *Listeria* species by the following tests
  - It shows a positive CAMP test when streaked at a right angle to *Staphylococcus aureus* (enhanced arrow head hemolysis, refer Chapter 22 for detail)
  - It ferments glucose, maltose, L-rhamnose and alpha methyl D-mannoside, producing acid without gas.
- **CSF examination:** It shows elevated pressure, increased protein, and increased lymphocyte count.

TREATMENT **Listeriosis**

- Ampicillin is the drug of choice, given for 2–3 weeks in combination with gentamicin for synergistic effect
- Cotrimoxazole is given for patients with penicillin allergy
- In febrile gastroenteritis, Amoxicillin is recommended in immunocompromised, elderly or pregnant patients
- Cephalosporins are not effective.

Prevention

Food-borne listeriosis can be prevented by thorough cooking of food, washing fresh vegetables, carefully cleaning utensils and avoiding consumption of unpasteurized dairy products.

ERYSIPELOTHRIX RHUSIOPATHIAE

Pathogenesis

Erysipelothrix rhusiopathiae (or *E. insidiosa*) is widely distributed in animals and fishes. It causes erysipelas especially in swine, turkeys, ducks and sheep.

- Human infection occurs by direct inoculation from animals or animal products
- High-risk groups are animal handlers (abattoir workers, butchers) and fishermen
- **Erysipeloid:** This is the most common form of human infection by *Erysipelothrix*; characterized by violaceous swelling with severe pain, but no pus (differentiates it from staphylococcal and streptococcal erysipelas where pus is seen at the infection site). Most common site is fingers (called "**seal finger**" **and** "**whale finger**")
- Rare clinical forms are diffuse cutaneous form and bacteremia with endocarditis.

Laboratory Diagnosis

- **Gram staining:** *E. rhusiopathiae* is a gram-positive bacillus that appears singly, in short chains or in long non-branching filaments
- **Culture:** On blood agar, it produces small transparent glistening alpha-hemolytic colonies
- The colony morphology and Gram stain appearance vary depending upon the growth medium, incubation temperature and pH
- **Biochemical reactions:** It is catalase negative, oxidase negative and indole-negative. The characteristic feature is it produces hydrogen sulfide on triple sugar iron agar, turning the color of the medium to black.

TREATMENT **Erysipeloid**

Erysipelothrix is highly susceptible to penicillin G. It is the drug of choice for severe infections. The organism is intrinsically resistant to vancomycin.

TROPHERYMA WHIPPLEI

Tropheryma whipplei is a gram-positive actinomycetes, not closely related to any known genus. It is the agent of **Whipple's disease** affecting the small intestine.

- Whipple's disease is characterized by fever, abdominal pain, diarrhea, weight loss and migratory polyarthralgia. Mesenteric lymph nodes of the small intestine are primarily involved
- **Laboratory diagnosis:**
  - Histopathological staining of intestinal biopsy shows prominent macrophage infiltration and fat deposition and characteristic pathognomonic vacuoles within the macrophage containing periodic acid-Schiff (PAS) stain positive bacilli
  - Culture of *T. whipplei* has been unsuccessful
  - Polymerase chain reaction (PCR) targeting 16S ribosomal RNA can be done to identify the bacilli.

TREATMENT **Whipple's disease**

- Recommended regimen: Doxycycline and hydroxychloroquine for 1 year, followed by lifelong doxycycline
- Alternate regimen include: IV ceftriaxone or IV meropenem for 14 days followed by doxycycline and hydroxychloroquine for 1 year
- Relapse: Any treatment lasting for less than one year has an approximate relapse rate of 40%.

EXPECTED QUESTIONS

I. Write short notes on:
1. Pathogenesis of *Listeria monocytogenes*.
2. Actinomycetoma.

II. Multiple Choice Questions (MCQs):

1. **Early onset neonatal disease caused by *Listeria monocytogenes* is characterized by all, *except*:**
   a. Acquired from maternal genital flora
   b. Presents as neonatal sepsis
   c. Mortality rate is less than 10%
   d. Does not cause nosocomial outbreaks
2. **In erysipeloid, the route of infection is:**
   a. Direct inoculation b. Ingestion
   c. Inhalation d. None of the above
3. **Which of the following actinomycete is acid fast?**
   a. *Streptomyces* b. *Actinomadura*
   c. *Nocardia* d. *Actinomyces*

Answers

1. c **2.** a **3.** c

Enterobacteriaceae-I

29
CHAPTER

FAMILY CHARACTERS (GENERAL PROPERTIES)

Members of the family Enterobacteriaceae should have the following properties:

- They are gram-negative bacilli
- Aerobes and facultative anaerobes
- Nonfastidious, can grow in ordinary media like nutrient agar
- Ferment glucose to produce acid with or without gas
- Reduce nitrate to nitrite
- They produce catalase (except *Shigella dysenteriae* type-1)
- They do not produce oxidase
- They are generally motile with peritrichous flagella, except some members which are nonmotile, such as *Shigella* and *Klebsiella*
- **Natural habitat:** Most of them are commensals in human intestine, called **coliform bacilli**, e.g. *Escherichia, Klebsiella, Proteus, Morganella, Providencia* and *Citrobacter*, etc. The exceptions are *Shigella, Salmonella* which are enteric pathogens, not commensals.

Classification

- **The oldest method of classification** of the family Enterobacteriaceae was based on fermentation of lactose on MacConkey agar (Table 29.1). It is still the most widely used classification, has a great practical application in laboratories to differentiate various members
- **Newer methods of classification:** After the availability of molecular methods, the taxonomy is greatly changed. Currently, three classifications of Enterobacteriaceae are in use:
  1. Bergey's manual (1984)
  2. Edwards-Ewing classification (1986)
  3. Farmer and Kelley classification (1991).

All the three classifications are similar, though not totally identical and have certain differences. However, the basic approach is the same. The family is further classified into → tribe → genus → species.

The tribe concept was proposed by Ewing. However other classifications directly classify family into genus and species. Use of tribe has a great impact in laboratory for easy

Table 29.1: Classification of family Enterobacteriaceae based on lactose fermentation

Groups	Lactose fermentation	Colonies on MacConkey agar	Examples
Lactose fermenters (LF)—all are coliform bacilli	Ferment lactose—producing acid	Produce pink-colored colonies, (acid changes the color of neutral red indicator to pink)	*Escherichia* *Klebsiella*
Non lactose fermenters (NLF)	Do not ferment lactose	Produce pale or colorless colonies	*Salmonella, Shigella, Proteus, Morganella, Providencia* and *Yersinia*
Late lactose fermenters (LLF or previously called **paracolon bacilli**)	Ferment lactose after 2–8 days of incubation	At 24 hours of incubation—produce pale or colorless colonies After 2 days—produce pink color colonies	*Shigella sonnei*

Table 29.2: Ewing's classification of family Enterobacteriaceae

Tribe	Genus
Tribe I: Escherichieae	*Escherichia* *Shigella*
Tribe II: Edwardsielleae	*Edwardsiella*
Tribe III: Salmonelleae	*Salmonella*
Tribe IV: Citrobactereae	*Citrobacter*
Tribe V: Klebsielleae	*Klebsiella* *Enterobacter, Hafnia* *Serratia, Pantoea*
Tribe VI: Proteeae	*Proteus* *Morganella* *Providencia*
Tribe VII: Yersinieae	*Yersinia*
Tribe VIII: Erwinieae	*Erwinia*

identification, as genera under each tribe share common properties. Hence, Ewing's classification is followed in this book (Table 29.2).

The biochemical reactions of various genera of the family Enterobacteriaceae are listed in Table 29.3.

TRIBE I: ESCHERICHIEAE

ESCHERICHIA COLI

It was described first by Escherich in 1885. *E. coli* is the most important species encountered as human pathogen.

- It is also the most common aerobe to be harbored in the gut of humans and animals
- After excreted in feces, it remains viable only for some days in the environment
- Hence, detection of *E. coli*, especially a variant called **thermotolerant *E. coli*** (survives at 44°C) is taken as an indicator of recent contamination of drinking water with human or animal feces
- Other species are less important as human pathogens. These include *E. fergusonii*, *E. hermannii* and *E. vulneris* which are rarely isolated from clinical specimens.

Virulence factors of *E. coli*

Virulence factors of *E. coli* may be grouped into surface antigens and toxins.

Surface antigens

E. coli possesses four surface antigens—(1) somatic (O), (2) flagellar (H), (3) capsular antigens (K), and (4) fimbrial antigen.

- Serotyping of *E. coli* is based on agglutination with the specific antisera directed against each surface antigen
- So far more than 174 O serotypes, 100 K serotypes and 53 H serotypes of *E. coli* have been recognized
- The strain of *E. coli* is designated based on the serotype number of its antigens; for example, O121: K37: H8.

Contd...

Contd...

1. **Somatic or O antigen:**
   - It is the lipopolysaccharide (LPS) antigen
   - It is heat-stable
   - Occasionally, it cross reacts with O antigens of other species
   - Early O serotypes are usually the commensals of intestine
   - Late O serotypes are the diarrhea producing strains
   - Serotyping is done by slide agglutination with specific O antisera
   - O antigen is the most important virulence factor, responsible for endotoxic activity; it protects the bacteria from phagocytosis and bactericidal effect of complement.
2. **Flagellar or H antigen** (H from *Hauch*, meaning film of breath).
   - It is heat labile
   - Presence of H antigen (flagella) makes the bacteria motile, hence contributing to their virulence.
3. **Capsular or K antigen** (K for Kapsel, German for capsule)-
   - It is the polysaccharide capsular antigen present on the envelope or microcapsule of a few strains of *E. coli*
   - When present, it encloses O antigen and renders the strain inagglutinable by O antiserum
   - It may also contribute to virulence by inhibiting phagocytosis
   - It is expressed by only few strains of *E. coli*; e.g. those causing neonatal meningitis, pyelonephritis and septicemia.
4. **Fimbrial antigen** (pilus) is the organ of adhesion, helps in attachment and colonization. It is expressed by a few strains of *E. coli*. Various fimbrial antigens are:
   - **CFA** (colonization factor antigen): It is a type of fimbriae expressed by enterotoxigenic *E. coli*
   - **Mannose resistant fimbriae** (e.g. P, M, S, F1C and Dr fimbriae): They hemagglutinate with RBCs that is not inhibited by mannose. These are expressed by uropathogenic *E. coli* and help in colonization of these strains onto uroepithelial cells
   - **P fimbriae** bind specifically to the P blood group antigens present on human RBCs and uroepithelial cells.

Toxins

The exotoxins secreted by *E. coli* are of several types:

- **Enterotoxins:** They are produced by diarrheagenic strains of *E. coli*. They are of three types; heat labile toxin, heat stable toxin and verocytotoxin (all have been described in detail in Table 29.4)
- **Hemolysins:** They are produced more commonly by virulent strains of *E. coli* (especially pyelonephritis strains); however, their role in pathogenesis is unclear
- **Cytotoxic necrotizing factor 1** (CNF1) and **secreted autotransporter toxin** (SAT): They are cytotoxic to bladder and kidney cells.

Others

Siderophores (i.e. aerobactin)—Helps in iron uptake.

Table 29.3: Biochemical reactions of Enterobacteriaceae

			ICU tests			Triple sugar iron (TSI)							LAO decarboxylation test				Sugar fermentation test			
Tribe	**Genus and species**	**Catalase**	**Indole**	**Citrate**	**Urease**	**TSI**	**Gas**	**H_2S**	**MR**	**VP**	**Motility**	**PPA**	**Lysine**	**Arginine**	**Ornithine**	**ONPG**	**Glucose**	**Sucrose**	**Lactose**	**Mannitol**
Tribe I	*Escherichia coli*	+	+	–	–	A/A	+	–	+	–	+	–	+	–/+	+/–	+	+	–/+	+	+
	Shigella																			
	S. dysenteriae[1]	–/+	–	–	–	K/A	–	–	+	–	–	–	–	–	–	–	+	–	–	–
	S. flexneri	+	–	–	–	K/A	–	–	+	–	–	–	–	–	–	–	+	–	–	+
	S. boydii	+	–	–	–	K/A	–	–	+	–	–	–	–	–	–	–	+	–	–	+
	S. sonnei	+	–	–	–	K/A	–	–	+	–	–	–	–	–	+	+	+	+ (late)	+ (late)	+
Tribe II	*Edwardsiella tarda*	+	+	–	–	K/A	+	+	+	–	+	–	+	–	+	–	+	–	–	–
Tribe III	*Salmonella*																			
	S. Typhi	+	–	–	–	K/A	–	+[2]	+	–	+	–	+	–	–	–	+	–	–	+
	S. Paratyphi A	+	–	–	–	K/A	+	–	+	–	+	–	–	–	+	–	+	–	–	+
	S. Paratyphi B	+	–	+	–	K/A	+	++	+	–	+	–	+	+	+	–	+	–	–	+
Tribe IV	*Citrobacter*																			
	C. freundii	+	–	+	–	A/A	+	++	+	–	+	–	–	+/–	–	+	+	+	+/–	+
	C. koseri	+	+	+	–	K/A	+	–	+	–	+	–	–	+/–	+	+	+	–/+	+/–	+
Tribe V	*Klebsiella*																			
	K. pneumoniae	+	–	+	+	A/A	++	–	–	+	–	–	+	–	–	+	+	–	+	+
	K. oxytoca	+	+	+	+	A/A	++	–	–	+	–	–	+	–	–	+	+	–	+	+
	Enterobacter																			
	E. aerogenes	+	–	+	–	A/A	++	–	–	+	+	–	+	–	+	+	+	+	+	+
	E. cloacae	+	–	+	+/–	A/A	++	–	–	+	+	–	–	+	+	+	+	+	+	+
	Hafnia	+	–	–	–	K/A	+	–	–	+	+	–	+	–	+	+	+	–	–	+
	Serratia[3]	+	–	+	–	K/A	+	–	–	+	+	–	+	–	+	+	+	+	–	+
	Pantoea	+	–	+/–	–/+	A/A	–/+	–	–	+	+	–	–	–	–	+	+	–	–/+	+
Tribe VI	*Proteus*																			
	P. vulgaris	+	+	–/+	+	K/A	+/–	++	+	–	Swar-	+	–	–	–	–	+	–	–	–
	P. mirabilis	+	–	+/–	+	K/A	+	++	+	+/–	ming[4]	+	–	–	+	–	+	–	–	–
	P. penneri	+	–	–	+	K/A	–/+	–/+	+	–	+	+	–	–	–	–	+	–	–	–
	Morganella morganii	+	+	–	+	K/A	+	–	+	–	+	+	–	–	+	–	+	–	–	–
	Providencia																			
	P. rettgeri	+	+	+	+	K/A	–	–	+	–	+	+	–	–	–	–	+	–	–	+
	P. stuartii	+	+	+	–/+	K/A	–	–	+	–	+	+	–	–	–	–	+	–	–	–
	P. alcalifaciens	+	+	+	–	K/A	+/–	–	+	–	+	+	–	–	–	–	+	–	–	–
Tribe VII	*Yersinia*																			
	Y. pestis	+	–	–	–	K/A	–	–	+	–	–	–	–	–	–	+/–	+	–	–	+
	Y. enterocolitica	+	+/–	–	+/–	K/A	–	–	+	–	+[5]	–	–	–	+	+	+	+	–	+
	Y. pseudotuberculosis	+	–	–	+	K/A	–	–	+	–	+[5]	–	–	–	–	+/–	+	–	–	+

>90% of strains positive (+), >90% of strains negative (–), 50–90% of strains positive (+/–), 50–90% of strains negative (–/+)

[1] *S. dysenteriae* type 1: It is the only exception in family Enterobacteriaceae which is catalase negative, [2] *S.* Typhi: Only speck of H_2S present at the junction of slant and butt, [3] *Serratia*: It produces red color pigment (prodigiosin), [4] Swarming motility: It is observed only in *Proteus mirabilis* and *Proteus vulgaris*, [5] *Y. enterocolitica* and *Y. pseudotuberculosis* exhibit differential motility (motile at 25°C, but not at 37°C), H_2S: Hydrogen sulfide, MR: Methyl red, VP: Voges-Proskauer test, PPA: Phenyl pyruvic acid test, ONPG: Ortho-Nitrophenyl-β-galactoside, TSI: Triple sugar iron test.

Table 29.4: Various properties of enterotoxins of *Escherichia coli*

LT (heat-labile toxin)	ST (heat-stable toxin)	Verocytotoxin or Shiga-like toxin
Produced by: Enterotoxigenic *E. coli*	**Produced by:** Enterotoxigenic *E. coli*	**Produced by:** Enterohemorrhagic *E. coli*
It resembles cholera toxin in its structure and function, but it is less potent than the latter **Mechanism of action:** It has 2 peptide fragments: A and B ***Fragment B:*** It is the binding fragment, has five subunits. It binds to GM1 ganglioside receptors present on the intestinal epithelium following which A fragment is internalized and cleaved into A1 and A2 peptides ***Fragment A:*** • Fragment A2 helps in tethering A and B subunits together • Fragment A1 is the active fragment (27 kDa), causes ADP ribosylation of G protein → upregulates the activity of adenylate cyclase → results in the intracellular accumulation of cyclic AMP (adenosine monophosphate) → leads to increased outflow of water and electrolytes into the gut lumen, with consequent diarrhea	ST is of two types: ST-I and ST-II **Mechanism of action:** ***ST-I:*** Binds to the guanylate cyclase C → increased production of cyclic guanosine monophosphate (cGMP) → fluid accumulation in gut lumen → diarrhea ***ST-II:*** Though it causes fluid accumulation (in young piglets), the mechanism is not known yet, it is not through cAMP or cGMP mediated	It is so named because it is cytotoxic to Vero cell lines Also called **Shiga-like toxin** as it resembles Shiga toxin in its structure and function **Mechanism of action:** It has two fragments: A and B ***Fragment B*** binds to a globotriosyl ceramide (Gb3) receptor on intestinal epithelium ***Fragment A*** is the active fragment. It inhibits protein synthesis by inhibiting 28S subunit of 60S subunit ribosome **Two types** of Shiga-like toxin are known to exist: Stx1 and Stx2
Plasmid-coded	Plasmid-coded	Bacteriophage-coded
Detection of LT: Toxin detection: by latex agglutination, ELISA Molecular methods: PCR detecting gene coding for LT Older methods which are obsolete now include: • Ligated rabbit ileal loop test • Tissue culture test (Y-1 mouse adrenal cell)	**Detection of ST:** Same as for LT	**Detection of VT:** Serologically—Latex agglutination, ELISA Molecular methods—using specific DNA probe Cytotoxicity on Vero and HeLa cell lines

Clinical Manifestations

E. coli is one of the most common pathogen encountered clinically and has been associated with various manifestations.

- **Urinary tract infection** (UTI): It is caused by uropathogenic *E. coli* (UPEC) (described later)
- **Diarrhea**: It is caused by six types of diarrheagenic *E. coli* (described later)
  1. Enteropathogenic *E. coli* (EPEC)
  2. Enterotoxigenic *E. coli* (ETEC)
  3. Enteroinvasive *E. coli* (EIEC)
  4. Enterohemorrhagic *E. coli* (EHEC)
  5. Enteroaggregative *E. coli* (EAEC)
  6. Diffusely adherent *E. coli* (DAEC)
- **Other infections:**
  - Abdominal infections: *E. coli* is the most common cause of both primary bacterial peritonitis (occurs spontaneously) and secondary bacterial peritonitis (occurs secondary to intestinal perforation leading to spillage of commensal *E. coli* from intestine). It also causes visceral abscesses, such as hepatic abscess
  - Pneumonia (especially in hospitalized patients—ventilator-associated pneumonia)
  - Meningitis (especially neonatal meningitis)
  - Wound and soft tissue infection such as cellulitis and infection of ulcers and wounds, especially in patient with diabetic foot
  - Bacterial prostatitis (most common cause)
  - Osteomyelitis
  - Endovascular infection and bacteremia.

LABORATORY DIA GNOSIS — ***Escherichia coli***

- **Sample collection:** Depends on the site of infection—urine, stool, pus, wound swab, etc.
- **Direct smear:** Gram-negative bacilli, and pus cells
- **Culture:**
  - Blood agar: Circular, gray, moist colonies, hemolysis variable
  - MacConkey agar: Flat, pink LF colonies
- **Culture smear and motility testing:** Motile gram-negative bacilli
- **Biochemical identification:**
  - Catalase positive and oxidase negative
  - Nitrate is reduced to nitrite
  - ICUT tests: Indole(+), Citrate (–), Urease(–), TSI: A/A, gas(+), H_2S(–)
  - Sugar fermentation test: Ferments most sugars
- **Antimicrobial susceptibility testing**

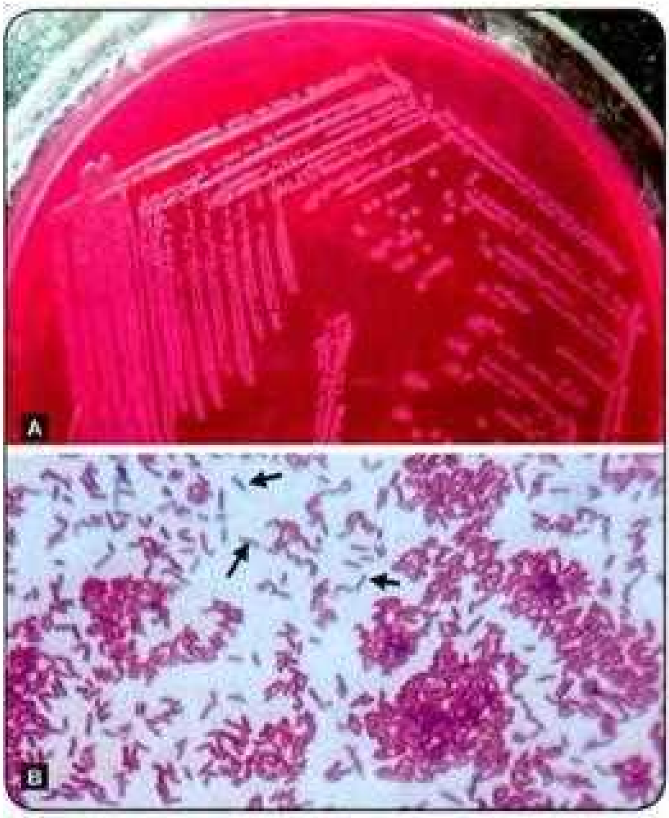
Laboratory Diagnosis

Diagnosis of UPEC and diarrheagenic *E. coli* has been described later in the chapter under UTI and diarrhea.

- **Sample collection:** It depends on the site of infection (Table 29.5)
- **Direct smear of specimen by Gram staining:** *E. coli* is gram-negative, straight rod measuring 1–3 µm × 0.4–0.7 µm, arranged singly. Plenty of pus cells are also found in direct smear

Table 29.5: Sample collection in *E. coli* infections

Specimens collected	Disease
Pus, exudates and wound swab	Cellulitis or wound infection
Urine (midstream)	Urinary tract infections (UTI)
Stool	Diarrhea
Cerebrospinal fluid (CSF)	Meningitis
Peritoneal exudate	Peritonitis
Sputum	Pneumonia
Tracheal aspirate	Ventilator-associated pneumonia
Blood	Bacteremia



Figs 29.1A and B: A. Flat pink lactose fermenting colonies of *E. coli* on MacConkey agar; **B.** Slender gram-negative bacilli (arrows showing)

Source: Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (with permission).

- **Culture:** *E. coli* is an aerobe and facultative anaerobe. It grows on ordinary culture media at optimum temperature of 37°C (ranges 10–40°C) in 18–24 hours. The culture media used are as follows:
  - **Blood agar:** Colonies are big, circular, gray, moist and occasionally β hemolytic (mainly UPEC strains)
  - **MacConkey agar:** Colonies are circular, moist, smooth with entire margin, **flat and pink** (due to lactose fermentation) (Fig. 29.1A)
  - **Liquid medium,** such as peptone water broth-produces uniform turbidity
- **Culture smear and motility testing:** Culture smear of the colonies shows gram-negative bacilli arranged singly (Fig. 29.1B). Hanging drop reveals motile bacilli (by peritrichate flagella)
- **Biochemical test:** Described below.

E. coli **shows the following Biochemical Properties**
- Catalase positive and oxidase negative
- Nitrate is reduced to nitrite
- **ICUT tests:**
  - Indole test: Positive (cherry red colored ring is formed)
  - Citrate test: Negative (citrate is not utilized)
  - Urease test: Negative (urea is not hydrolyzed)
  - TSI (triple sugar iron agar) test: Shows acid/acid, gas present, H_2S absent
- **Sugar fermentation test:** *E. coli* ferments most of the sugars, such as glucose, lactose, mannitol, maltose (but not sucrose), with production of acid and gas.
- **MR** (methyl red) test: Positive
- **VP** (Voges-Proskauer) test: Negative.

- **Antimicrobial susceptibility testing:** It is necessary to administer proper antibiotics.
  - It is done on Mueller-Hinton agar by using disk diffusion method
  - *E. coli* can rapidly develop resistance to multiple drugs. Many strains of *E. coli* are producers of β lactamases such as ESBL (extended spectrum β lactamases) and MBL (metallo-β-lactamases).

Urinary Tract Infection (UTI)

E. coli (uropathogenic *E. coli* or UPEC) is the single most common pathogen, accounting for 70–75% of all cases of UTI. **UPEC** serotypes O1, O2, O4, O6, O7 and O75 are responsible for most UTIs.

Route of spread: *E. coli* reaches the urinary tract by two routes.
1. **Ascending route:** After colonizing the periurethral area, *E. coli* ascends the urinary tract to reach bladder resulting in cystitis and urethritis. Further ascension, leads to pyelonephritis.
2. **Descending route:** It is due to hematogenous seeding of *E. coli* into kidneys resulting in pyelonephritis.

Types: Depending on the site involved, there are two types of UTIs—1. lower UTI and 2. upper UTI (Table 29.6).

Predisposing factors that Promote UTI:
- **Females:** Due to short urethra and close proximity to anus, there is more chance of getting lower UTI than males.
- Presence of urinary catheters
- **Urinary obstruction:** As occurs in prostate enlargement or urinary stones; leads to urine stasis
- **Pregnancy:** Physiological obstruction in urinary tract due to growing fetus may lead to prolonged stasis of urine. Asymptomatic bacteriuria (urinary infection without symptoms) is common in pregnant women.

Table 29.6: Differences between lower UTI and upper UTI

Characteristics	Lower UTI	Upper UTI
Sites involved/ syndromes	Bladder (cystitis), and urethra (urethritis)	Kidney and ureter (pyelonephritis)
Symptoms	Local manifestations—Dysuria, urgency, frequency	Local and systemic manifestations (such as fever, vomiting, abdominal pain)
Route of spread	Ascending route	Both ascending (common) and descending route
Occurrence	More common	Less common
Virulence factors of *E. coli*	Fimbriae (e.g. P fimbriae)	Capsular K antigen

Virulence factors of *E. coli* responsible for both upper and lower UTI:
- Cytotoxins (CNF 1: cytotoxic necrotizing factor 1 and SAT: Secreted autotransporter toxin)
- Hemolysins

Laboratory Diagnosis of UPEC

Specimen Collection

- **Clean voided midstream urine:** It is the most common specimen for UTI; collected after properly cleaning the urethral meatus or glans
- **Suprapubic aspiration** of urine from the bladder: It is the most ideal specimen. It is recommended for patients in coma or infants
- In catheterized patients, urine should be collected from the catheter tube (after clamping and disinfecting); but not from the bag.

Transport

Urine sample should be processed immediately. If delay is expected for more than 1-2 hours, then it can be stored in refrigerator or stored by adding boric acid, glycerol or formate for maximum 24 hours.

Direct Examination

The screening tests done are as follows:
- **Wet mount examination:** It is done to demonstrate the pus cells in urine. Pyuria of more than 8 pus cells/mm^3 is taken as significant
- **Leukocyte esterase test:** It is rapid and cheaper method, detects leukocyte esterases secreted by pus cells present in urine
- **Nitrate reduction test (Griess test):** Nitrate reducing bacteria like *E. coli* give a positive result
- **Gram staining** of urine is not a reliable indicator as—(1) the bacterial count in urine is usually low, (2) pus cells rapidly deteriorate in urine and may not be seen well. Gram staining may be limited to pyelonephritis and invasive UTI cases and a count of ≥1 bacteria/oil immersion field is taken as significant.

Culture

- **Culture media:** Urine sample should be inoculated onto MacConkey agar and blood agar or CLED agar (cysteine lactose electrolyte deficient agar)
- **Kass concept of significant bacteriuria:** This is based on the fact that, though the normal urine is sterile it may get contaminated during voiding, with normal urethral flora. However, the bacterial count in contaminated urine would be lower than that caused by an infection
  - A count of $\geq 10^5$ **colony forming units (CFU)/mL** of urine is considered as significant—indicates infection (referred as 'significant bacteriuria' developed by Kass)
  - Count between 10^4 to 10^5 CFU/mL indicates doubtful significance; should be be clinically correlated
  - **Low count of $\leq 10^4$** CFU/mL is due to commensal bacteria (due to contamination during voiding and of no significance). However, low counts may be significant in the following conditions:
    - Patient on antibiotic or on diuretic treatment
    - Infection with some gram-positive organisms such as *S. aureus* and *Candida*
    - Pyelonephritis and acute urethral syndrome
    - Sample taken by suprapubic aspiration.
- **Quantitative culture:** This is done to count the number of colonies. Each colony on plate corresponds to one bacterium in urine sample. Quantitation is done by:
  - Semi-quantitative method such as standardized loop technique
  - Quantitative method such as pour plate method.

Antibody Coated Bacteria Test

This test is done to differentiate upper and lower UTI.
- In **upper UTI**, as the route of spread is hematogenous, bacteria coated with specific antibodies are found in urine. Such bacteria coated with specific antibody are detected by immunofluorescence method using fluorescent labeled antihuman globulin
- In **lower UTI**, bacteria found in urine are never coated with specific antibodies.

Diarrhea (Diarrheagenic E. coli)

Diarrheagenic *E. coli* are antigenically distinct from the commensal *E. coli* which colonize the intestine. Only few serotypes of *E. coli* which express the enterotoxin or other virulence mechanisms can cause diarrhea. There are six types of diarrheagenic *E. coli*.

Enteropathogenic E. coli (EPEC)

EPEC frequently causes **infantile diarrhea** (outbreaks) and occasionally cause sporadic diarrhea in adults. Person-to-person spread is seen
- It is nontoxigenic and noninvasive
- Mechanism of diarrhea:
  - **Adhesion** to intestinal mucosa, mediated by plasmid coded **bundle-forming pili**, which form cup-like projections called **pedestals**
  - **A/E lesions** (attaching and effacing lesions): These are typical lesions produced on the intestinal epithelium (coded by chromosomal **LEE gene**, i.e. locus for enterocyte effacement); which leads to disruption of brush border epithelium causing increased secretion and watery diarrhea.

Enterotoxigenic E. coli (ETEC)

ETEC is the most common cause of **traveler's diarrhea** causing 25–75% of cases.
- It causes acute watery diarrhea in infants and adults
- Common serotypes associated are—O6, O8, O15, O25, O27, O153, O159, etc.
- It is toxigenic, but not invasive
- **Pathogenesis of ETEC is by:**
  - **Attachment** to intestinal mucosa is mediated by fimbrial protein called **CFA** (colonization factor antigen)
  - **Toxin production**—(1) heat-labile toxin or LT (acts by ↑cAMP), (2) heat-stable toxin or ST (acts by ↑cGMP).
- **Diagnosis:** Detection of toxins is the mainstay of diagnosis. (refer to Table 29.4).

Enteroinvasive E. coli (EIEC)

Common serotypes associated with EIEC are O28, O112, O114, O124, O136, O152, etc.
- **Pathogenesis:** EIEC is not toxigenic, but invasive. The epithelial cell invasion is mediated by a plasmid-coded antigen called virulence marker antigen **(VMA)**
- EIEC is biochemically, genetically and pathogenically closely related to *Shigella*
- **Manifestations:** These include ulceration of bowel, dysentery (diarrhea with mucus and blood, called bacillary dysentery resembling shigellosis)
- **Diagnosis:**
  - Detection of VMA by ELISA
  - HeLa cell invasion assay
  - **Sereny test** (inoculation of bacterial suspension into guinea pig eyes produces conjunctivitis)
  - Compared with other *E. coli* strains, EIEC are biochemically atypical being non motile, lactose non-fermenters and negative for lysine decarboxylase.

Enterohemorrhagic E. coli (EHEC)

EHEC is prevalent mainly in industrialized countries; in contrast to other diarrheagenic *E. coli* which are common in developing regions.
- **Serotypes** associated with EHEC are:
  - O157:H7 (most common serotype)
  - Other serotypes are rarely associated such as O26:H11, O6, O55, O91, O103, O111 and O113.
- EHEC is usually transmitted by contaminated food, i.e. consumption of lettuce, spinach, sprouts and undercooked **ground beef**
- **Low infective dose:** The infective dose of EHEC is very low. Only few organisms (<10^2 bacilli) are required to initiate the infection
- **Pathogenesis:** EHEC secretes a toxin called **verocytotoxin** or **Shiga-like toxin** (refer Table 29.4 and the highlight box below).

> **Shiga-like Toxin**
>
> **Mechanism of action:** Shiga like toxin acts by inhibiting the protein synthesis by inhibiting the 28S subunit of 60S ribosome.
>
> **Shiga-like toxin is of two types—Stx1 and Stx2**
> - Stx1 is indistinguishable from shiga toxin produced by *Shigella dysenteriae* type 1
> - Stx2 is more commonly associated with development of HUS than Stx1.

- **Manifestations:** Shiga-like toxin has predilection for endothelial cells causing capillary microangiopathy which leads to:
  - **HC (hemorrhagic colitis):** It manifests as gross bloody diarrhea, abdominal pain and fecal leukocytosis but no fever
  - **Hemorrhagic uremic syndrome (HUS):** It is characterized by injury to small vessels of the kidney and brain, which can lead to bloody diarrhea, thrombocytopenia, renal failure and encephalopathy but without fever. It is more common in children.
- **Diagnosis:**
  - **Sorbitol MacConkey agar:** EHEC, in contrast to other *E. coli*, does not ferment sorbitol and produces pale colonies
  - **Rainbow agar:** O157 strains appear as black colonies on this medium as they are negative for β-glucuronidase.
  - **Toxin detection:**
    - Demonstration of cytotoxicity in Vero cell lines (gold standard method)
    - Fecal toxin detection by ELISA or rapid tests.
  - **PCR** can be used to differentiate genes coding for Stx1 and Stx2.

Enteroaggregative E. coli (EAEC)

It is so named because it adheres to HEp-2 cells in a distinct pattern, layering of the bacteria aggregated in a stacked-brick fashion. Most strains are "O" untypeable but "H" typeable.

- **Pathogenesis:**
  - Intestinal colonization is mediated by aggregative adhesion fimbriae I (regulated by *aggR* gene)
  - It also produces **EAST 1 toxin** (enteroaggregative heat stable enterotoxin 1).
- **Manifestations:** Persistent and acute diarrhea are commonly seen; especially in developing countries.

> ***E. coli* O104: H4**
>
> It is an enteroaggregative strain that has caused major outbreaks in Germany in 2011. One peculiar feature of this strain is, it produces Shiga-like toxin and can cause HUS.

Diffusely-adherent E. coli (DAEC)

It is characterized by:

- Ability to adhere to HEp-2 cells in a diffuse pattern
- Expresses diffuse adherence fimbriae which contribute to the pathogenesis
- DAEC is capable of causing diarrheal disease, primarily in children aged 2–6 years.

> **TREATMENT** ***E. coli***
>
> **Extra-intestinal *E. coli***
>
> - Treatment is essentially based up on antimicrobial susceptibility test report
> - Majority of *E. coli* acquired in hospitals are multi-drug resistant (MDR); resistant to cephalosporins, quinolones, cotrimoxazole, etc. However, if found susceptible, then these agents may be preferred
> - They often produce ESBLs (extended spectrum β-lactamases) or AmpC β-lactamases and become resistant to most β-lactams except carbapenems
> - Carbapenems, amikacin or β-lactam/β-lactamase inhibitor combinations (BL/BLIs) such as piperacillin-tazobactam or cefoperazone-sulbactam are usually the agent of choice for hospital acquired MDR *E. coli* infection
> - Polymyxins, fosfomycin or tigecycline are the next line antimicrobials for carbapenem resistant isolates (e.g. carbapenamase producing isolates).
>
> **Diarrheagenic *E. coli***
>
> The mainstay of treatment is fluid replacement. The use of antimicrobials should generally be avoided.

SHIGELLA

Shigella, the most important agent of bacillary dysentery, is named after Japanese microbiologist Kiyoshi Shiga who isolated the first member, *S. dysenteriae* serotype-1 (the *Shiga bacillus*) in 1896 from epidemic dysentery. Shigellae differ from *E. coli* being nonmotile and not fermenting most sugars except mannitol.

Classification

Based on a combination of biochemical and serological characteristics, shigellae are classified into four species—*S. dysenteriae, S. flexneri, S. boydii* and *S. sonnei* which are also designated as serogroups A, B, C and D respectively.

Antigens and Serotyping of *Shigella*

Based on somatic O polysaccharide antigen, *Shigella* species are further typed into four groups.

1. ***S. dysenteriae* (group A):** It has 15 serotypes. It does not ferment mannitol, in contrast to other species of *Shigella*, which are mannitol fermenters.
   - Serotype 1 (*S. shigae*) is the only *Shigella*, which produces Shiga toxin. It is the only member of family Enterobacteriaceae to be catalase negative. It is indole negative
   - Serotype 2 (*S. schmitzi*) forms indole and ferments sorbitol and rhamnose
   - Serotype 3–7 were formerly called Large-Sachs group.
2. ***S. flexneri* (group B):** Based on type specific antigen, it is further typed into 8 serotypes.
   - *S. flexneri* is the most complex species antigenically
   - Serotype 8 is always indole negative and is further typed to three biotypes—Manchester, Newcastle and Boyd 88 based on gas production from sugars
   - *S. flexneri* can also be typed by bacteriophage typing into 123 phage types.
3. ***S. boydii* (group C):** It has 19 serotypes. It was first described by Boyd. *S. boydii* is isolated least frequently from cases of bacillary dysentery
4. ***S. sonnei* (group D):** It is antigenically homogeneous and has only one serotype. It can be typed by colicin typing into 26 colicin types.

Other Antigens of Shigella

- K antigen may be present in some serotypes which may not be visible as capsule, but it covers the O antigen and makes it inagglutinable by homologous O antisera
- Fimbrial antigens may be found in some strains, especially in *S. flexneri*
- Flagellar H antigen is absent
- Cross reactivity:
  - Antigens of many *Shigella* serotypes (other than *S. sonnei*) cross-react with serotypes of *E. coli*
  - Antigens of *S. sonnei* cross react with *Plesiomonas shigelloides*.

> **Pathogenesis**
>
> *Shigella* is one of the important cause of bacillary dysentery.
>
> - **Mode of transmission:** Infection occurs by ingestion through contaminated fingers (most common), food, and water or rarely flies. It can also be transmitted sexually (homosexuals)
> - **Minimum infective dose:** As low as 10–100 bacilli are capable of initiating the disease, probably because of their ability to survive in gastric acidity
>
> *Contd...*

Contd...

- **Entry via M cell:** Bacilli enter the mucosa via M cells and are then engulfed by macrophages. Subsequently, the macrophages release the bacilli, which induce the recruitment of inflammatory cells to the infected site releasing cytokines which inturn cause acute colitis—the hallmark of shigellosis
- **Invasion:** Once inside the submucosa, shigellae induce their own uptake into the adjacent epithelial cells. Invasion is determined by a **large virulence plasmid** of 214 kb size, which codes for important virulence factors such as **ipa proteins** and **type III secretion system**. They inturn help in uptake of the shigellae into the host cells
- **Direct cell-to-cell spread:** Shigellae spread directly from one host cell to the other by inducing actin polymerization of host cells, mediated by ***IcsA*** proteins
- **Exotoxins:**
  - ***Shigella* enterotoxin (ShET1 and 2)**
    - **ShET1** is structurally similar to cholera toxin and is found essentially in *S. flexneri* 2a
    - **ShET2** is present in all *S. flexneri* isolates. It helps in iron uptake.
  - **Shiga toxin:** It is a cytotoxin, produced by *S. dysenteriae* type 1. It is structurally and functionally similar to verocytotoxin of EHEC. It inhibits protein synthesis by inhibiting 28S RNA of 60S ribosome. It enhances local vascular damage of intestine as well as internal organs, such as kidney and brain.
- **Endotoxin:** It acts similar to any other gram-negative endotoxin and induces intestinal inflammation and ulcerations.

Clinical Manifestations

Shigellosis typically evolves through five phases:

1. **Incubation period:** It usually lasts for 1–4 days.
2. **Initial phase** is characterized by watery diarrhea with fever, malaise, anorexia and vomiting.
3. **Phase of dysentery:** It is characterized by frequent passage of bloody mucopurulent stools with increased tenesmus and abdominal cramps. Endoscopy shows an edematous and hemorrhagic mucosa, with ulcerations and overlying exudates. Most of the cases are self-limiting.
4. **Phase of complication:** It is commonly seen with children less than 5 years age.
   - **Intestinal** complications such as toxic megacolon, perforations and rectal prolapse
   - **Metabolic** complications, such as hypoglycemia, hyponatremia, and dehydration
   - **Ekiri syndrome** or toxic encephalopathy: It is a metabolic complication of shigellosis; manifests as altered consciousness, seizures, delirium, abnormal posturing and cerebral edema
   - Bacteremia is rare and can lead to meningitis and pneumonia. Rarely, cases of vaginitis and keratoconjunctivitis have been reported.
5. **Postinfectious phase:** Patients expressing HLA-B27, develop an autoimmune reaction months after shigellosis; characterized by reactive arthritis, ocular inflammation and urethritis. It is seen only after *S. flexneri* infection (occurs in 3% of cases).

Epidemiology

Risk factors for shigellosis include overcrowding, poor hygiene and children less than 5 years.

- It tends to occur as epidemics in developing countries such as Indian subcontinent and sub-Saharan Africa
- *S. flexneri* accounts for maximum number of cases (60%) in the developing areas including India, whereas *S. sonnei* is more prevalent in developed and industrialized world, accounting for 77% of cases
- Cases caused by *S. dysenteriae* type-1 are associated with high mortality. It usually causes epidemics of dysentery, particularly in refugee camps
- Humans are the natural host and cases are the only source of infection. Chronic carriage is rare except in malnourished children or AIDS patients
- **World:** Shigellosis is the most communicable disease among bacterial causes of diarrhea. Worldwide, 80 million cases of bacillary dysentery with 7 lakh deaths occur annually, of which majority are from developed countries. Children (<5 years) accounts for nearly 60% of the cases
- With improved sanitation, the incidence of shigellosis is decreasing. However, the worrisome part of the present day is development of drug resistance among the *Shigella* strains.

LABORATORY DIAGNOSIS — ***Shigella dysentery***

- **Specimen:** Fresh stool
- **Transport media:** Sach's buffered glycerol saline broth
- **Wet mount preparation:** Pus cells, erythrocytes
- **Culture**
  - Enrichment broth such as—Selenite F broth, tetrathionate broth and gram-negative broth
  - **Selective media such as:**
    - Mildly selective media, e.g. MacConkey agar- translucent NLF colonies
    - Highly selective medium, e.g. DCA, XLD agar and SS agar
- **Culture smear and motility testing:** Gram-negative bacilli and non-motile
- **Biochemical reactions:**
  - Catalase positive and oxidase negative (except *S. dysenteriae* type 1 which is catalase negative)
  - Nitrate is reduced to nitrite
  - **ICUT tests:** Indole (–), Citrate (–), Urease (–), TSI: K/A, gas (–), H_2S (–)
  - **Sugar fermentation test:**
    - Ferments glucose and mannitol (except *S. dysenteriae*)
    - Does not ferment lactose and sucrose (except *S. sonnei* which is a late fermenter for both lactose and sucrose).
- **Typing**
  - Slide agglutination test with specific antisera can differentiate between four *Shigella* species
  - Bacteriocin or colicin typing is done for *S. sonnei*
- **Antimicrobial susceptibility testing**

Laboratory Diagnosis

- **Specimen collection:** Fresh stool is collected. Rectal swabs are not satisfactory
- **Transport media:** Specimens should be transported immediately. If delay is inevitable, specimens should be transported in a suitable medium, such as Sach's buffered glycerol saline
- **Wet mount preparation** of feces shows large number of pus cells, erythrocytes and macrophages
- **Culture:** To inhibit the commensals, fecal specimen is inoculated simultaneously into enrichment broth and selective media
- **Enrichment broth** such as Selenite F broth, tetrathionate broth and gram-negative broth are used. Uniform turbidity appears in 24 hours, from which again subcultures are made onto selective media
- **Selective media such as:**
  - **Mildly selective media:** On MacConkey agar, the growth appears as small (2 mm), circular, translucent and non-lactose fermenting pale or colorless colonies
  - **Highly selective medium** contains higher concentration of bile salts as inhibitory agent
    - **DCA** (Deoxycholate citrate agar): Colonies are similar to those on MacConkey agar-translucent and NLF (colorless) colonies
    - **XLD agar** (Xylose lysine Deoxycholate): Colonies of *Shigella* appear red without black center. It is superior to DCA as it is less inhibitory to *S. dysenteriae* and *S. flexneri*
    - **SS agar** (*Salmonella Shigella* agar)
    - **Hektoen enteric agar.**
- **Culture smear and motility testing:** Gram stain of colonies reveal short, gram-negative bacilli measuring 1–3 μm × 0.5 μm. They are nonmotile, noncapsulated and non-sporing
- **Biochemical reactions:** Biochemically *Shigella* is an organism of exceptions. *Shigella* species and their serotypes can be differentiated by an array of biochemical tests
  - **Catalase:** All shigellae are catalase positive except *S. dysenteriae* serotype-1 and *S. flexneri* serotype-4a
  - **Oxidase test** is negative for all species
  - **Mannitol fermentation:** All species ferment mannitol except *S. dysenteriae*, Newcastle biotype of *S. flexneri* serotype-6 and rabaulensis biotype of *S. flexneri* serotype-4a
  - **Lactose and sucrose fermentation:** Shigellae are lactose and sucrose non-fermenters except *S. sonnei* which is a late fermenter of both lactose and sucrose
  - **Gas production:** All shigellae are **anaerogenic** (do not produce gas) except—Manchester and Newcastle biotypes of *S. flexneri* type 6
  - **Indole production:** Though most shigellae do not produce indole, it is consistent with only *S. dysenteriae* serotype-1, *S. flexneri* serotype-6 and *S. sonnei*. For others, it is variable. *S. dysenteriae* serotype-2 is always indole positive
  - All are urease and citrate negative
  - TSI shows alkaline/acid, no gas and no H_2S
  - **Decarboxylase test:** All shigellae are negative for lysine, arginine and ornithine except *S. sonnei* which decarboxylates ornithine
  - ONPG test is negative for all shigellae, except *S. sonnei*.
- **Slide agglutination test:** Because of biochemical variations, identification of *Shigella* is always confirmed by slide agglutination with polyvalent antisera (genus specific). Then, the species identification can be done by using group specific antisera specific for serogroups A, B, C or D. Serotypes under each species are further detected by using type specific antisera
- **Bacteriocin or colicin typing** is done for *S. sonnei*. This is based on ability of a strain to produce particular colicin which inhibits the growth of a set of selected indicator strains. There are 26 colicin types of *S. sonnei*
- **Antimicrobial susceptibility testing** is done on Mueller Hinton agar by disk diffusion test.

TREATMENT ***Shigella***

Because of the prompt transmissibility, current recommendation is that every case of shigellosis should be treated with antibiotics.

- Ciprofloxacin is the drug of choice
- Alternative drugs which are effective are ceftriaxone, azithromycin, pivmecillinam and some fifth-generation quinolones
- Duration of treatment is about 3 days except for:
  - *S. dysenteriae* type 1 infection—5 days
  - Infections in immunocompromised patients—7–10 days.
- Oral rehydration solution (ORS) should be started for correction of dehydration and nutrition should be started as soon as possible after the completion of initial rehydration.

Prevention

Being highly infectious; strict infection control measures of contact precaution should be followed (Chapter 53).

- Handwashing after handling of children's feces and before handling of food is highly recommended
- Stool decontamination (e.g. with sodium hypochlorite) has proven useful
- No vaccine against shigellosis is currently available, though several clinical trials are being conducted.

TRIBE II: EDWARDSIELLEAE

Edwardsiella is a commensal in the gut of reptiles and fishes. Human infection is rare; associated with ingestion of inadequately cooked aquatic animals or due to snake-related injury.

- *E. tarda* is the most frequently isolated species of *Edwardsiella* in clinical specimens
- It is associated with gastroenteritis (most common presentation) followed by extraintestinal manifestations such as septic shock, liver abscess and infections related to trauma and aquatic environment
- *E. tarda* is so named because it is biochemically slow. It is motile and biochemical properties are similar to *E. coli* with some exceptions:
    - Ferments fewer sugars (only glucose and maltose)
    - Non-lactose fermenter
    - Produces H_2S.

Treatment: Unlike *E. coli*, *E. tarda* is usually susceptible to most antimicrobials used for gram-negative bacilli such as quinolones or cephalosporins.

TRIBE III: SALMONELLEAE

Tribe Salmonelleae comprises of genus *Salmonella* which is discussed separately in detail in Chapter 30.

TRIBE IV: CITROBACTEREAE

Citrobacter species are mostly environmental contaminants isolated from water, soil, food and feces of man and animals.

They occasionally cause urinary tract, gallbladder and middle ear infections and neonatal meningitis (*C. koseri*).

Identification: *Citrobacter* species are motile, lactose fermenters like *E. coli*, but differ from the latter in being citrate positive and lysine decarboxylase negative. Various species can be differentiated by:

- *C. freundii*—indole negative and H_2S positive
- *C. koseri* (previously, *C. diversus*)—indole positive and H_2S negative
- *C. amalonaticus*—indole positive, H_2S negative and grows in KCN medium.

Some strains of *Citrobacter freundii* (formerly called **Ballerup-Bethesda** group) possess Vi antigen, which is antigenically similar to that of salmonellae and may lead to confusion in identification.

Treatment: Most *Citrobacter* isolated are MDR; and the guideline for treatment is same as that for *E. coli*.

TRIBE V: KLEBSIELLEAE

The tribe Klebsielleae consists of genera *Klebsiella*, *Enterobacter*, *Hafnia* and *Serratia*. They differ from all other tribes being VP positive but MR negative.

KLEBSIELLA

Klebsiella species are usually found as commensals in human intestines and as saprophytes in soil. Genus *Klebsiella* has two species—*K. pneumoniae* and *K. oxytoca*. Similar to *E. coli*, *Klebsiella* species are also lactose fermenters; however, they differ in being non-motile and capsulated (possess capsular polysaccharide).

Pathogenesis

K. pneumoniae has three subspecies:

1. *K. pneumoniae* subspecies *pneumoniae*: It is the most pathogenic among all.
    - It is responsible for severe lobar pneumonia, urinary tract infections, meningitis (neonates), septicemia and pyogenic infections such as abscesses and wound infections
    - It frequently colonizes the oropharynx of hospitalized patients and is a common cause of nosocomial infections. Most of the hospital strains are multidrug resistant
    - Pneumonia tends to be destructive with production of thick, mucoid, brick red sputum. Some time, the sputum has a thin and currant jelly-like appearance
    - Some strains can rarely cause diarrhea and have been shown to produce an *E. coli* like heat stable enterotoxin.
2. *K. pneumoniae* subspecies *ozaenae* is associated with **atrophic rhinitis** (or *ozena*), characterized by foul smelling nasal discharge. It is biochemically inactive.
3. *K. pneumoniae* subspecies *rhinoscleromatis* causes **rhinoscleroma**: a chronic granulomatous hypertrophy of the nose, prevalent in southeastern Europe, India and in Central America. It is biochemically inactive.

Laboratory Diagnosis

K. pneumoniae shows the following biochemical properties:

- **Gram staining:** *Klebsiella* is short, plump, straight capsulated gram-negative rods, about 1-2 μm × 0.5-0.8 μm in size (Fig. 29.2)
- **Culture:** On MacConkey agar, it produces large dome-shaped mucoid (due to capsule) sticky, pink color, lactose fermenting colonies (Fig. 29.3A)
- **Biochemical identification:** See the following box.

Fig. 29.2: Direct smear (sputum) showing pus cells with gram-negative bacilli with clear halo (capsule) (arrows showing)

Source: Department of Microbiology, JIPMER, Puducherry (with permission).

Biochemical identification of *Klebsiella*

K. pneumoniae can be identified by the following properties.

- **ICUT test:**
  - Indole test: Negative
  - Citrate test: Positive (citrate is utilized)
  - Urease test: Positive (urea is hydrolyzed)
  - TSI (triple sugar iron agar test): Shows acid/acid, gas present, H_2S absent
- **Sugar fermentation test:** Ferments most of the sugars such as glucose, lactose, mannitol, maltose (but not sucrose), with production of acid and gas
- **VP** (Voges-Proskauer) test: Positive
- **MR** (methyl red) test: Negative

K. oxytoca is biochemically similar to *K. pneumoniae*, but differs from the latter by being indole positive.

Treatment: Most of the clinical isolates of *Klebsiella* are MDR; and the guideline for treatment is same as that for *E. coli*.

ENTEROBACTER

Enterobacter species are similar to *Klebsiella* in most biochemical reactions (VP positive and lactose fermenter) but differs from the latter in being motile and ornithine decarboxylase positive.

- *E. aerogenes* and *E. cloacae* are the most commonly isolated species from the clinical specimens. *E. aerogenes* has been recently renamed as *Klebsiella aerogenes*
  - They are also widely distributed in water, sewage, soil and feces of healthy persons
  - They are opportunistic pathogens, implicated in infected wounds and urinary and respiratory tract infections and occasionally septicemia and meningitis.
- *E. asburiae* differs from other *Enterobacter* in being non-motile and VP negative. It has been isolated from blood, wound and feces
- *E. sakazakii* strains are biochemically similar to *E. cloacae* except that they produce yellow pigment, do not ferment sorbitol and may give PPA test positive. They have been isolated from cases of neonatal meningitis and septicemia.

Most *Enterobacter* isolated are MDR; and the guideline for treatment is same as that for *E. coli*.

HAFNIA

H. alvei, the only species under genus *Hafnia*, which is rarely isolated from wounds, abscess, sputum, urine and blood.

- It is lactose non-fermenter and positive for lysine and ornithine decarboxylase
- Like *Serratia*, the biochemical reactions are most reliable when tested at 30°C.

Figs 29.3A to C: A. *Klebsiella* on MacConkey agar (Mucoid dome-shaped pink-colored lactose-fermenting colonies); **B.** *Serratia marcescens* (red-colored colonies); **C.** Swarming growth of *Proteus* on blood agar

Source: Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (with permission).

SERRATIA

The characteristic property of *Serratia* is production of a red non-diffusible pigment called **prodigiosin**, which is formed optimally at 30°C (Fig. 29.3B).

- *S. marcescens* is the medically most important species. Human infection with other species is rare
- It is a saprophyte found in water, soil and food. It may grow in sputum after collection and makes the sputum red (due to pigment production). This condition is known as 'pseudohemoptysis'
- *S. marcescens* is being increasingly reported in various **nosocomial infections**, such as meningitis, endocarditis, septicemia, urinary, respiratory and wound infections
- The hospital strains are often non-pigmented and multiple drug resistant (produce AmpC β-lactamases)
- The biochemical properties of *S. marcescens* include: production of lipase, gelatinase and DNase.

Treatment: Most *Serratia* isolated are MDR; and the guideline for treatment is same as that for *E. coli* except that it is intrinsically resistant to polymyxins and nitrofurantoin.

PANTOEA

Pantoea (Greek word, meaning-of all sources) has been isolated from diverse geographical and ecological sources. *P. agglomerans* was associated with a nationwide outbreak of septicemia due to contaminated intravenous fluid.

TRIBE VI: PROTEEAE

Tribe Proteeae comprises of three genera: *Proteus, Morganella* and *Providencia.*

- **PPA positivity** is the unique tribe character of Proteeae. They produce an enzyme phenylalanine deaminase which converts phenylalanine to phenylpyruvic acid
- All are motile and lactose nonfermenter
- They have a fishy pungent odor
- They are part of commensals in human intestine. However, they can cause nosocomial outbreaks of UTI, wound infections, etc.

PROTEUS

Historical Importance

Proteus species show pleomorphism, i.e. they vary in size. It is named after the Greek God 'Proteus' who was able to assume any shape.

Naming of H and O antigens: In general, the terms H and O antigens are used to denote the flagellar and somatic antigens of any organism respectively. However, the naming of these antigens were linked historically to the properties of *Proteus*.

- The **H antigen is named** from the ability of flagellated strains of *Proteus* to grow on agar as a thin film resembling the film of breath on glass (from German word '*Hauch*', meaning 'film of breath').
- **Naming of O antigen:** The thin film is not observed when strains carrying only the somatic antigen (non-flagellated strains) grow on media (from German word '*Ohne Hauch*' meaning 'without film of breath').

Pathogenesis

Proteus mirabilis and *P. vulgaris* are the most commonly encountered species.

- **Saprophytes:** Most of the species are widely distributed in nature and are isolated from decomposing animal matter, sewage and soil
- **Commensals:** They are also frequently present on the moist areas of the skin, intestine of humans and animals
- **Infections produced:** They are opportunistic pathogens, commonly associated with urinary, wound and soft tissue infections and septicemia
  - *Proteus* species are often involved in nosocomial outbreaks
  - **Struvite stones in bladder:** *Proteus* produces urease enzyme, which breaks down urea to form ammonia that damages the renal epithelium and makes the urine alkaline. Alkaline urine predisposes to the deposition of phosphate, which leads to the formation of renal calculi
  - Other *Proteus* species such as *P. penneri* and *P. myxofaciens* are rarely encountered in clinical specimens.

Proteus as the basis of Weil–Felix Reaction

Somatic antigen of certain non-motile *Proteus* strains (called **X strains**) cross react with the alkali-stable antigen of some *Rickettsia* species.

- Thus, *Proteus* antigens can be used to detect heterophile antibodies in sera of patients suffering from rickettsial infections
- Three non-motile *Proteus* strains: OX2, OX19 (from *P. vulgaris*) and OXK (from *P. mirabilis*) are used in this agglutination test.

Laboratory Diagnosis

- **Pleomorphism:** *Proteus* species are gram-negative coccobacilli occasionally appear bacillary and in filamentous forms
- **Odor:** They produce characteristic putrid fishy or seminal odor in cultures

- **Swarming:** *Proteus* has an ability to swarm (or spread) on solid media (see Fig. 29.3C).

Swarming

The swarming of *Proteus* on solid media (e.g. blood agar) appears in two patterns:
- Uniform film of growth extended on the whole plate (continuous swarming)
- Concentric circles of growth surrounding the point of inoculum (discontinuous swarming) (Fig. 29.3C).

Mechanism of swarming: At the margin of a colony, the sparsely flagellated, short (2–4 µm) vegetative cells of *Proteus* become multinucleated, densely flagellated, nonseptate, elongated cells of 20–80 µm in length, known as a **swarm cells**.
- Swam cells have a property to migrate to surrounding uninoculated areas resulting in spreading of the colonies
- This transition from vegetative cells to swam cells takes place when the cells receive a number of extracellular and intracellular signals.

Common problems in the laboratory: When mixed growth is present in the culture plate, swarming of *Proteus* overgrows other bacterial colonies. Hence, swarming is a common problem in the laboratory in isolating other bacterial colonies which are present along with *Proteus* swarming. Several methods have been used to inhibit swarming:
- Increased agar concentration to 6%
- Incorporation of alcohol, boric acid, chloral hydrate, sodium azide, sulfonamide, surface active agents, etc.
- Swarming does not occur on MacConkey agar, CLED agar and phenyl ethyl agar.

Organisms exhibiting swarming:
- Swarming is seen only with *P. mirabilis* and *P. vulgaris* but not with other members of tribe Proteeae
- Swarming can also be observed in other bacteria, such as *Serratia marcescens, Vibrio parahaemolyticus* and *Clostridium tetani*.

Biochemical Properties

Both *P. mirabilis* and *P. vulgaris* (see Table 29.3) are:
- Urease positive
- Citrate variable
- TSI shows alkaline/acid, gas present and H_2S present
- MR positive but VP negative.

However, *P. mirabilis* and *P. vulgaris* can be differentiated by:
- Indole test—positive for *P. vulgaris*, negative for *P. mirabilis*
- Ornithine decarboxylase test—positive for *P. mirabilis*, negative for *P. vulgaris*.

Typing of *Proteus* can be done by:
- Bacteriocin typing
- Bacteriophage typing
- Ribotyping
- Dienes phenomenon (see box).

Dienes phenomenon: When two strains of *Proteus* are inoculated at different areas on a culture plate:
- If swarming of two strains merge incompletely, and remain separated by a narrow line of demarcation—indicates two strains are different
- If swarming of two strains merge completely without any line of demarcation—indicates two strains are identical.

MORGANELLA

Morganella has only one species, *M. morganii*.
- It is commonly found in human and animal feces
- It is rarely associated with urinary tract infection, pneumonia and wound infection. Most of the infections are nosocomial
- It does not swarm in culture. It is indole and urease positive but citrate negative (see Table 29.3)
- TSI shows alkaline/acid, gas present but no H_2S.

Scombroid food poisoning: *Morganella morganii*, a commensal in sea fish can breakdown histidine present in sea fish into histamine, which can cause food poisoning following seafood intake. Proper storage of fish in freezer (<16°C) can prevent this condition.

PROVIDENCIA

Providencia species are associated with nosocomial infections of the urinary tract, wounds and burns.

It consists of five species; *P. rettgeri, P. stuartii, P. alcalifaciens, P. rustigianii* and *P. heimbachae*.
- *P. rettgeri* and *P. stuartii* are common pathogens. *P. alcalifaciens* has been proposed to cause diarrhea, but not proved
- They are motile but do not show swarming. Common biochemical properties are shown in Table 29.3.

TREATMENT **Tribe Proteeae**

Members of the tribe Proteeae are often multidrug resistant. They are also resistant to many disinfectants.
- They show intrinsic resistance to nitrofurantoin, tigecycline and polymyxin
- They produce various β lactamases such as extended spectrum β lactamases (ESBL) and AmpC β-lactamases. As a result, they are resistant to most of the β-lactam drugs
- Drug of choice depends on the antimicrobial susceptibility testing. In general, aminoglycosides, fourth generation cephalosporins (cefepime), carbapenems and fosfomycin are effective in treatment
- In general, *P. mirabilis* is more susceptible to antibiotics than *P. vulgaris*.

TRIBE VII: YERSINIEAE

The tribe Yersinieae comprises of genus *Yersinia* which contains three well-established human pathogens.

- *Yersinia pestis:* It is responsible for rodent-borne zoonotic disease called **plague**
- *Yersinia pseudotuberculosis* and *Y. enterocolitica*—both cause **yersiniosis**, a self-limiting gastrointestinal illness that may occasionally have serious complications in special circumstances.

YERSINIA PESTIS

Yersinia pestis was isolated for the first time by Alexandre Yersin in 1894 in Hong Kong.

- It is the agent of plague, a fulminant systemic zoonosis, transmitted from rodents by arthropod vector (the rat flea)
- *Y. pestis* is gram-negative oval coccobacillus, often shows **bipolar staining** (ends of the bacilli stain darker than the central part) and pleomorphism (variable size and shapes) in older cultures. It is non-motile and capsulated.

Epidemiology of Plague

Plague is the one of the greatest killer known to mankind.

Plague Pandemics

There were three pandemics reported in the history, each was associated with a different biotype of *Y. pestis*, differentiated by glycerol fermentation and nitrate reduction (Table 29.7).

- **First pandemic (in AD541):** It occurred in the period of Roman Emperor Justinian
- **Second pandemic** (in 14th century) was called **black death**, which had killed up to one-third of the European population
- **Third pandemic (1894):** It started in Hong Kong. It mainly affected India and China causing more than 10 million deaths by 1918.

Timeline of Plague in India

- **1896 to 1918:** Hong Kong pandemic entered India and millions of people were killed

Table 29.7: Biotypes of *Y. pestis* and associated pandemics

Biotypes	Nitrate reduction	Glycerol fermentation	Pandemics associated
Medievalis	-	+	First pandemic Justinian, AD541
Antiqua	+	+	Second pandemic Black death, Europe (1347–52)
Orientalis	+	-	Third pandemic (1894–1918) Hong Kong, China, India

- **1918 to 1967:** Plague gradually declined, occasional cases continued to be reported from endemic foci
- **1967 to 1994:** No plague cases were reported
- **1994 (Surat epidemic):** It started as bubonic plague from Beed-Latur belt in Maharashtra. But, it soon became pneumonic plague and spread to Surat and adjoining regions of Gujarat. More than 6000 suspected plague cases with 60 deaths were reported over a period of two months (August-September 1994)
- **In 2002 (Shimla outbreak):** A short outbreak occurred at Rohru, near Shimla. Four deaths were reported
- **In 2004 (Uttarkashi outbreak):** Localized outbreak of bubonic plague (8 cases and 3 deaths) was reported from Dangud village of Uttarkashi district, Uttaranchal
- **Four potential endemic foci** are there in India at present which include—(1) region near Kolar, Karnataka (2) Beed-Latur belt in Maharashtra, (3) Rohru in Himachal Pradesh, and (4) Dangud village, Uttaranchal.

Current Situation in World

- Over five years (2004 to 2009), a total of 12,503 cases were reported (with 843 deaths) worldwide, mainly confined to 16 countries of Africa, Asia and America
- Africa accounts for highest number of cases (97%) worldwide.

Epidemiological Factors

- **Reservoir:** Wild rodents, such as gerbils (*Tatera indica*), field mice and the bandicoot found in forests are the main reservoirs of infection in India than the domestic rats *Rattus rattus* as once thought
- **Source of infection** are infected wild rodents, rat fleas and cases of pneumonic plague
- **Vector:** Rat flea is the commonest vector of *Y. pestis*, which acquires infection by feeding on infected wild rodents
  - Several species of rat flea may act as vectors such as *Xenopsylla cheopis* (the most efficient vector, found in North India) and *Xenopsylla astia* (less efficient, found in South India) and *Xenopsylla brasiliensis*
  - Human flea (*Pulex irritans*) may rarely serve as vector.
- **Plague cycles:** Plague exists in two natural cycles:
  1. Domestic cycle: It occurs between humans, rat fleas and rodents.
  2. Wild or sylvatic cycle: It occurs in nature among wild rodents, independent of human beings.
- **Mode of transmission:** Human plague is frequently contracted from:
  - Bite of an infected rat flea (most common)
  - Direct contact with tissues of infected animal (rodents)
  - Droplet inhalation (man to man) from cases of pneumonic plague
  - Bite of an infected human flea (*Pulex irritans*).

❖ **Blocked flea:** In a blood meal, the fleas suck about 0.5 mL blood containing 5000 bacilli from infected rodents
  - In the gut of the flea, the bacilli multiply enormously and may block the proventriculus. Such blocked flea eventually dies as it cannot obtain a blood meal
  - However, while making efforts to suck, it regurgitates the blood mixed bacteria into the bite, thus transmitting the infection
  - Infection may also be transmitted by contamination of the bite wound with the feces of infected fleas
  - A partially blocked flea is more dangerous than a completely blocked flea as it survives longer inside burrows, may be up to 4 years in certain species
  - **Extrinsic incubation period** is the interval between the flea acquiring infection through blood meal and becoming a blocked flea; which is usually about two weeks for *Xenopsylla cheopis*.

❖ **Cheopis index** (Average number of *X. cheopis* per rat) is the most significant flea index. Plague outbreak is likely to occur in places having cheopis index of more than 1

❖ **Seasonality:** Plague is seasonal in North India (September to May). However, in South India, it occurs throughout the year which may be attributed to the climatic conditions of South favoring the rodents to breed.

Virulence factors of *Y. pestis* (Table 29.8)

- **Fraction 1 (F1) antigen:** It is a plasmid (pFra) encoded heat labile capsular protein antigen. It is expressed at 37°C or inside human body. It inhibits macrophage phagocytosis. It is highly antigenic and is used as immunodiagnostic marker of infection
- **Phospholipase D/*Yersinia* murine toxin (Ymt):** It promotes *Y. pestis*' survival in the midgut of the infected flea
- **Surface proteases (*Pla* gene-encoded):** They activate mammalian plasminogen and also degrade complements. They adhere to the extracellular matrix component laminin, thus promoting *Y. pestis* to disseminate from the site of inoculation
- **pH 6 antigen:** It is a fibrillar surface protein, binds to host lipoproteins
- **Lipopolysaccharide:** It possesses endotoxin activity
- **Pigmentation:** Virulent strains produce brown colonies on hemin-containing media by storing hemin in the outer membrane. Pigments promote biofilm formation and is essential for flea blocking by *Y. pestis*
- **Low calcium response plasmid:** It codes for type III secretion system (which injects F1 proteins into the host cells) and adhesins (help in attachment)
- **Siderophore:** It helps in acquisition of iron and reduces production of reactive oxygen species by phagocytes, thereby decreasing bacterial killing.

Human Plague: Clinical Types

Human plague occurs in three clinical forms—(1) bubonic (most common form), (2) pneumonic and (3) septicemic.

Table 29.8: Virulence factors of *Yersiniae*

Yersiniae	Virulence antigens
Common to all *Yersinia*	Lipopolysaccharide Pigmentation Low calcium response plasmid Siderophore
Y. pestis-specific	Fraction 1 (F1) antigen Phospholipase D/murine toxin Surface proteases
Y. pestis and *Y. pseudotuberculosis*	pH 6 antigen
Y. enterocolitica and *Y. pseudotuberculosis*	Inv protein Ail protein *Yersinia* adhesin A (YadA)
Y. enterocolitica-specific	Myf antigen Heat stable toxin (ST toxin)
Y. pseudotuberculosis-specific	Super antigen mitogen

Bubonic Plague

It is the most common type, transmitted by the bite of an infected rat flea.

❖ Bacilli pass through the local lymphatics to reach the regional lymph nodes, where they multiply

❖ Incubation period is about 2–7 days

❖ The onset is sudden and is characterized by fever, malaise, headache and painful lymphadenitis

❖ **Buboes:** Regional lymph nodes appear as tense, tender swellings called buboes; the most common site being inguinal (Fig. 29.4A), but can also be crural, axillary, cervical, or submaxillary, depending on the site of the bite. Children are most likely to present with cervical or axillary buboes

❖ Bubonic plague cannot spread from person to person as the bacilli are locked up in buboes

❖ Without treatment, dissemination occurs leading to pneumonia (secondary) and meningitis.

Pneumonic Plague

Primary pneumonic plague results from inhalation of bacilli in droplets expelled from another person or an animal with plague pneumonia.

❖ Incubation period is short, about 1–3 days

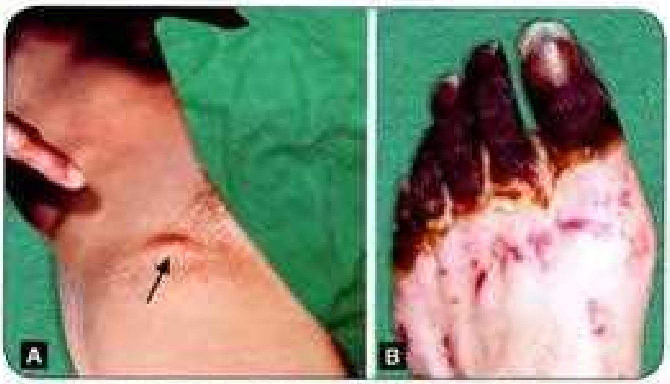
❖ The onset is sudden and is characterized by fever, headache and respiratory symptoms (productive cough or hemoptysis, dyspnea, and chest pain)

❖ Though pneumonic plague is rare (<1%), it is highly infectious and highly fatal

❖ Agent of bioterrorism—aerosolized *Y. pestis* is a possible source of bioterrorism attack, especially in non-endemic regions.

Septicemic Plague

❖ Primary septicemic plague is rare except for accidental laboratory infections.



Figs 29.4A and B: Plague manifestations. **A.** Swollen inguinal lymph node (bubo); **B.** Gangrene of the toes turned the dead digits black (black death)

Source: Public Health Image Library, A. ID#2044, B. ID#16550, Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

- Secondary septicemic plague is more common. It develops from spread of bubonic or pneumonic plague
- Incubation period is about 2–7 days
- Massive involvement of blood vessels results in hemorrhages in the skin and mucosa which may lead to gangrene of the affected site; hence disease was named in the past as **black death** (Fig. 29.4B).

Laboratory Diagnosis

Specimen Collection

Depending upon the type of plague, the specimens collected are:

- Bubonic plague—pus or fluid aspirated from buboes
- Pneumonic plague—sputum and blood
- Septicemic plague—blood and splenic aspirate (post mortem).

Transport medium (e.g. Cary-Blair medium) can be used if delay in transportation is expected.

Direct Microscopy

- **Gram staining:** Reveals presence of pus cells and gram-negative oval coccobacilli with rounded ends surrounded by capsule
- **Wayson stain** or methylene blue staining demonstrates the typical **bipolar** or **safety pin** appearance. Two ends are darkly stained with clear central area (Fig. 29.5).

Culture

Y. pestis is aerobic and facultatively anaerobic. The optimum temperature for growth (unlike most pathogens) is **27°C** but the capsule develops best at **37°C**. It is not fastidious and grows on ordinary media. Various media used are as follows:

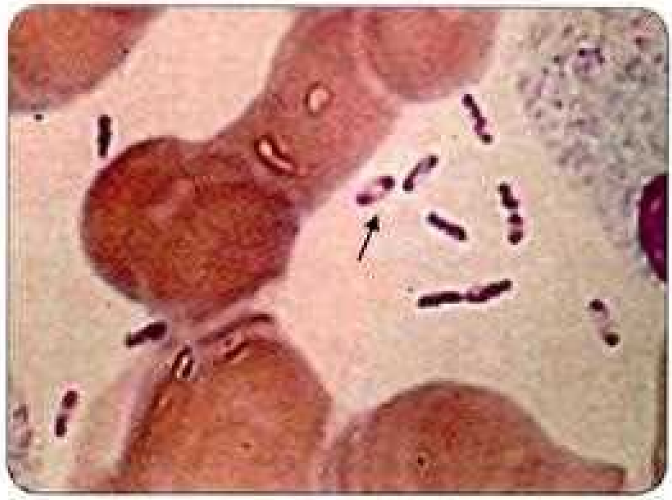


Fig. 29.5: Wayson staining demonstrates bipolar appearance of *Yersinia pestis* (arrow showing)

Source: Centers for Disease Control and Prevention (CDC) Atlanta, USA and Jawetz, Melnick, & Adelberg's Medical Microbiology; McGraw-Hill Education (with permission).

- Blood agar: Colonies are non-hemolytic and ***dark brown*** pigmented due to the absorption of the hemin pigment
- MacConkey agar: Lactose non-fermenting colorless colonies are formed
- Nutrient broth: Granular turbidity occurs with surface pellicles formed later
- Nutrient broth with **oil** or **ghee** floated on top-characteristic **stalactites-like growth** occurs hanging down from the under surface of the oil
- *Yersinia*-specific **CIN agar** (cefsulodin, irgasan, novobiocin): It is a selective medium, useful for culture of specimens containing normal flora, e.g. sputum.

Culture Smear and Motility Testing

- Gram staining of culture smear reveals **pleomorphism**-coccid, coccobacillary, bacillary, filamentous and giant forms. Involution forms are seen in older cultures
- *Y. pestis* is nonmotile both at 25°C and 37°C; in contrast to other *Yersinia* species which are motile at 25°C and nonmotile at 37°C.

Biochemical Reactions

Y. pestis shows the following reactions:

- **Sugar fermentation:** It ferments glucose, mannitol and maltose with the production of acid but no gas. Lactose and sucrose are not fermented
- It is catalase positive, oxidase negative
- Indole, urease and citrate tests are negative
- MR positive, but VP test is negative
- Biotyping is done based on glycerol fermentation and nitrate reduction (see Table 29.7).

F1 Antigen Detection

It may be detected from bubo aspirate or sputum by direct immunofluorescence test, ELISA or immunochromatographic test (ICT) by using monoclonal antibodies.

Antibodies to F1 Antigen Detection

Antibodies may be detected by ELISA, passive agglutination or CFT.
- Antibodies have a limited diagnostic value as they appear late. Only a retrospective diagnosis can be made if fourfold rise of titer is noted
- However, antibodies are useful epidemiological markers, as they remain positive for several years.

Molecular Methods

PCR is available targeting gene coding F1 antigen, *pesticin* gene, and the *plasminogen activator* gene.

Animal Inoculation

It is carried out by using Guinea pigs or white rats.

TREATMENT ***Yersinia pestis***

Early start of antibiotics is crucial for reducing mortality.
- Streptomycin has been the choice of treatment for plague in the past, given for 10 days
- Gentamicin is superior to streptomycin and currently recommended for treatment
- Levofloxacin has recently been approved for treatment and post-exposure prophylaxis
- Alternative drugs, such as doxycycline and chloramphenicol are also effective
- β-lactams and macrolides are generally not recommended as the response is poor.

Prevention of Plague

Prevention and control of plague involves:
- Control of cases by early diagnosis, isolation and treatment of cases
- Control of fleas by use of effective insecticides, such as DDT or BHC (β-hexachloro-cyclohexane)
- Control of rodents
- **Chemoprophylaxis** should be given to all contacts of pneumonic plague. Doxycycline (100 mg twice a day) or tetracycline (500 mg 6 hourly) is the drug of choice, given for 7 days
- **Vaccine:** WHO recommends using vaccine only for prevention of an anticipated outbreak and not for general use
  - **Formalin killed vaccine** (Sokhey's modification of original Haffkine vaccine): It is prepared in Haffkine institute, Mumbai
    - It is given subcutaneously, two doses 4 weeks apart and a booster given after 6 months. It is contraindicated in infants <6 months
    - Protection is short-lasting (<6 months)
    - It is not protective against pneumonic plague and has considerable side effects.
  - **Live attenuated vaccine** based on strain EV76 is still used in countries of the former Soviet Union but has significant side effects
  - **Subunit recombinant F1** (rF1) vaccine is under trial.

Plague in Rodents

Plague is primarily a disease of rodents in which man becomes accidently involved. The plague bacillus is naturally parasitic in rodents and the disease spreads among rodents by rat fleas. When a diseased rat dies (rat fall), the fleas leave the carcass and, in the absence of another rat, may bite human beings, causing bubonic plague. Plague in rodents is similar to that in man. The disease is mild or inapparent in resistant species.

YERSINIOSIS

Yersiniosis is a zoonotic infection caused by enteropathogenic *Yersinia* species, i.e. *Y. enterocolitica* or *Y. pseudotuberculosis*.
- Pigs and other wild and domestic animals are the usual hosts
- Human infection occurs due to consumption of contaminated food such as raw pork, milk, etc.
- Yersiniosis is most common in childhood and in colder climates
- Patients present with abdominal pain and sometimes with diarrhea.

Geographical Distribution

- *Y. enterocolitica* is found worldwide, most commonly in Northern Europe and America
- Outbreaks of *Y. pseudotuberculosis* are generally rare, have been reported from Finland.

Serogrouping

- *Y. enterocolitica* is further characterized biochemically (six biotypes) and antigenically (60 serotypes, based on somatic O antigen). Worldwide, most clinical infections are associated with serogroups O:3 and O:9
- *Y. pseudotuberculosis* can be further differentiated into six serotypes (1 to 6) based on somatic O and flagellar H antigens.

Virulence Factors

There are several groups of virulence factors such as:
- **Virulence factors common to both the species**:
  - Invasin (Inv) protein: It binds to β-1 integrins on M cells of GI mucosa which helps in invasion

- Ail protein (attachment and invasion locus): It helps in attachment, invasion and inactivates complements
- *Yersinia* adhesin A (Yad A)—(1) it binds to extracellular matrix proteins, such as collagen and fibronectin and helps in invasion, (2) inactivates complements.

❖ ***Y. enterocolitica* specific virulence factors:**
- Myf antigen is a fimbrial antigen, helps in adhesion
- Heat-stable toxin: Similar to that of *E. coli*, produced only at temperature <30°C
- pH6 antigen: Fimbrial surface protein, helps in adhesion. It is also expressed by *Y. pestis*.

❖ ***Y. pseudotuberculosis* specific virulence factors:**
- Super antigen—binds to T cells non-specifically leading to massive cytokine release.

❖ **Virulence factors common to all yersiniae:** Such as LPS, pigments, siderophores and low calcium response plasmid (described earlier in Table 29.8).

Clinical Manifestations

Overall, *Y. enterocolitica* is more frequently reported clinically than *Y. pseudotuberculosis*.

❖ **Self-limited gastroenteritis** (diarrhea with or without blood) occurs in younger children

❖ **Intestinal complications** occur in older children, characterized by terminal ileitis (mostly in *Y. enterocolitica*) and mesenteric adenitis. Patients present with acute pain abdomen, may mimic ***pseudoappendicitis***

❖ **Septicemia:** It is seen typically in adults, characterized with fever and leukocytosis. It usually occurs in patients with coexisting diabetes mellitus, liver disease and iron overload

❖ **Post-infective phenomena** (in adults) occurs commonly with *Y. enterocolitica*. It occurs as a result of autoimmune activity, initiated by the deposition of bacterial non-viable components in joints and other sites. Manifestations include:
- *Reactive arthritis*—mostly associated in persons positive for HLA-B 27
- *Erythema nodosum:* It occurs independently without any link to HLA-B 27 phenotype
- *Graves' disease*—*Y. enterocolitica* contains an antigen similar to thyroid-stimulating hormone (TSH) binding site. However, whether this cross-reactivity has any significant role in Graves' disease remains unclear.

❖ **Super antigen:** Some strains of *Y. pseudotuberculosis* express a super antigen **mitogen**, which has caused scarlet-like fever in Russia, similar illness in Japan (**Izumi-fever**) and has been linked to the pathogenesis of idiopathic acute systemic vasculitis of childhood called **Kawasaki's disease**.

Laboratory Diagnosis

Culture Isolation

❖ **For isolation from blood:** Blood culture bottles (BHI broth) should be used

❖ **For isolation from lymph nodes aspirate:** Culture is done on conventional media (blood agar, nutrient agar and MacConkey agar)
- **Blood agar:** They produce granular translucent colonies with a beaten copper surface, non-hemolytic colonies
- **MacConkey agar:** Growth of *Y. pseudotuberculosis* is poor. *Y. enterocolitica* grows well and produces lactose non-fermenting pale colonies.

❖ **For isolation from feces, food or soil:** Selective media should be used, such as:
- Deoxycholate citrate agar
- MacConkey agar
- **Yersinia CIN agar** (Cefsulodin-irgasan-novobiocin): Typical **dark red bull's eye** appearing colonies are formed in 24 hours

❖ **Incubation:** Plates should be incubated at 25°C and 37°C to differentiate from most of the other pathogens which grow only at 37°C

❖ **Cold enrichment** can also be done by incubating in phosphate-buffered saline at 4°C for 3 weeks.

Biochemical Tests

Y. enterocolitica and *Y. pseudotuberculosis* show the following properties by which they can be differentiated from *Y. pestis*:

❖ **Differential motility:** They are motile at 22°C (but not at 37°C)

❖ **Cold enrichment:** Growth improves on refrigeration (4°C)

❖ Urease positive

Tests to differentiate *Y. enterocolitica* from *Y. pseudotuberculosis* include:

❖ Sugar fermentation
- Sucrose, cellobiose and sorbitol are fermented only by *Y. enterocolitica*
- Rhamnose, salicin and melibiose are fermented only by *Y. pseudotuberculosis*

❖ Ornithine decarboxylase-positive only for *Y. enterocolitica*

❖ VP test is positive only for *Y. enterocolitica*.

Serology

Antibodies can be detected by agglutination or ELISA using serotype specific O-antigen types. In *Y. pseudotuberculosis* infection, antibodies appear early during acute phase of illness; whereas *Y. enterocolitica* specific agglutinating antibodies are more likely to be found in convalescent sera.

TREATMENT **Yersiniosis**

Most cases of diarrhea are self-limiting. Treatment is required only for systemic infections such as in case of septicemia.

- Fluoroquinolone (ciprofloxacin) or third-generation cephalosporins (cefotaxime) are effective
- *Y. enterocolitica* strains nearly always produce β-lactamases but not *Y. pseudotuberculosis* strains.

TRIBE VIII: ERWINIEAE

Tribe Erwinieae comprises of genus *Erwinia*. The *Erwinia* species are primarily plant pathogens and are also saprophytes in soil. *E. persicinus* had occasionally been isolated from urinary infections in hospitalized patients.

NEWLY INCLUDED GENERA

Based on DNA hybridization studies, several new genera are recently included in the family Enterobacteriaceae such as:

- ***Klebsiella granulomatis*** (previously, *Calymmatobacterium granulomatis*): It is the agent of sexually transmitted disease called **granuloma inguinale** or **donovanosis**. It is described in detail in Chapter 36.
- ***Plesiomonas:*** It is an oxidase positive, motile fermenting gram-negative bacillus, previously classified under Vibrionaceae family
  - *P. Shigelloides* is the only species. It is so named because it is antigenically related to *S. sonnei*
  - However, it differs from *Shigella* in being positive for oxidase, utilizes lysine, arginine and ornithine and is motile with polar lophotrichous flagella
  - It is the only member of Enterobacteriaceae which is oxidase positive
  - It is found as saprophyte in water and soil, and also as a commensal in animal and rarely in human intestine
  - Human infection: It rarely causes gastroenteritis which may be severe in immunocompromised patients. Extraintestinal manifestations include rare cases of meningitis, septicemia, cellulitis and septic arthritis.
- **Other new genera include:** *Ewingella, Buttiauxella, Budvicia, Cedecea, Kluyvera, Rahnella* and *Tatumella.*

EXPECTED QUESTIONS

I. Essays:

1. List the diarrheagenic *Escherichia coli*. Discuss the pathogenesis and laboratory diagnosis of diarrheagenic *E. coli*.
2. A 24-year-old female was admitted with fever, dysuria and frequency of micturition for the past 3 days. Urine microscopy revealed pyuria.
   a. What is the clinical diagnosis of this condition?
   b. Which is the most common etiological agent for this condition?
   c. What are the various methods to collect the specimen?
   d. Describe the laboratory diagnosis of this condition in detail.

II. Write short notes on:

1. *Klebsiella pneumoniae*.
2. Pathogenesis of shigellosis.
3. Virulence factors of *Yersinia pestis*.
4. Laboratory diagnosis of plague.

III. Multiple Choice Questions (MCQs):

1. **Traveler's diarrhea is caused by:**
   a. ETEC b. EHEC
   c. EPEC d. EIEC
2. **Culture media used for EHEC O157: H7 is:**
   a. O7 culture b. Sorbitol MacConkey media
   c. XLD agar d. Deoxycholate media
3. **Most common cause of community-acquired urinary tract infection is:**
   a. *E. coli* b. *Proteus*
   c. *Pseudomonas* d. *Klebsiella*
4. **Phenyl alanine deaminase test is characteristic of which of the following tribes of Enterobacteriaceae:**
   a. Escherichieae b. Salmonelleae
   c. Yersinieae d. Proteeae
5. **Which of the following *Shigella* species is mannitol nonfermenter?**
   a. *S. sonnei* b. *S. boydii*
   c. *S. dysenteriae* d. *S. flexneri*
6. **Plague is transmitted by:**
   a. Rat flea b. Soft tick
   c. Hard tick d. Louse
7. **Bipolar staining is characteristic of:**
   a. *Yersinia pestis* b. *Shigella*
   c. *Klebsiella* d. *Proteus*
8. **Most common cause of Scombroid poisoning is:**
   a. *Escherichia coli* b. *Klebsiella*
   c. *Morganella* d. *Pseudomonas*

Answers

1. a **2.** b **3.** a **4.** d **5.** c **6.** a **7.** a **8.** c

Enterobacteriaceae II: Salmonella

30
CHAPTER

Chapter Preview

- Classification and nomenclature
- Typhoidal salmonellae
- Non-typhoidal salmonellae

Diseases caused by various members of the genus *Salmonella* are extremely important public health problems worldwide. The credit of discovery of '*Salmonella*' goes to Salmon and Smith (1885).

The most important member of the genus is *Salmonella* Typhi, the causative agent of typhoid fever. It was first observed by Eberth (1880) and Gaffky (1884) and hence was formerly called Eberth-Gaffky bacillus or *Eberthella* Typhi.

CLASSIFICATION AND NOMENCLATURE

Salmonella is antigenically complex. The classification and nomenclature of salmonellae have undergone several modifications over the past years. There are several classifications proposed so far.

Clinical Classification

It is the oldest, user friendly classification which is still widely used. It divides salmonellae into two groups:

1. **Typhoidal *Salmonella*:** It includes serotypes *S.* Typhi and *S.* Paratyphi. They are restricted to human hosts, in whom they cause enteric fever (typhoid/paratyphoid fever).
2. **Non-typhoidal *Salmonella* or NTS:** The remaining serotypes can colonize the intestine of a broad range of animals, including mammals, reptiles, birds and insects. They also infect humans causing food-borne gastroenteritis and septicemia.

Antigenic Classification (Kauffmann–White Scheme)

The classification within the genus is based on the presence of different somatic (O) and flagellar (H) antigens which can be detected by agglutination with the respective antisera (Table 30.1).

- **Serogroups:** Based on O antigen, salmonellae are initially classified into serogroups
  - Earlier, serogroups were named as letters, e.g. A, B, C and so on
  - However, as the numbers increased, the serogroups are re-designated as numbers, e.g. 1, 2, 3 and so on
  - Currently, there are up to 67 serogroups, each containing group-specific O antigen, for example:

Table 30.1: Kauffmann–White antigenic classification for *Salmonella*

Serogroup		Serotype name	O Ag*	Vi Ag	H Ag*	
New	Old				Phase 1	Phase 2
2	A	*S.* Paratyphi A	1, 2, 12	–	a	[1,5]
4	B	*S.* Paratyphi B	1, 4, [5], 12	–	b	1,2
		S. Typhimurium	1, 4, [5], 12	–	i	1,2
		S. Agona	1, 4, 12	–	f,g,s	–
		S. Heidelberg	1, 4, [5], 12	–	r	1,2
7	C1	*S.* Paratyphi C	6, 7	+	c	1,5
		S. Choleraesuis	6, 7	–	c	1,5
		S. Thompson	6, 7, 14	–	k	1,5
8	C2-C3	*S.* Muenchen	6, 8	–	d	1,2
		S. Newport	6, 8, 20	–	e,h	1,2
9	D1	*S.* Typhi	9, 12	+	d	–
		S. Enteritidis	1, 9, 12	–	g,m	[1,7]
		S. Gallinarum	1, 9, 12	–	–	–
		S. Dublin	1, 9, 12	+	g,p	–
3,10	E1	*S.* Anatum	3, 10, [15], [34]	–	e,h	1,6

Abbreviation: Ag, antigen.

Note: *Antigen in brackets are not always present. Only some representative serotypes are given in the table.

- Serogroup-2 (formerly, serogroup A)—contains group specific O antigen type 2
- Serogroup-4 (formerly, serogroup B)—contains group-specific O antigen type 4
- Serogroup-9 (formerly, serogroup D)—contains group specific O antigen type 9.

❖ **Serotypes:** Each serogroup is further differentiated into serotypes, based on the type of flagellar antigens present. Currently, there are more than 2,500 serotypes of salmonellae.

Molecular Classification

Based on DNA hybridization studies, the genus *Salmonella* consists of two species—(1) *Salmonella enterica* and (2) *S. bongori*.

❖ Within the species *S. enterica,* there are six subspecies differentiated by biochemical variations; namely *enterica, salamae, arizonae, diarizonae, houtenae* and *indica*

❖ Each subspecies is further differentiated into serotypes (based on O and H antigens as described in the Kauffmann-White scheme) (Table 30.1)

❖ Most of the pathogenic typhoidal and non-typhoidal *Salmonella* serotypes are placed under species *enterica* and subspecies *enterica*.

Nomenclature

Taxonomically, the correct nomenclature of the members of salmonellae is very much complicated, e.g. ***Salmonella* species *enterica* subspecies *enterica* serotype Typhi**.

❖ However, for routine use a simplified format is followed where only the genus and serotype names are included, for example *Salmonella* serotype Typhi or in short, *S.* Typhi.

ANTIGENIC STRUCTURE

Salmonellae possess three important antigens on their cell wall, based on which they are classified. The antigens are:

1. Somatic antigen (O)
2. Flagellar antigen (H)
3. Surface envelope antigen (Vi)—found in some species.

Note: Fimbrial antigens may be present in some strains. They are nonspecific, widespread among other members of Enterobacteriaceae and may cause confusion in identification.

The O and H antigens are described in Table 30.2.

Table 30.2: Difference between somatic (O) and flagellar (H) antigen

O antigen	H antigen
Somatic antigen It is a part of cell wall lipopolysaccharide (LPS)	**Flagellar antigen** • Made up of proteins flagellin • It confers motility to the bacteria
Heat stable, alcohol stable	Heat labile, alcohol labile
Formaldehyde labile	Formaldehyde stable
In Widal test, O antigen of *S.* Typhi is used	In Widal test, H antigens of *S.* Typhi, *S.* Paratyphi A and B are used
O Ag is less immunogenic	H Ag is more immunogenic
O antibody appears early, disappears early: indicates recent infection	H antibody appears late, disappears late: indicates convalescent stage
When O antigen reacts with O antibody forms compact, granular, chalky clumps • Agglutination takes place slowly • Optimum temperature for agglutination is 55°C	When H antigen reacts with H antibody forms large, loose, fluffy clumps • Agglutination takes place rapidly • Optimum temperature for agglutination is 37°C
Serogrouping of salmonellae is based on the O antigen	Serogroups are differentiated into serotypes based on H antigen
O antigen is also called the Boivin antigen because it can be extracted from the bacterial cell by treatment with trichloracetic acid—this property was first shown by Boivin	• Flagellar antigens exist in two alternative phases—Phase I and II • Most of them are biphasic except *S.* Typhi which is monophasic

Vi Antigen

Vi antigen is a surface polysaccharide envelope or capsular antigen covering the O antigen. The naming is due to the belief that Vi antigen is related to virulence.

❖ It is expressed in only few serotypes, such as *S.* Typhi, *S.* Paratyphi C, *S.* Dublin and some stains of *Citrobacter freundii* (the Ballerup-Bethesda group)

❖ When Vi antigen is present, it renders the bacilli inagglutinable with the O antiserum. However, the strain becomes agglutinable after boiling or heating at 100°C for 1 hour, which removes Vi antigen and exposes the O antigen. Vi antigen is also destroyed by 1 N HCl and 0.5 N NaOH, but not by alcohol or 0.2% formaldehyde

❖ As Vi antigen is **poorly immunogenic** and antibody titers are low, it is not helpful in the diagnosis of cases. Hence, the Vi antigen is not employed in the Widal test

❖ However, it is believed that the complete absence of the Vi antibody in a proven case of typhoid fever indicates poor prognosis

❖ The Vi antibody usually disappears early in convalescence, but if persists, indicates the development of the carrier state

❖ Phage typing of *S.* Typhi can be done by using Vi specific bacteriophages

❖ Vi antigens can also be used for vaccination.

Antigenic Variations

The antigens of salmonellae can undergo several types of phenotypic and genotypic variations.

Variation in O Antigen

- **S-R variation** (smooth to rough) is due to loss of the O antigen side chain from LPS, leading to exposure of core polysaccharide portion (or R antigen) of LPS
  - Smooth colonies are produced by virulent strains carrying the O antigen
  - Rough strains form large, rough, and irregular colonies and are avirulent due to loss of O antigen. Colonies are autoagglutinable in saline suspensions, and lack O serotype specificity, hence are not suitable for antisera testing.
- **Lysogenic conversion:** Infection with a bacteriophage to *Salmonella* may cause loss or gain or change of an O antigen. *S.* Anatum is converted into *S.* Newington by infection with one phage (gaining O15 antigen) and the latter into *S.* Minneapolis by another phage infection (gaining O34 antigen).

Variation in H Antigen

- **OH-O variation:** It is associated with the loss of flagella which can be induced by:
  - **Phenol agar:** Growing the cultures on agar containing phenol (1:800) causes inhibition of flagella temporarily which can be regained by sub-culturing on the media without phenol
  - **By mutation:** This is seen in non-motile mutant of *S.* Typhi 901-O strain, employed in the Widal test. Here also, the loss of flagella is not total. The flagellated cells which are found in small numbers in such cultures can be revived by subculturing the culture in ***Craigie's tube*** (explained in Figure 30.1). Alternatively, a U-tube containing soft agar may be used.
- **Phase variation:** The flagellar antigens exist in two phases. Each phase comprises of a distinct set of flagellar antigens
  - Phase 1 antigens are serotype specific and designated as a, b, c, etc. Phase 2 antigens are nonspecific or group antigens. They are few in number and are designated as 1, 2, etc.
  - **Serotypes can be classified as:**
    - Diphasic: Most salmonellae possess antigens of both phases
    - Monophasic: Some serotypes possess only phase 1 antigens, e.g. *S.* Typhi, *S.* Agona, *S.* Dublin, and *S.* Senftenberg
    - Aphasic: *S.* Gallinarum does not have any flagellar antigens. Hence, it is non-motile.
  - For serotyping, it is essential to identify the antigens of both the phases
  - Though flagellar genes for antigens of both phases are present in cultures of diphasic strains, generally only one phase antigen is expressed and gets agglutinated by its phase antisera

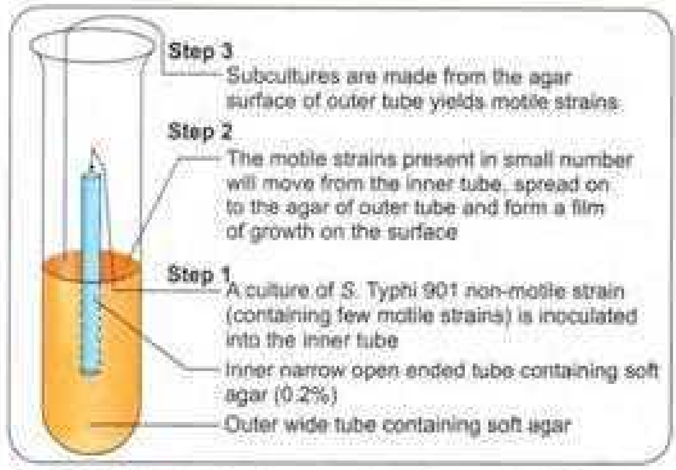


Fig. 30.1: Craigie's tube demonstrating the reviving of the motile strains from a mixture of motile/non-motile strains of *S.* Typhi 901

  - Hence phase conversion has to be done to express the other phase antigens
  - Phase conversion—a culture in phase 1 can be converted to phase 2 by passing it through a Craigie's tube containing specific phase 1 antiserum.

TYPHOIDAL SALMONELLA

Typhoidal salmonellae include *S.* Typhi and *S.* Paratyphi A, B and C which cause enteric fever. Pathogenesis is described below.

Pathogenesis

Salmonellae are transmitted by oral route, through contaminated food or water.

- **Infective dose of** *Salmonella* is higher than that of *Shigella*. Minimum 10^3–10^6 bacilli are needed to initiate the infection
- **Risk factors** that promote transmission include the conditions that decrease:
  - Stomach acidity (<1 year age, antacid ingestion, or achlorhydria or prior *Helicobacter pylori* infection)
  - Intestinal integrity (inflammatory bowel disease, prior GIT surgery or suppression of the intestinal flora by antibiotics)
- **Entry through epithelial cells (M cells)** lining the intestinal mucosa—Salmonellae can trigger the formation of membrane ruffles on the cell membrane of M cells. These ruffles reach out and enclose the adherent bacteria within the large vesicles. This process of uptake is called **bacteria-mediated endocytosis (BME)**; which is mediated by a specialized type III secretion system. Following entry, the bacilli remain inside the vacuoles in the cytoplasm
- **Entry into macrophages:** Salmonellae containing vacuoles cross the epithelial layer to reach submucosa, where they are phagocytosed by the macrophages
- **Survival inside the macrophages:** *S.* Typhi induces certain alterations on its surface so that the bacilli are no longer susceptible to the lysosomal enzymes of macrophages

Contd...

Contd...

This is mediated by organism's regulatory systems such as **PhoP/PhoQ** system which triggers the expression of outer-membrane proteins and mediates modifications in LPS

- **Primary bacteremia:** Salmonellae contained inside the macrophages spread via the lymphatics to enter the blood stream (transient primary bacteremia)
- **Spread:** Then, the bacilli disseminate throughout the reticuloendothelial tissues (liver, spleen, lymph nodes and bone marrow) and other organs, such as gallbladder, kidneys and lungs where further multiplication takes place
- **Secondary bacteremia** occurs from the seeded organs, which leads to the onset of clinical disease.

Clinical Manifestations of Enteric Fever

Incubation period is about 10-14 days. Enteric fever is a misnomer as the manifestations are more extraintestinal than intestinal. Various manifestations are as follows.

- **Fever (step ladder pattern of remittent fever):** Fever rises gradually to a higher level with every spike; then falls down, but does not touch normal
- **Other symptoms:** Headache, chills, cough, sweating, myalgia and arthralgia
- **Rashes** (called **rose spots):** Faint, salmon-colored, blanching, maculopapular rash on the trunk and chest seen in 30% of patients at the end of the first week
- **Early intestinal manifestations** such as abdominal pain, nausea, vomiting and anorexia
- **Important signs** include hepatosplenomegaly, epistaxis and relative bradycardia
- **Complications:** Gastrointestinal bleeding and intestinal perforation can occur mostly in the third and fourth weeks of illness
- **Neurologic manifestations** occur rarely which include meningitis, cerebellar ataxia and neuropsychiatric symptoms (described as "muttering delirium" or "coma vigil") such as paranoid psychosis, hysteria, delirium and aggressive behavior.

Epidemiology

- **Host:** Humans are the only natural hosts for typhoidal salmonellae
- **Mode of transmission:** It is by ingestion of contaminated water and food. Rarely homosexual and laboratory acquired transmissions have been reported
- **Prevalence:** Worldwide, an estimated 27 million cases of enteric fever with 2-6 lakh deaths occur annually
- **Incidence** is:
  - Highest (>100 cases per 100,000 population per year) in south central and southeast Asia
  - Medium (10-100 cases per 100,000) in the rest of Asia, Africa, Latin America
  - Low (<10 cases per 100,000) in other parts of the world.
- **Locality and age:** Enteric fever is:
  - More common in urban than rural areas
  - More common among young children and adolescents than in adults.
- **Factors** that favor transmission include:
  - Poor sanitation and improper cleaning of drinking water
  - Contaminated water, food and drinks
  - Lack of hand washing and toilet access, and evidence of prior *Helicobacter pylori* infection.
- **Typhi vs Paratyphi:** *S.* Typhi infection is more common than *S.* Paratyphi A (ratio is 4:1). However, *S.* Paratyphi A appears to be increasing, especially in India; may be due to increased vaccination for *S.* Typhi
- **Carriage:** Up to 10% of untreated patients become carriers and excrete *S.* Typhi in feces or urine.
  - **Carriers are of two types:**
    1. Fecal carriers: Typhoid bacilli multiply in the gall bladder and are excreted in feces. Fecal carriers are more common
    2. Urinary carriers: Multiplication takes place in kidneys and bacilli are excreted in urine. Urinary carries are rare.
  - **Duration of shedding:** Carriers continue to shed the bacilli in feces and urine for:
    - Convalescent carriers 3 weeks to 3 months (after clinical cure)
    - Temporary carriers 3 months to 1 year
    - Chronic carriers for more than 1 year.
  - **Chronic carriers** occur in about 1-4% of infected people. Chronic carriage is more common in:
    - Women, infants and old age
    - Biliary tract abnormalities which leads to increased fecal excretion
    - Abnormalities of urinary tract and associated *Schistosoma haematobium* infection of bladder—leads to increased urinary excretion.
  - **Food handlers or cooks** who become chronic carriers are particularly dangerous, can excrete the bacilli for many years. The best known example of such typhoid carrier was Mary Mallon **('Typhoid Mary'),** a New York cook who gave rise to more than 1300 cases during her lifetime causing several outbreaks.
- **Reference centers** for *Salmonella* in India:
  - National *Salmonella* Phage Typing Center—at Lady Hardinge medical college, New Delhi
  - National *Salmonella* Reference Centre—at Central Research Institute, Kasauli
  - National *Salmonella* Reference Centre for animal origin—at Izatnagar.

LABORATORY DIAGNOSIS — Enteric fever

- **Culture isolation**
  - Blood and bone marrow culture
    - Conventional: BHI broth/agar
    - Automated blood culture systems such as BACTEC or BacT/ALERT).
  - Stool culture (in 3–4 weeks of illness):
    - Enrichment broth such as Selenite F broth, tetrathionate broth and gram-negative broth
    - Low selective medium: MacConkey agar (translucent NLF colonies)
    - Highly selective media: DCA, XLD agar, SS agar and Wilson Blair's Bismuth sulphite medium (jet black colonies).
  - Urine culture (in 3–4 weeks of illness)—done on MacConkey agar.
- **Culture smear and motility:** Motile, gram-negative bacilli
- **Biochemical identification**
  - Catalase positive and oxidase negative
  - Nitrate is reduced to nitrite
  - ICUT: Indole(–), Citrate(+/–), Urease(–)
    TSI:K/A, gas(+) except in *S.* Typhi, H_2S (*S.* Typhi- small speck, *S.* Paratyphi A-absent, *S.* Paratyphi B-abundant).
- **Slide agglutination test:** To confirm the serotype
- **Serum antibody detection (Widal test):** 2–3 weeks of illness Antibodies are detected against TO, TH, AH, BH antigens
  - In *S.* Typhi infection: ↑TO and TH antibodies
  - In *S.* Paratyphi A infection: ↑TO and AH antibodies
  - In *S.* Paratyphi B infection: ↑TO and BH antibodies.

  Result and interpretation
  - O antibodies: Appear early, disappear early, and produce granular chalky clumps when react with O Ag
  - H antibodies: Appear late, disappear late and produce cottony woolly clumps when react with H Ag.
- **Antigen detection (serum and urine):** By ELISA, CIEP
- **Molecular methods:** PCR detecting *flagellin* gene, *iro B* and *fliC* gene
- **Nonspecific findings**—For example, neutropenia
- **Antimicrobial susceptibility testing.**

Laboratory Diagnosis

Type of specimen to be collected depends on the duration of illness (Table 30.3). Blood, stool and urine are collected for culture and serum for serology.

Culture and Identification

Blood Culture

- **Blood culture** is the ideal method for diagnosis in the first week of fever, which becomes positive in about 90% of cases. There after the positivity declines to 75% in the second week and 60% in the third week and 25% till the fever subsides
- **Clot culture:** Blood is centrifuged, and then the serum is separated and used for Widal test and the clot is used for culture. Clot culture has shown a higher isolation rate than blood culture
- **Culture medium:** Both conventional and automated blood culture bottles are available
  - Conventional: (i) Monophasic medium containing 50–100 mL of brain heart infusion (BHI) broth (Fig. 30.2A; (ii) Castaneda's biphasic medium consisting of BHI agar slope and 50–100 mL of BHI broth (Fig. 30.2B)
  - Automated blood culture systems such as BACTEC or BacT/ALERT (Fig. 30.2C).
- **Procedure:** 10–20 mL of fresh blood is directly injected into the blood culture bottle, containing 50–100 mL of broth in 1:5 dilution. This is needed to overcome the effect of inhibitory substances present in the blood.

Table 30.3: Various tests used for diagnosis of enteric fever depending upon duration of illness

Duration of illness	Specimen used and test(s) done
First week	Culture of: • Blood • Bone marrow aspirate • Duodenal aspirate
Second week and Third week	Serum: • For antibody detection by Widal test • For antigen detection Stool and urine culture
Fourth week	Stool and urine culture
Carriers	Stool and urine culture Serum for detection of antibodies to Vi antigen Sewage culture—indirect way

Figs 30.2A to C: Blood culture bottles. **A.** Monophasic medium (BHI broth); **B.** Biphasic medium (Castaneda's), containing BHI broth and BHI agar; **C.** BacT/ALERT bottle

Source: A to C. Department of Microbiology, JIPMER, Puducherry (*with permission*).

- **Sodium polyanethol sulfonate (SPS):** It is added to the medium as anticoagulant. It also counteracts the bactericidal action of blood
- **Incubation:** Blood culture bottles are incubated at 37°C. Salmonellae are nonfastidious, growth occurs within 24 hours. However, for isolation of other possible fastidious organisms in blood, e.g. for *Brucella*, incubation is carried out up to 1 week.
- **Repeat subcultures:**
  - From monophasic BHI medium: Repeat subcultures are made onto blood agar and MacConkey agar periodically for 1 week. There is a risk of contamination due to opening of the cap of the bottle every time when subcultures are made
  - Biphasic BHI medium is preferred over monophasic, as the subcultures can be made just by tilting the bottles so that the broth runs over the agar slope. Bottle is incubated in upright position. The colonies grown over the agar slant are used for further identification
  - Automated blood culture systems are much superior than conventional as they monitor the growth continuously once in every 15–20 minutes. Following growth, the bottles become flagged positive automatically by the system and then the subcultures can be done.
- **Colony appearance:**
  - Blood agar: Nonhemolytic moist colonies
  - MacConkey agar: Colonies are round (1–3 mm size), translucent, pale and non-lactose fermenting.

Stool and Urine Culture

It is useful for isolation of *Salmonella* in the third and fourth weeks of illness. They remain positive even after antibiotic treatment. Stool and urine culture are also done for detection of carriers.

Urine culture seldom becomes positive as salmonellae are shed in urine infrequently. Urine is centrifuged and the deposit is inoculated onto MacConkey agar.

Stool culture is done similar to the method followed for *Shigella* (Described in Chapter 29). Appropriate media should be used to inhibit the commensals in the stool.

- **Enrichment broth** such as Selenite F broth, tetrathionate broth and gram-negative broth are used
- **Selective media** such as:
  - **Low selective media** such as MacConkey agar
  - **Highly selective media:** Growth of *S.* Typhi occurs as follows (Figs 30.3A and B):
    - **DCA** (deoxycholate citrate agar): Produces non-lactose fermenting pale colonies with black center
    - **XLD agar** (xylose lysine deoxycholate): Produces red colonies with black center
    - **SS agar** (*Shigella Salmonella* agar): Colonies are colorless with black centers
    - **Hektoen enteric agar:** Colonies are typically blue-green with black centers
    - **Wilson Blair's** brilliant green Bismuth sulfite medium is particularly useful for the isolation of *S.* Typhi from heavily contaminated specimens. *S.* Typhi produces characteristic **jet black** colored colonies with a **metallic sheen** due to production of H_2S. *S.* Paratyphi A and others that do not form H_2S produce green colored colonies.

Other Specimens

- **Bone marrow** culture is employed during the first week of illness (55–90% sensitive) when blood culture is negative, especially when patient is on antibiotics
- **Duodenal aspirate** culture is recommended during first week of illness if both blood and bone marrow cultures turn negative
- **Combination** of blood, bone marrow, and intestinal secretions culture is the best method in the first week, which shows a sensitivity of more than 90%
- **Other specimens** from which salmonellae can be isolated are rose spots, pus from suppurative lesions, cerebrospinal fluid (CSF), sputum and autopsy specimens such as gallbladder, liver and spleen.

Culture Smear and Motility Testing

Gram-stain of colonies reveals gram-negative (2–4 × 0.6 μm size), non-sporing and non-capsulated bacilli. They are motile with peritrichous flagella except for *S.* Gallinarum and *S.* Pullorum. However, non-motile variants (OH → O) may be occasionally found within the serotypes.

Figs 30.3A and B: Colonies of *S.* Typhi **A.** DCA (Deoxycholate citrate agar) showing pale colonies with black center and **B.** XLD agar (Xylose lysine deoxycholate) showing red colonies with black center
Source: Department of Microbiology, JIPMER, Puducherry (with permission).

Biochemical Identification

Serotypes of salmonellae show the following biochemical properties (see below and refer Table 29.3 of Chapter 29).

Biochemical Identification of Salmonellae
- Catalase positive and oxidase negative

ICUT test:
- Indole test—negative
- Citrate test—positive (except for *S.* Typhi and *S.* Paratyphi A, which are citrate negative)
- Urease test—negative
- TSI (triple sugar iron test) shows:
  - Alkaline/acid
  - Gas present (except for *S.* Typhi, which is anaerogenic),
  - Abundant H_2S present except for:
    - *S.* Paratyphi A and *S.* Choleraesuis: H_2S not produced
    - *S.* Typhi: Speck of H_2S present at the junction of slant and butt
- MR positive and VP negative
- Sugar fermentation test: Gluocse, mannitol, arabinose, maltose, dulcitol and sorbitol are fermented
- **Decarboxylation test**
  - *S.* Typhi—only lysine is decarboxylated
  - *S.* Paratyphi A—only ornithine is decarboxylated
  - *S.* Paratyphi B—positive for all, i.e. lysine, arginine and ornithine

Slide Agglutination Test

Identification of *Salmonella* at genus level can be confirmed by slide agglutination using polyvalent O antisera. Then, the serotypes can be identified by using type specific O antisera.
- *S.* Typhi: Agglutinates with O9 antisera
- *S.* Paratyphi A: Agglutinates with O2 antisera
- *S.* Paratyphi B: Agglutinates with O4 antisera

Flagellar antigens can also be determined by using type-specific H antisera.

Masking effect of Vi antigen: Sometimes, fresh isolates of *S.* Typhi are inagglutinable with O antisera due to presence of Vi antigen. Such strains should be either (1) tested with Vi antisera or (2) boiled for 60 minutes at 100°C to remove Vi antigen followed by testing with O antisera.

Antimicrobial Susceptibility Testing

It is done on Mueller-Hinton agar by disk diffusion method.

Demonstration of Serum Antibodies

Widal Test

Widal test is one of the oldest and most widely used serological tests for diagnosis of enteric fever. It was discovered by Fernand Widal in 1896.

- **Principle:** It is an agglutination test where H and O antibodies against *S.* Typhi and *S.* Paratyphi A and B are detected and measured in the patient's sera by using O and H antigens.
- **Antigens used:** Four antigens are used.
  1. O antigens of *S.* Typhi (TO)
  2. H antigens of *S.* Typhi (TH)
  3. H antigens of *S.* Paratyphi A (AH)
  4. H antigens of *S.* Paratyphi B (BH)

  (The paratyphoid O antigens cross-react with the typhoid O antigen due to their sharing of factor 12, hence they are not used in the test.)
- **Preparation of antigens:** Commercial Widal kits using stained antigens are available which are commonly used now. In house antigen preparation is seldom followed.
  - The preparation of antigen relies on the principle that—O antigen is heat-stable, alcohol-stable but formaldehyde-labile. H antigen is heat-labile, alcohol-labile but stable to formaldehyde
  - **Strains used:** *S.* Typhi 901 'O' and 'H' strains and laboratory maintained strains for *S.* Paratyphi A and B
  - **O antigen preparation:** The *S.* Typhi 901 O strain is cultured on 1:800 phenol agar (to inhibit H antigen) and the growth is scraped off in saline → mixed with 20 times its volume of absolute alcohol → heated at 40–50°C for 30 minutes → centrifuged → deposit is re-suspended in saline → chloroform is added as a preservative
  - **H antigen preparation:** By adding 0.1% formalin to a 24 hour broth culture or saline suspension of an agar culture.
- **Procedure of Widal test:**
  - Patient's serum is serially diluted in normal saline in test tubes from 1 in 10 to 1 in 640 dilutions. Four such sets are made
  - To each set of diluted sera, respective four antigen suspensions (TO, TH, AH, BH) are added
  - Control tubes containing the antigens and normal saline should be kept to check for autoagglutination
  - Test tubes are incubated in water bath at 37°C overnight. Some authors recommend initial incubation at 50–55°C for 2 hours, followed by overnight incubation at room temperature.
- **Results:**
  - O agglutination appears as compact granular chalky clumps (disk-like pattern), with clear supernatant fluid
  - H agglutination appears as large loose fluffy cotton-woolly clumps, with clear supernatant fluid
  - If agglutination does not occur, *button* formation occurs due to deposition of antigens and the supernatant fluid remains hazy

- *Titer:* The highest dilution of sera, at which agglutination occurs, is taken as the antibody titer.

❖ **Interpretation (Table 30.4):**
- **Significant titer:** Any titer is not significant. In endemic countries like India, due to prior exposure, people will always have some baseline antibodies. Higher titers are only significant. The cut-off varies from place to place depending on endemicity of the disease
  - Significant titer in most of the places in India is taken as:
    - H agglutinin titer more than 200 and
    - O agglutinin titer more than 100.
  - Low titers should be ignored and considered as baseline titers in endemic areas.
- **False-positive:** Widal test may occur due to:
  - ***Anamnestic response:*** It refers to a transient rise of titer due to unrelated infections (malaria, dengue) in persons who have had prior enteric fever
  - If bacterial antigen suspensions are not free from fimbriae
  - Persons with inapparent infection or
  - Persons with prior immunization (with TAB vaccine).
- **Fourfold rise** in antibody titer demonstrated by testing paired sera at 1 week interval is more meaningful than a single high titer. Rise in titers in anamnestic responses is transient that usually falls after 1 week whereas, in true infection, the titer increases by fourfold after 1 week
- **False-negative:** Widal test may occur in:
  - Early stage (1st week of illness)
  - Late stage (after fourth week)
  - Carriers
  - Patients on antibiotics
  - Due to prozone phenomena (antibody excess)—this can be obviated by serial dilution of sera.
- O agglutinins appear early and disappear early and indicate recent infection. H agglutinins appear late and disappear late

Table 30.4: Interpretation of Widal test

Widal test result	Suggestive of
Rise of TO and TH antibody	Enteric fever due to *S.* Typhi
Rise of TO and AH antibody	Enteric fever due to *S.* Paratyphi A
Rise of TO and BH antibody	Enteric fever due to *S.* Paratyphi B
Rise of only TO antibody	Recent infection: Due to any serotype—*S.* Typhi or *S.* Paratyphi A or B
Rise of only TH antibody	? Convalescent stage/anamnestic response
Rise of all TH, AH, BH antibodies	Post TAB vaccination

- O antibodies are serotype nonspecific. They are raised in all infections, i.e. *S.* Typhi, *S.* Paratyphi A and B
- H antibodies are specific. TH, AH and BH antibodies are raised in *S.* Typhi, *S.* Paratyphi A and B infections respectively.

Other Antibody Detection Tests

Various commercial methods available are:

❖ **Typhidot test:** 50 kDa OMP (outer membrane protein) antigen is used; it uses a dot ELISA format to detect both IgM and IgG separately after 2-3 days of infection

❖ **IDLTubex test:** O9 antigen is used, detects only IgM antibodies against *S.* Typhi by a semiquantitative colorimetric method

❖ **IgM dip stick test** and **ELISA** detect anti-LPS IgM antibodies

❖ **Dot blot assay:** Flagellar antigen is used, detects only IgG antibodies.

Demonstration of Serum Antigens

Antigens of typhoidal salmonellae are consistently present in the blood in the early course of the disease, and also in the urine of patients during the late phase. Several methods are available for antigen detection:

❖ ELISA

❖ Coagglutination test and CIEP were used earlier, now not in use.

Molecular Methods

Several polymerase chain reaction (PCR) based methods (e.g. nested PCR) are available to detect and differentiate typhoidal salmonellae by targeting various genes, such as *flagellin* gene, *Iro B* and *fliC* gene.

Other Nonspecific Methods

❖ WBC count: Neutropenia is seen in 15–25% of cases. Leukocytosis is more common among children, during early phase and in cases complicated by intestinal perforation or secondary infection

❖ Liver function tests moderately deranged

❖ Muscle enzyme levels moderately elevated.

Antimicrobial Susceptibility Testing

It is done on Mueller Hinton agar by disk diffusion method.

Detection of Carriers

❖ **Culture:** By stool and bile culture (detects fecal carriers) and urine culture (detects urinary carriers)

❖ **Detection of Vi antibodies:** It is done by tube agglutination test by using *S.* Typhi suspension carrying Vi antigen (Bhatnagar strains). Even a titer of 1:10 is also considered as significant. Though Vi antibody detection is useful, confirmation should always be made by culture

- **Isolation of salmonellae from sewage:** It is carried out to trace the carriers in the communities. It can be done by:
  - Sewer-swab technique: Gauze pads left in sewers are cultured on highly selective media, such as Wilson and Blair media
  - Filtration: Sewage can be filtered through millipore membranes and the membranes are cultured on highly selective media.

Typing of Salmonellae (Typhoidal and Non-typhoidal)

For adequate surveillance and determining the source of food-borne infections and outbreaks in hospitals (caused by non-typhoidal salmonellae), several typing methods are used such as:

- **Phenotypic methods:** In general, phenotypic methods have low discriminatory power which limits their use as epidemiological tool
  - **Phage typing** is done for *S.* Typhi, by using specific bacteriophage called Vi phage II which enters into the cell by using Vi antigen as receptor. The phage types most widespread and abundant throughout the world are E1 and A, followed by B2, C1, D1, and F1. It was in use before, now obsolete
  - **Bacteriocin typing and biotyping:** They were in use before, now obsolete
  - **Antibiogram typing:** It is based on antimicrobial resistance pattern.
- **Genotypic methods:** They have good discriminating ability. Widely used methods are as follows:
  - Insertion sequence (IS) 200 typing: It has been shown to be more useful for certain serotypes (e.g. *S.* Infantis and *S.* Heidelberg)
  - Pulse field gel electrophoresis (PFGE)
  - Ribotyping
  - PCR-based methods, such as random amplified polymorphic DNA typing (RAPD) and PCR-RFLP (Restriction fragment length polymorphism)
  - Sequence-based typing (most widely used).

TREATMENT **Enteric Fever**

Treatment of enteric fever (Table 30.5) depends on the susceptibility of the strains.

The currently recommended drugs are as follows:

- Third generation cephalosporins, e.g. ceftriaxone
- Azithromycin
- Fluoroquinolones, e.g. ciprofloxacin

Drugs that were used in the past were:

- Chloramphenicol
- Amoxicillin
- Cotrimoxazole.

Table 30.5: Treatment of enteric fever

	Drug of choice	Alternate drug
Empirical treatment	This is the treatment given before antimicrobial susceptibility report is available	
	Ceftriaxone[a]	Azithromycin[b]
Fully susceptible	Susceptible to all the drugs given for enteric fever	
	Ciprofloxacin[c]	Amoxicillin Chloramphenicol Cotrimoxazole
MDR strains (Multi-drug resistant)	Defined as resistant to chloramphenicol, ampicillin and cotrimoxazole-antibiotics used to treat enteric fever long back	
	Ciprofloxacin	Ceftriaxone Azithromycin
NAR strains (Nalidixic acid resistant)	Defined as strains resistant to nalidixic acid with reduced susceptibility to ciprofloxacin	
	Ceftriaxone	Azithromycin, Ciprofloxacin[d] (higher dose and longer course)
Carriers	Ampicillin or Amoxicillin plus probenecid for 6 weeks	Cotrimoxazole or Ciprofloxacin

[a] 1–2 g/day (IV) for 10–14 days
[c] 500 mg twice a day oral for 5 days
[b] 1g/day oral for 5 days
[d] 750 mg twice a day per oral for 10–14 days

Drug Resistance in Typhoidal Salmonellae

- **Multidrug-resistant (MDR) *S.* Typhi:** It is defined as resistant to chloramphenicol, ampicillin and cotrimoxazole-antibiotics used to treat enteric fever long back. MDR strains emerged in 1989 in China and Southeast Asia including India and since then they have disseminated widely
- **NAR strains (Nalidixic acid resistant):** Due to the increased use of fluoroquinolones to treat MDR strains in 1990s, strains with reduced susceptibility to ciprofloxacin have emerged in India, other regions of southern Asia and sub-Saharan Africa (most recently)
- **Resistance to ceftriaxone:** It is very rare, has been reported recently. Both extended spectrum β-lactamases (ESBLs) and AmpC β-lactamase producing *S.* Typhi have been detected
- **Old is gold:** Interestingly, it is noticed that many strains reverted susceptible to the olden days drugs (amoxicillin, chloramphenicol, cotrimoxazole) as they were not in use for long time.

Prophylaxis

Theoretically, it is possible to control or eliminate enteric fever since the agents survive only in the human hosts and are spread by contaminated food and water. Many developed countries have proven this. However, in developing countries, this goal is currently unrealistic due to lack of adequate sewage disposal and water treatment. There are three lines of prophylactic measures.

Control of Reservoir

Control of Cases

- By early diagnosis and prompt effective treatment
- Disinfection of stool or urine soiled clothes with 5% cresol, 2% chlorine or by steam sterilizer
- Follow up examination of stool and urine culture to detect carriers (twice, at 3–4 months and at 12 months).

Control of Carriers

- Early detection of carriers by stool/urine culture or by detection of Vi antibodies
- Effective treatment of carriers by:
  - Ampicillin or amoxicillin (4–6 g/day) plus probenecid (2 g/day) for 6 weeks. These drugs get concentrated in bile and may eliminate 70% of carriers
  - Surgery: Cholecystectomy plus ampicillin is regarded as the most effective approach for carrier state elimination (80% cure rate).

Sanitation measures

Sanitation measures include the following:

- Protection and purification of drinking water supplies
- Hand washing and improvement of basic sanitation
- Promotion of food hygiene
- Health education.

Vaccine

Immunization provides short time protection. It is indicated in following situations:

- Travelers going to endemic areas
- People attending *melas* and *yatras*
- Household contacts
- People at increased risk (school children)
- People living in endemic area (optional).

There are three types of vaccines available (see below).

Vaccines for Typhoid Fever

There are two types of typhoid vaccines available currently.

Parenteral Vi polysaccharide vaccine:

It is composed of purified Vi capsular polysaccharide antigen derived from *S.* Typhi strain Ty2.

- Dosage: Single dose containing 25 μg of Vi antigen is given IM or subcutaneously
- Vaccine confers protection for 2 years; booster is given every 2 years
- Age: It is given only after 2 years of age. Capsular antigen being T independent antigen, is poorly immunogenic to children < 2 years
- **Vi-rEPA:** Vi antigen is conjugated with recombinant *Pseudomonas aeruginosa* Exotoxin A. Conjugation increases immunogenicity of the Vi antigen; therefore this vaccine can be given to children less than two years.

Contd...

Contd...

Typhoral (oral live attenuated S. Typhi Ty2 1a vaccine):

- Typhoral is a stable live attenuated mutant of *S.* Typhi strain Ty2 1a, which lacks the enzyme UDP-galactose-4-epimerase (Gal E mutant)
- On ingestion, it multiplies for some time, initiates the immune response but self-destructs (dies of its own after 4–5 cell divisions, due to lack of Gal E enzyme) and therefore cannot induce any pathogenesis
- It is indicated only after 6 years of age
- The vaccine is available in lyophilized form as **enteric coated capsules**
- It is given orally before food, on alternate days- 1, 3, 5, 7 with booster every 5 years. No antibiotics should be given during this period
- **Protective immunity** starts after 7 days of the last dose and lasts for 4 years.

Parenteral TAB vaccine (heat-killed whole cell *S.* Typhi/ *S.* Paratyphi A and B): It is no longer in use because of significant side effects.

NON-TYPHOIDAL SALMONELLAE

Non-typhoidal salmonellae (NTS) include the pathogenic salmonellae other than *S.* Typhi and *S.* Paratyphi A, B and C. Majority of infections due to NTS are caused by *S.* Typhimurium and *S.* Enteritidis followed by *S.* Newport, *S.* Javiana, *S.* Heidelberg, *S.* Choleraesuis and *S.* Dublin.

Non-typhoidal Salmonellae vs Typhoidal Salmonellae

Non-typhoidal salmonellae differ from typhoidal salmonellae in many respects:

- **Zoonotic:** NTS can be acquired from multiple animal reservoirs (whereas the typhoidal salmonellae are strictly human pathogens)
- **Transmission** of NTS is most commonly associated with animal food products, especially eggs, poultry, undercooked ground meat and dairy products (typhoidal salmonellae are mainly water-borne)
- **Resistance:** Compared to other enteric gram-negative pathogens, salmonellae are relatively resistant to many environmental factors, such as drying, salting, smoking and freezing. This explains why they survive in diverse range of foods
- **Seasonality:** Transmission of NTS is highest during the rainy season in tropical climates and during the warmer months in temperate climates, coinciding with the peak in food-borne outbreaks
- **Prevalence:** NTS are widely prevalent in developed as well as developing countries (typhoidal salmonellae are mainly confined to developing countries)
- **Outbreaks** of NTS are common in hospitals (typhoidal salmonellae outbreaks are community based)
- **Pathogenesis** is similar to that of enteric fever except that in NTS gastroenteritis, there is massive neutrophil infiltration

into intestinal mucosa (in contrast to enteric fever, where there is mononuclear cells infiltration).

Clinical Manifestations

- **Gastroenteritis:** Infection with NTS most often results in gastroenteritis—characterized by nausea, vomiting, watery diarrhea, fever and onset of abdominal cramps 6–48 hours after the ingestion of contaminated food (gastroenteritis is uncommon in typhoidal salmonellae)
- **Bacteremia:** Up to 8% of patients with NTS gastroenteritis develop into bacteremia which leads to either endovascular infection or seedling to various organs leading to metastatic localized infection. Risk factors for bacteremia include:
  - NTS serotype: Most common being *S.* Choleraesuis (source-pig) and *S.* Dublin (source—cattle)
  - Age: Infants and elderly people are at higher risk
  - HIV and other conditions with low immunity.
- **Endovascular infections**, such as endocarditis and arteritis, occur rarely in people with pre-existing valvular heart disease.
- **Metastatic localized infections** such as:
  - Intra-abdominal infections, such as hepatic or splenic abscesses or cholecystitis
  - NTS meningitis (commonly in infants)
  - Pulmonary infections, such as lobar pneumonia and lung abscess
  - UTI (pyelonephritis and cystitis) in people with underlying renal stones or urinary tract abnormality
  - Genital tract infections include ovarian, testicular abscesses, prostatitis and epididymitis
  - *Salmonella* osteomyelitis: It is commonly associated with sickle cell disease
  - **Reactive arthritis** (Reiter's syndrome) seen in persons with HLA-B27 histocompatibility antigen.

TREATMENT — Non-typhoidal salmonellae

In contrast to enteric fever where antibiotics are started as early as possible, in uncomplicated NTS gastroenteritis, the treatment is conservative with fluid replacement.

- Antibiotic use is associated with increased rates of carriers and relapse, hence it is limited only to invasive NTS infection or severe gastroenteritis having higher risks of developing invasive infection
- Drugs given are similar to that of enteric fever
  - Ciprofloxacin is given for preemptive treatment or severe gastroenteritis
  - Ceftriaxone is indicated for bacteremia and invasive infections.

Drug Resistance

NTS are more drug resistant than typhoidal salmonellae.

- MDR strains of NTS are resistant to more than 5 drugs-ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracyclines (abbreviated as **ACSSuT**)
- Increased use of ceftriaxone and ciprofloxacin to treat MDR strains leads to emergence of resistance to ceftriaxone (due to production of AmpC β-lactamases) and ciprofloxacin (due to point mutation in DNA gyrase genes).

EXPECTED QUESTIONS

I. Essay:

1. Meena, a young adult female was admitted to the hospital with intense headache, abdominal discomfort for the past 5 days. She had also developed fever which is of remittent type with gradual rise in a step ladder fashion. On examination, she was toxic with temperature of 101° F, tongue was coated and mild splenomegaly was present.
   a. What is the most probable etiological diagnosis?
   b. Describe the pathogenesis of this condition.
   c. Mention sample collection and laboratory diagnosis in detail.
   d. Add a note on treatment and vaccination available for this clinical condition.

II. Write short notes on:

1. Typhoid carriers.
2. Drug resistance in salmonellae.
3. Non-typhoidal salmonellae.

III. Multiple Choice Questions (MCQs):

1. **All of the following salmonellae are motile, *except*:**
   a. S.Typhi b. S. Enteritidis
   c. *S.* Gallinarum d. *S.* Chester
2. **S. Typhi is the causative agent of typhoid fever. The infective does of *S.* Typhi:**
   a. One bacillus b. 10^3–10^6 bacilli
   c. 10^8–10^{10} bacilli d. 1–10 bacilli
3. **In a patient with typhoid, diagnosis after 15 days of onset of fever is best done by:**
   a. Blood culture b. Stool culture
   c. Urine culture d. Widal test
4. **Antibodies against which of the following antigen appear early following infection with S.Typhi?**
   a. Vi antigen b. O antigen
   c. H antigen d. Capsular antigen
5. **In Widal test, rise of TO and AH indicates infection with:**
   a. S.Typhi b. *S.* Paratyphi A
   c. *S.* Paratyphi B d. *S.* Paratyphi C
6. **Antibodies against which of the following antigen appear in typhoid carrier?**
   a. Vi antigen b. O antigen
   c. H antigen d. Capsular antigen
7. **Bacteria mediated endocytosis (BME) is observed in:**
   a. *Shigella* b. *Listeria*
   c. *Salmonella* d. *Campylobacter*
8. **Currently the drug of choice for enteric fever is:**
   a. Ciprofloxacin b. Azithromycin
   c. Ceftriaxone d. Chloramphenicol

Answers

1. c **2.** b **3.** d **4.** b **5.** b **6.** a **7.** c **8.** c

Vibrio and Aeromonas

31
CHAPTER

Chapter Preview

- Vibrio
  - *Vibrio cholerae*
  - Halophilic vibrios
- *Aeromonas* species

Earlier, family Vibrionaceae comprised of four genera—(1) *Vibrio*, (2) *Aeromonas*, (3) *Plesiomonas* and (4) *Photobacterium*. However, the taxonomy has been greatly changed.

- *Aeromonas* has now been given its own family status, the Aeromonadaceae
- The taxonomy of *Plesiomonas* remains controversial; it is closely related to *Proteus* and recently has been placed into family Enterobacteriaceae (described in Chapter 29)
- *Photobacterium* is a plant pathogen.

VIBRIO

Vibrios are curved gram-negative bacilli that are actively motile by means of single polar flagellum.

- The organism was first described and named by Filippo Pacini in 1854. The name '*Vibrio*' is derived from its characteristic vibratory motility
- Robert Koch isolated the organism in 1886, and named it as Komma bacillus (due to its characteristic **curved** or **comma-shaped** appearance)
- They are fermentative, strongly aerobic, oxidase positive (except *V. metschnikovii*), non-sporing and non-capsulated (except *V. parahaemolyticus* and *V. vulnificus* which are capsulated)
- Growth is stimulated in presence of salt—a unique property exhibited by all vibrios. However, the optimum salt concentration required, varies among different vibrios
- **Habitat:** Vibrios are ubiquitous, found worldwide. Being salt loving, the natural habitat of vibrio is the in marine environments (sea water and sea food), surface waters, river and sewage
- Of the 35 *Vibrio* species recognized, only 12 have been associated with human infections
- Among them, the most important is *V. cholerae* that causes a devastating acute diarrheal disease **'cholera'** and has been responsible for seven global pandemics and several epidemics over the past two centuries.

VIBRIO CHOLERAE

Classification

Based on Salt Requirement

Based on salt requirement, vibrios can be classified into:

- **Nonhalophilic vibrios:** They can grow without salt, but 1% salt is optimum for their growth. They cannot grow at higher salt concentrations. Examples include *V. cholerae* and *V. mimicus*
- **Halophilic vibrios:** They cannot grow in the absence of salt. They can tolerate and grow at higher salt concentration of up to 7–10%. Examples include *V. parahaemolyticus*, *V. alginolyticus* and *V. vulnificus*.

Heiberg Classification (1934)

Heiberg classified vibrios into eight groups based on fermentation of three sugars—(1) mannose, (2) arabinose and (3) sucrose. *V. cholerae* was placed in Group I.

Gardner and Venkatraman Classification

This classification of *V. cholerae* (1935) was based on serogrouping, biotyping, serotyping and phage typing. This classification was later on updated by several researchers. Such typing schemes are of great epidemiological importance in tracking the outbreaks by finding out the relatedness between the isolates in different clinical specimens (Fig. 31.1).

Serogrouping

Based on somatic O antigen, *V. cholerae* can be grouped into more than 200 serogroups or serovars (updated by the most widely used Sakazaki typing scheme).

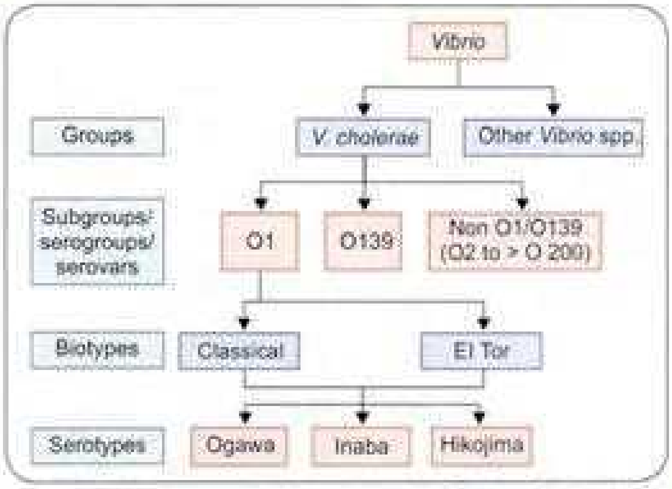


Fig. 31.1: Gardner and Venkatraman classification of *V. cholerae*

- **O1 serogroup:** Among all serogroups, O1 was responsible for all pandemics and most of the epidemics of cholera. Strains belonging to O1 serogroup are agglutinated by O1 antisera.
- **NAG vibrios:** Serogroups other than O1 were not agglutinated by O1 antiserum and were called non-agglutinable or NAG vibrios. They were thought to be non-pathogenic, hence also named as **non-cholera vibrios (NCV)**. Later on, it was observed that many other serogroups are pathogenic to man and are agglutinable with their respective antisera. Hence these terms such as NAG or NCV are no longer in use.
- **O139 serogroup:** It was identified in 1992 and since then it has caused several epidemics and outbreaks of cholera in the coastal regions of India and Bangladesh.
- **Non O1/O139 serogroups:** They have occasionally caused sporadic outbreaks of diarrhea and extraintestinal manifestations, but have never caused epidemic cholera so far.

Biotyping

Serogroup O1 has two biotypes—(1) classical and (2) El Tor; differentiated by various biochemical reactions and their susceptibility to polymyxin B and bacteriophages (Table 31.1).

- **Classical biotype:** It was responsible for the first six pandemics of cholera worldwide. It was highly virulent and had caused several deaths
- **El Tor biotype** replaced the classical biotype by 1961 and caused the seventh pandemic of cholera. It was first identified by Gotschlich (1905) at a quarantine camp on the Sinai Peninsula in El-Tor, Egypt
- **Currently,** almost all outbreaks or epidemics of cholera are due to biotype El Tor, although occasional classical isolates are still seen. However, many isolates of current days do not fit into both the biotypes and are called as **El Tor variants**.

Variants of El Tor Biotype

Several variants of El Tor biotype have been described recently in Bangladesh and in few other places of Asia and Africa. They show properties overlapping with both El Tor and classical biotypes. Variants of El Tor biotype include the following types:

- **Matlab variants (El Tor hybrid):** These strains could not be biotyped because they have a mixture of both classical and El Tor properties, were described first in Bangladesh in 2002
- **Mozambique variant** (2004–2005): It has typical phenotypic properties and genome of El Tor, except that the cholera toxin and its gene (CTX) are of classical type.

Serotyping

O1 serogroup can further be divided into three serotypes—(1) Inaba, (2) Ogawa, and (3) Hikojima; based on minor antigenic differences of O antigen (Table 31.2).

- Ogawa is the most common serotype isolated from clinical samples followed by Inaba
- However, during epidemics, shifting between serotypes can take place, more common being Ogawa to Inaba shift which occurs due to mutations in *rfbT* gene
- Hikojima represents an unstable transitional state; where both Inaba and Ogawa antigens are expressed

Phage Typing

El Tor and classical biotypes can also be differentiated based on their susceptibility to different lytic bacteriophages.

- Basu and Mukherjee phage typing was the most widely used scheme to differentiate O1 biotypes

Table 31.1: Differences between classical and El Tor *V. cholerae*

Biotypes of *V. cholerae* O1	Classical biotype	El Tor biotype
β hemolysis on sheep blood agar	Negative	Positive
Chick erythrocyte agglutination	Negative	Positive
Polymyxin B (50 IU)	Susceptible	Resistant
Group IV phage susceptibility	Susceptible	Resistant
El Tor Phage V susceptibility	Resistant	Susceptible
VP (Voges-Proskauer) test	Negative	Positive
CAMP test	Negative	Positive
Cholera toxin gene	CTX-1	CTX-2

Abbreviation: CAMP test, Christie Atkins Munch-Petersen test

Table 31.2: Serotypes of *V. cholerae* O1 and their distinct O antigen types

Serotype	O antigen types
Ogawa	A, B
Inaba	A, C
Hikojima	A, B, C

- It was later updated by Chattopadhyay (1993), and then it has been expanded to include O139 strains (Chakrabarti, 2000).

Pathogenesis of Cholera

Pathogenesis of cholera is toxin-mediated. Both *V. cholerae* O1 and O139 are capable of producing cholera toxin, thus resulting in cholera.

- **Mode of transmission:** *V. cholerae* is transmitted by ingestion of contaminated water or food
- **Infective dose:** Since *V. cholerae* is extremely acid-labile; a high infective dose of 10^8 bacilli is required to bypass the gastric barrier
- **Factors promoting transmission:** These include all those conditions where gastric acidity is reduced, such as hypochlorhydria, use of antacids, etc.
- **Crossing of the protective layer of mucus:** In the small intestine, vibrios penetrate the mucous layer and reach near the epithelial cells, which may be achieved by:
  - Its highly active motility
  - Secreting mucinase and other proteolytic enzymes
  - Secreting hemagglutinin protease (cholera lectin): It cleaves the mucus and fibronectin. It also helps in releasing vibrios bound to intestinal mucosa, facilitating their spread to other parts of the intestine and also their fecal shedding.
- **Adhesion and colonization:** The next step in the pathogenesis is, its adhesion to the intestinal epithelium which is facilitated by a special type IV fimbria called **toxin coregulated pilus** (TCP). It is so named because the synthesis of TCP, cholera toxin and several other virulence factors are coregulated by a single gene called *ToxR*
- **Cholera toxin (CT):** Once established in the human small intestine, the organism produces a powerful enterotoxin called cholera toxin. It resembles heat-labile toxin (LT) of *E. coli* in its structure and function, but it is more potent than the latter

Mechanism of action of cholera toxin

The toxin molecule is about 85 kDa; consists of two peptide fragments—A and B (Fig. 31.2)

- **Fragment B** is the binding fragment and is pentameric, contains **five subunits** weighing 11.7 kDa each. It binds to GM1 ganglioside receptors present on the intestinal epithelium, following which A fragment is internalized and cleaved into A1 and A2 peptides
- **Fragment A2** helps in tethering A and B subunits together
- **Fragment A1** is the active fragment (27 kDa), causes ADP ribosylation of G protein → upregulates the activity of adenylate cyclase → results in the intracellular accumulation of cyclic adenosine monophosphate (cAMP).

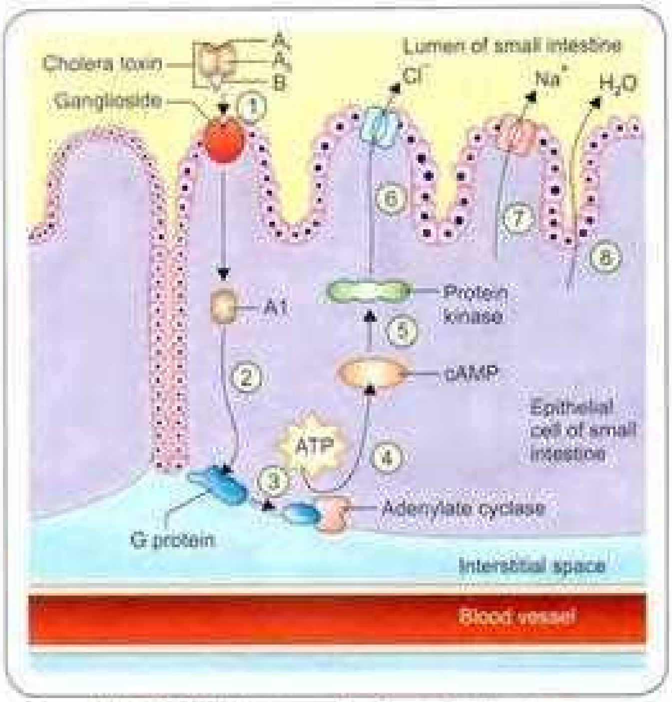


Fig. 31.2: Mechanism of action of cholera toxin

Abbreviations: CAMP, cyclic adenosine monophosphate; ATP, adenosine triphosphate.

- **Increase in cyclic AMP leads to:**
  - In small intestine, cyclic AMP inhibits the absorptive sodium transport system in villus cells and activates the secretory chloride transport system in crypt cells, which lead to the accumulation of sodium chloride in the intestinal lumen
  - Water moves passively into the bowel lumen to maintain osmolality which leads to the accumulation of isotonic fluid that results in watery diarrhea
  - Loss of fluid and electrolytes leads to shock (due to profound dehydration) and acidosis (due to loss of bicarbonate).
- **Gene for cholera toxin** (CTX): It is a part of pathogenicity island of the organism, encoded by genome of a filamentous bacteriophage (CTXϕ) which is integrated as prophage into the bacterial chromosome. TCP present on the surface of *V. cholerae* is the receptor for this bacteriophage
  - In this phage coded pathogenicity island, many other genes important for pathogenicity are clustered together such as genes encoding the biosynthesis of TCP, accessory colonization factors, and regulator genes
  - It is believed that the pathogenicity islands are acquired by horizontal gene transfer through bacteriophage which may account for the emergence of new toxigenic *V. cholerae* serogroups such as O139 (probably derived from an El Tor O1 strain).

- ***ToxR* gene:** It regulates the expression of CT, TCP and other virulence factors and is itself regulated by environmental factors, such as heat-shock response.
- **Chromosomes:** *V. cholerae* has two circular chromosomes, one large and one small.
  - Large chromosome bears the pathogenicity island, which is needed for its growth and intestinal survival
  - Small chromosome bears the gene essential for regulatory and metabolic pathways needed for environmental survival.
- **Other virulence factors include:**
  - Zona occludens toxin: It disrupts the tight junctions between mucosal cells
  - Accessory cholera enterotoxin: It is associated with phage packaging and secretion
  - Vero cell toxin: It is analogous to the toxin produced by *Shigella dysenteriae* 1. Its role in the intestine is not clear; however, it is important for the survival of the bacilli in aquatic environment
  - Accessory colonization factors: They help in adhesion and colonization
  - Siderophore: It is required for iron acquisition
  - Bacterial endotoxin (LPS): Unlike other gram-negative bacilli, the LPS of *V. cholerae* does not contribute to the pathogenesis of cholera. However, it is immunogenic, and is included as a component in killed vaccines.

Clinical Manifestations of Cholera

V. cholerae O1 or O139 infections produce a range of clinical manifestations such as:

1. Asymptomatic infection (75% of cases)
2. Mild diarrhea or cholera (20% of cases)
3. Sudden onset of explosive and life-threatening diarrhea (*cholera gravis*, in 5% of cases).

Incubation period varies from 24 to 48 hours. The usual manifestations include:

- **Watery diarrhea:** Cholera characteristically begins with the sudden onset of painless watery diarrhea that may quickly become voluminous
- **Rice water stool:** The stool is typically non-bilious, slightly cloudy and watery with mucus flakes and a fishy, inoffensive odor. Being noninvasive, there is no associated blood or pus cells in stool. It often resembles the water in which rice has been washed
- Vomiting may be present but fever is usually absent
- Muscle cramps may occur due to electrolyte imbalance
- **Complications** are directly proportional to the fluid loss, which results in loss of body weight (Table 31.3).

Table 31.3: Complications of cholera

Loss of body weight by	Symptoms
<5%	Increased thirst
At 5–10%	• Postural hypotension • Weakness • Tachycardia • Decreased skin turgor
At >10%	Renal failure (due to acute tubular necrosis) and fluid loss result in: • Oliguria • Weak or absent pulses • Sunken eyes • Sunken fontanelles in infants • Wrinkled ("washerwoman") skin • Somnolence and coma

Epidemiology

History of Pandemics

Cholera can occur in many forms—sporadic, limited outbreaks, endemic, epidemic or pandemic.

- **Home land:** The **delta region** of the Ganges and Brahmaputra in West Bengal (India) and Bangladesh was known to be the homeland of cholera since ancient times
- **Till early nineteenth century**, cholera was virtually confined to its home land, causing large epidemics periodically
- **First six pandemics** occurred between 1817 and 1923. All were caused by the classical biotype of *V. cholerae* which had spread from Bengal to involve most of the world; resulted in several thousands of deaths
- After the end of the 6th pandemic, **from 1923 to 1961** cholera was largely restricted to its home land
- **Seventh pandemic:** It had started in 1961 and it differed from the first six pandemics in many ways
  - It was the only pandemic that originated outside India, i.e. from Indonesia (Sulawesi, formerly Celebes Island) in 1961. India was affected in 1964 and the whole world was encircled by 1991
  - It was the only pandemic to be caused by El Tor biotype which had largely replaced the classical biotype by that time
  - El Tor produced a much milder cholera; however, El Tor infection was associated with more carrier rate than the classical. This is due to the fact that El Tor is much harder than the classical vibrios and capable of surviving in the environment much longer
  - This accounts for rapid spread of El Tor, involving the entire globe including some parts, such as Central

and South American countries, Australia and other affluent countries which were never affected before.

O139 (Bengal strain)

It was isolated first from Chennai in 1992. Since it was not agglutinated by any of the antisera available at that time (O1 to O138), it was designated as a new serogroup O139 or the **Bengal strain** as it spread rapidly along the coastal region of Bay of Bengal up to West Bengal, then to the adjacent areas of Bangladesh.

- O139 appears to be a derivative of O1 El Tor, but differs from the latter in having a distinct LPS and being **capsulated**. As a result, it is invasive, can cause bacteremia and extraintestinal manifestations
- There is no cross protection between O1 and O139
- O139 had caused large-scale outbreaks of clinical cholera and spread rapidly across almost 11 Asian countries and became a threat to cause the next pandemic
- However, by 1994 the fear had come down and once again the O1 El Tor became dominant and largely replaced O139
- Currently, O139 still causes a minority of cases in India and Bangladesh.

Current Situation

In the World

Cholera is a notifiable disease. However, it is often under reported, hence the true incidence is unknown.

- It is possible that more than 1.3-4 million cases of cholera occur every year, resulting in 21 000 to 1.4 Lakh deaths annually
- But in 2016, only 1,32, 121 cholera cases and 2420 deaths were reported to WHO worldwide; out of which, 54% of cases were reported from Africa, 13% from Asia and 32% from Hispaniola
- Several outbreaks have been recently reported such as from Yemen in the year 2016-2018 (large scale outbreak with >6 Lakh cases and >2000 deaths reported), Nigeria in 2017, Somalia in 2018
- Majority of cases are due to O1 El Tor. However, occasional cases may occur due to O139 and classical biotype, especially in Bangladesh.

In India

The situation has greatly changed in India both geographically as well as in terms of number of cases and deaths.

- West Bengal is no longer the home land; almost all the states have been affected
- Both morbidity (number of cases) and mortality (deaths) have greatly reduced. In 2011, about 2,341 cases were reported with 10 deaths, in contrast to more than 1,76,307 cases with 86,997 deaths in 1950
- El Tor dominance continues, while O139 causes minority of cases
- **NICED:** National reference Center for cholera in India is located at National Institute of Cholera and Enteric Diseases (NICED), Kolkata.

Epidemiological Determinants

- **Reservoir:** Humans are the only reservoir of infection. There is no known animal reservoir
- **Source:** The source of the infection may be either asymptomatic cases or carriers
- **Carriers:** They are apparently healthy people who shed the bacilli in feces. Carriers may be:
  - Incubatory carriers: They are less common, as cholera has a short incubation period of 1-2 days
  - Convalescent carriers: They are the recovered patients who shed the bacilli for 2-3 weeks
  - Contact or healthy carriers: They contract the infection from subclinical cases and in turn shed the bacilli for less than 10 days
  - Chronic carriers: Minority of convalescent carriers become chronic carriers.

  In general, biotype El Tor has more carrier rate than classical. The case-carrier ratio is 1: 50 for the classical biotype and 1:90 for the El Tor biotype
- **Cholera season:** Maximum transmission is associated with high temperatures, heavy rainfall and flooding, but cholera can occur throughout the year
- **Other factors** that promote transmission include poor sanitation, poverty, overcrowding, population mobility (as occurs in pilgrimages, fairs, festivals and marriages).
- **Factors determining severity** of the disease include:
  - Lack of pre-existing immunity
  - Persons with **'O' blood group** are at greater risk of severe disease if infected, while those with type AB blood group are at least risk. The reason is not clear.
  - Malnutrition
  - People with low immunity (e.g. HIV infected people).
- **Age:** During inter epidemic period, all the age groups are affected equally, however during epidemics it affects more number of children
- **Habitat:** *V. cholerae* is a natural inhabitant of coastal sea salt water and brackish estuaries, where the organism can persist for long periods, particularly in association with small crustaceans, such as copepods, crabs or plankton
- **Persistence** of *V. cholerae*:
  - During epidemics, it is maintained by carriers and subclinical cases
  - In inter epidemic period, it is maintained in sea water, crustaceans and planktons.
- **Resistance**
  - *V. cholerae* is acid-labile but stable to alkali

- It is heat-labile (killed within 30 minutes by heating at 56°C or within few seconds by boiling), but stable to refrigeration and can remain in ice for 4–6 weeks
- Drying and sunshine can kill the bacilli in few hours
- It is susceptible to disinfectants, such as cresol and bleaching powder (6 mg/L)
- In general, biotype El Tor is more resistant than classical.

LABORATORY DIAGNOSIS *Vibrio cholerae*

- **Specimens:** Watery stool or rectal swab (for carriers)
- **Transport media:** VR medium, Cary-Blair medium
- **Direct microscopy**
  - Gram-negative rods, short curved comma-shaped (fish in stream appearance)
  - Hanging drop-demonstrates darting motility.
- **Culture**
  - Enrichment broth: Alkaline peptone water, Monsur's taurocholate tellurite peptone water
  - Selective media: Bile salt agar, Monsur's GTTT agar, TCBS agar (yellow colonies)
  - MacConkey agar-produces translucent NLF colonies.
- **Culture smear and motility testing**—reveals
  - Short curved gram-negative bacilli and
  - Darting motility.
- **Biochemical identification**
  - Catalase and oxidase positive
  - ICUT: Indole (+), Citrate (+/–), Urease (–), TSI:A/A, gas (–), H_2S (–)
  - Cholera red reaction positive
  - String test positive
  - Salt tolerance test positive
  - Ferments glucose and sucrose.
- **Biotyping:** To differentiate classical and El Tor
- **Serogrouping:** To differentiate O1 and O139
- **Serotyping:** To differentiate Ogawa, Inaba and Hikojima serotypes of serogroup O1
- **Antimicrobial susceptibility testing.**

Laboratory Diagnosis

Specimens

- Freshly collected watery stool is the specimen of choice for acute cases. Ideally, it should be collected before starting the antibiotics
- Rectal swab is the preferred specimen for convalescent patients or carriers.

Transport/Holding Media

Specimens should be transported immediately to the laboratory. If delay is expected, stool or rectal swabs may be inoculated in transport media, where the bacilli do not multiply but remain viable for several weeks. 1–3 mL of stool is mixed in 10–20 mL of various transport media such as:

- **Venkatraman-Ramakrishnan (VR) medium:** It is composed of crude sea salt (20 g) and peptone (5 g) in one liter of distilled water at pH 8.6–8.8
- **Alkaline salt transport medium** is same as VR medium; in addition, it has boric acid, NaOH and KCl and has a pH of 9.2
- **Cary-Blair medium:** This is a buffered solution of sodium chloride, sodium thioglycollate, disodium phosphate and calcium chloride, at a pH 8.4. It is also useful for *Salmonella* and *Shigella*
- **Autoclaved sea water** can also be used when other transport media are not available.

Direct Microscopy

- **Gram-staining** of mucus flakes of feces reveals short curved **comma-shaped** gram-negative rods, arranged in parallel rows, which is described by Koch as **fish in stream** appearance (Fig. 31.3)
- **Motility testing by hanging drop method:** They are actively motile frequently changing their direction, described as **darting motility** (dart means a small, slender, pointed missile which shows sudden, rapid movement when thrown at a target). It is also described as shooting star or swarming gnats motility
  - At times, it is difficult to differentiate darting motility of *V. cholerae* from active motility of other bacteria such as *Pseudomonas*. More so few other bacteria can also produce darting motility (e.g. *Campylobacter* and *Aeromonas*)
  - **Motility testing after adding H-antisera:** *V. cholerae* becomes non-motile when a drop of the watery stool specimen is added with flagellar (H) antiserum. This differentiates it from other actively motile organisms.

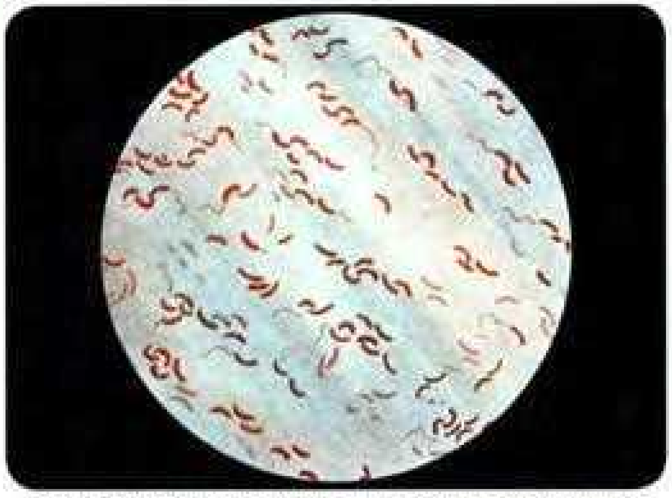


Fig. 31.3: *Vibrio cholerae* (Gram-stain): Curved comma-shaped gram-negative rods (fish in stream appearance).

Source: Public Health Image Library, ID#5324/Centers for Disease Control and Prevention (CDC) (with permission).

It is a simple and reliable method which confirms the diagnosis.

Culture

Cultural Conditions

V. cholerae is non-fastidious, grows well on ordinary media, such as:

- Nutrient agar (produces glistening translucent colonies with a bluish tinge in transmitted light) or
- Peptone water (produces uniform turbidity with formation of surface pellicle).

It is strongly aerobic (growth being scanty anaerobically). Other properties include:

- Hemodigestion: *V. cholerae* produces hemodigestion on blood agar which refers to the nonspecific lysis of blood cells by metabolic by products of bacteria. This can be seen on a blood agar plate, as greenish clearing around the main inoculum (Fig. 31.4A)
- It grows optimally at 37°C (range 16–40°C)
- Growth is better in alkaline medium. The optimum pH is 8.2 (range 7.4–9.6)
- NaCl (0.5–1%) stimulates the growth, however, high concentrations of NaCl (>6%) are inhibitory.

Culture Medium

To inhibit the commensals, fecal specimen should be inoculated simultaneously onto enrichment broth and selective media.

- **Enrichment broths:** They are incubated for 4–6 hours and thereafter a subculture is made onto another selective medium. Prolonged incubation of the broths should be avoided as the commensals may overgrow
- **Selective media:** Stool specimen is directly inoculated on to a selective medium and the plate is incubated at 37°C for 24 hours.

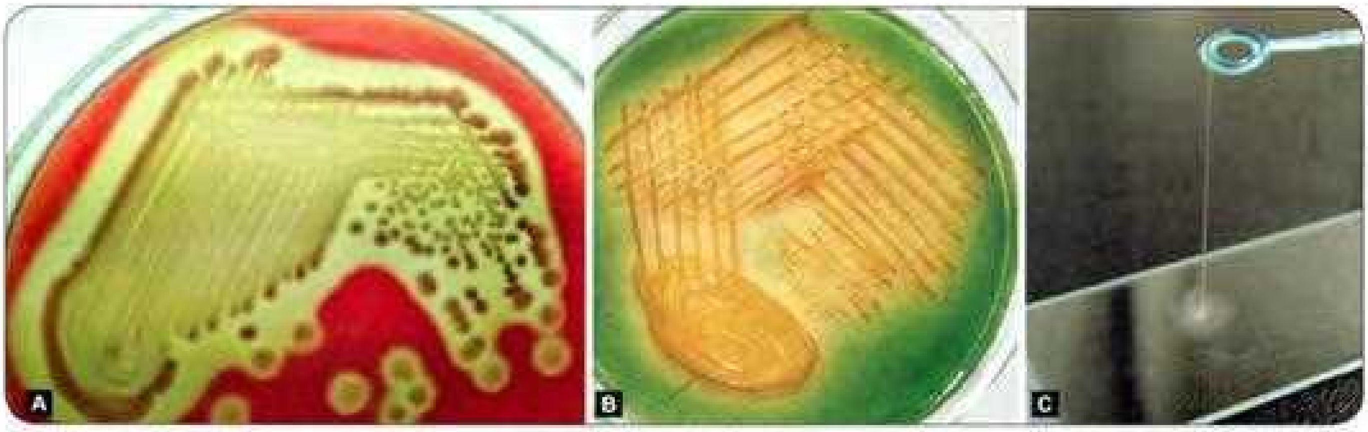
Enrichment Broth

- **Alkaline peptone water (APW)** contains peptone, NaCl in distilled water at a pH of 8.6. Stool sample is inoculated into APW at 1:10 ratio
- **Monsur's** taurocholate tellurite peptone water (pH 9.0).

Both can also be used as transport media.

Selective Media

- **Alkaline bile salt agar (BSA)** at pH **8.2:** This was the medium of choice in the past, still used in some laboratories. The colonies are typically glistening, **oil drop**, translucent, similar to those on nutrient agar.
- **Monsur's** gelatin taurocholate trypticase tellurite agar (GTTTA): *V. cholerae* produces small (1–2 mm), translucent colonies with a **grayish black Center and a turbid halo**. The alkaline pH (8.5) and potassium tellurite are inhibitory to most of the commensals. Classical biotypes grow better on it than on TCBS agar.
- **TCBS agar:** It contains thiosulfate, citrate, bile salts (as inhibitor), sucrose and has pH of 8.6. Bromothymol blue and thymol blue are used as indicators. This is widely used at present (Fig. 31.4B).
  - *V. cholerae* and other sucrose fermenting vibrios produce large (3–5 mm) yellow colored colonies; whereas sucrose non-fermenters (*V. mimicus* and *V. parahaemolyticus*) produce green colored colonies
  - Though it is inhibitory to most of the bacteria, some strains of *Aeromonas*, *Proteus* and *Enterococcus* may occasionally grow.
- **MacConkey agar:** When not sure about the type of enteric pathogen present in feces, MacConkey agar can be included in the panel. As it is mildly selective, it also supports other enteric pathogens such as *Shigella* and



Figs 31.4A to C: A. *Vibrio cholerae* on blood agar (hemodigestion); **B.** TCBS agar with yellow colored colonies of *Vibrio cholerae*; **C.** String test

Source: Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (with permission).

Salmonella. Colonies of *V. cholerae* are translucent and pale which may become pink on prolonged incubation (due to late lactose fermentation).

Culture Smear and Motility Testing

- Culture smear of the colonies reveals short curved gram-negative bacilli. The typical comma-shaped bacilli arranged in fish in stream appearance, which is observed in smears made from fresh samples are often lost on repeat subcultures
- Hanging drop shows typical darting motility.

Biochemical Reactions

V. cholerae shows the following biochemical properties:

- Catalase and oxidase positive
- **ICUT test:**
  - Indole test—positive
  - Citrate test—variable
  - Urease test—negative
  - TSI (triple sugar iron agar test)—shows acid/acid, gas absent, H_2S absent
- Nitrate reduction test is positive
- **Cholera red reaction:** Indole and nitrate reduction properties can be tested together by adding few drops of sulfuric acid to a peptone water culture of *Vibrio cholerae*. A reddish-pink color nitroso-indole ring is formed.
- **MR** (methyl red) test—positive
- **VP** (Voges-Proskauer) test—positive for El Tor, negative for classical biotype
- **Sugar fermentation test:** *V. cholerae* ferments glucose, sucrose and mannitol with production of acid but no gas. Mostly, it does not ferment lactose except some strains that may ferment late
- **String test:** When a colony of *Vibrio* is mixed with a drop of 0.5% sodium deoxycholate on a slide, the suspension loses its turbidity, and becomes mucoid. When tried lifting the suspension with a loop, it forms a string (Fig. 31.4C)
- **Decarboxylase tests:** These can differentiate *Vibrio* from related genera *Aeromonas* and *Plesiomonas*:
  - *Vibrio* utilizes lysine and ornithine
  - *Aeromonas* utilizes only arginine
  - *Plesiomonas* utilizes all, i.e. lysine, arginine and ornithine.
- **Susceptible to O/129** (vibriostatic agent): *Vibrio* species are susceptible to 10 µg of O/129 disk while *Aeromonas* and *Plesiomonas* are resistant
- **Salt tolerance test:** Peptone water broths with graded concentrations of NaCl are used to differentiate between *V. cholerae* from halophilic vibrios. *V. cholerae* tolerates maximum up to 6% NaCl.

Biotyping

The classical and El Tor biotypes can be differentiated by various biochemical tests, susceptibility to polymyxin B and bacteriophages (refer Table 31.1).

Serogrouping

Species identification is always confirmed by agglutination test done on a slide with *V. cholerae* polyvalent O antisera:

- **Specific serogroups** can be identified by using group-specific antisera. First the colony is tested with O1 antisera → If found negative, then tested with O139 antisera
- **Serotyping**-If agglutinated with O1 antisera, then the serotyping is done by testing simultaneously with Ogawa and Inaba antisera:
  - If agglutinated with Ogawa antisera—it is designated as Ogawa serotype
  - If agglutinated with Inaba antisera—it is designated as Inaba serotype
  - If agglutinated with both Ogawa and Inaba antisera—it is designated as Hikojima serotype.

Antimicrobial Susceptibility Testing

It is done on Mueller Hinton agar by disk diffusion test.

TREATMENT — Cholera

- **Fluid replacement:** It is the most important measure for management of the cholera patient. It should be prompt and adequate to correct hypovolemia and thereafter to be maintained to replace the ongoing fluid losses
  - **In mild to moderate fluid loss:** Oral rehydration solution (ORS) should be given
  - **In severe cases:** Intravenous fluid replacement with Ringer's lactate (or normal saline) should be carried out till the consciousness arrives, thereafter replaced by ORS.
- **Antibiotics** have a minor role as the pathogenesis is toxin mediated
  - Although not necessary for cure, use of antibiotic may decrease the duration and volume of fluid loss and hastens clearance of the organism from the stool; thus prevents the development of carrier stage
  - The WHO recommends the use of antibiotics to only severely dehydrated patients, although wider use is not contraindicated
  - **Drug of choice:** Macrolides such as erythromycin or azithromycin are the drugs of choice for adults, children and also in pregnancy. Alternatively, doxycycline, tetracycline or ciprofloxacin can be given in areas with confirmed susceptibility.

Prevention

General Measures

General measures include:

- Provision of safe water

- Improved sanitary disposal of feces
- Proper food sanitation
- Prompt outbreak investigation and taking necessary steps to reduce transmission
- Notification: Cholera is a notifiable disease locally and nationally, hence the cases should be notified
- Infection control measures (of contact precaution) such as hand hygiene is crucial to limit the spread of the disease (see Chapter 53).

Chemoprophylaxis

Tetracycline is the drug of choice (Table 31.4). It is indicated to household contacts, only during epidemic. Mass chemoprophylaxis is not advised as the duration of protection is short.

Vaccine

Injectable Killed Vaccines

They are no longer in use, as they provide little protection, cause adverse effects and fail to induce a local intestinal mucosal immune response.

Oral Cholera Vaccines

Oral cholera vaccines (OCV) are currently in practice. Two types of oral vaccines are available.

Killed Whole-cell vaccine:

- Two preparations are available:
  1. Whole-cell (WC) vaccine: It is composed of killed whole cells of *V. cholerae* O1 (classical and El Tor, Inaba and Ogawa).
  2. Whole-cell recombinant B subunit cholera vaccine (WC/rBS) (Dukoral): Composition is same as that of WC vaccine, in addition it has recombinant cholera toxin B subunit.
- **Schedule:** Two doses are given orally, at 7 days gap except for children 2–5 years (3 doses). It is not licensed for children less than 2 years

Table 31.4: Antibiotic therapy for cholera

	Drug of choice	Alternate drugs
Treatment of acute cases		
Adult	Doxycycline (single dose of 300 mg) or Tetracycline (12.5 mg/kg four times a day for 3 days)	Ciprofloxacin Erythromycin Azithromycin
Children/ pregnancy	Erythromycin or Azithromycin (10 mg/kg)	Cotrimoxazole
Chemoprophylaxis		
	Tetracycline (12.5 mg/kg four times a day for 3 days)	Doxycycline (single dose of 300 mg)

- **Protection** is short lived. For the first 6 months after vaccination, the protection rate is around 58% for WC vaccine and 85% for WC/rBS vaccine. However, it falls rapidly to 50% by 3 years of vaccination
- Children are better protected than adults
- WHO recommends for using vaccine during epidemics and outbreaks in the community but not during inter epidemic period.

Oral live attenuated vaccines (OCV): They use mutant strains that lack the gene encoding for cholera toxin.

- Several live OCV are under trial such as:
  - CVD 103-HgR, Peru-15 and *V. cholerae* 638 for classical and/or El Tor biotypes of *V. cholerae* O1
  - CVD-112 and Bengal-15 vaccine trials are ongoing for *V. cholerae* O139.
- CVD 103-HgR vaccine (*Orochol*) contains a live attenuated strain derived from reference strain 569 B (classical, O1, Inaba), given as single dose. Its protection starts after 8 days
- OCV is recommended to limit the risk of:
  - Occurrence of cholera outbreaks in displaced populations in endemic areas
  - Spread and incidence of cholera during an outbreak.

Non O1/O139 *V. Cholerae*

They may resemble biochemically to *V. cholerae* O1/O139, but do not agglutinate with O1 or O139 antisera.

Clinically, they differ from O1/O139 strains as follows:

- **Gastroenteritis:** Several outbreaks of gastroenteritis following sea food consumption (raw oysters) have been reported from Mexico and other places
  - Stool is watery or partly formed, less voluminous and bloody or mucoid
  - Many cases have abdominal cramps, nausea, vomiting and fever
  - However, they never cause epidemic cholera
  - Treatment is same as that of cholera. Fluid replacement is the most crucial step. Antibiotics can be given in severe dehydration.
- **Extraintestinal manifestations:** Such as otitis media, wound infection and bacteremia (in patients with liver disease) have been reported sporadically
  - Most of these infections are acquired by occupational or recreational exposure to seawater
  - Antibiotics are often indicated. Most strains are sensitive to tetracycline, ciprofloxacin and third-generation cephalosporins.

HALOPHILIC VIBRIOS

Halophilic vibrios can withstand higher salt concentration (>6%) in contrast to *V. cholerae*, which can tolerate up to 6%. They are widespread in marine environments. Cases tend to occur during late summer and early rain fall, when the bacterial counts are highest in the water.

Vibrio parahaemolyticus

Though *V. parahaemolyticus* was first reported from Japan (1953), the incidence of infection has greatly increased in several countries including Japan since 1993. In India, it has been reported from Kolkata.

Clinical Manifestations

- Food-borne gastroenteritis is the most common presentation, occurs following raw or uncooked sea food (e.g. oyster) intake. It commonly presents as watery diarrhea or rarely as dysentery with abdominal cramps.
- Extraintestinal manifestations are rare, such as wound infection, otitis and sepsis.

Pathogenesis

Pathogenesis of *V. parahaemolyticus* is related to the presence of the following virulence factors:

- Polysaccharide capsule which prevents the bacilli from phagocytosis
- Hemolysin (thermo-stable)
- Urease enzyme (in few strains): It breaks down urea from food to release ammonia that buffers gastric acidity
- It possesses two type III secretion systems in cell wall, which directly inject toxic bacterial proteins into host cells.

Serotype: *V. parahaemolyticus* has 13 O somatic antigens and more than 60 K capsular antigens. Most of the infections are caused due to serotypes O3:K6, O4:K68, and O1:K-untypable.

Laboratory Diagnosis

Laboratory diagnosis of *V. parahaemolyticus* is carried out similar to that followed for *V. cholerae*. The distinct properties are as follows:

- **Morphology:** It is capsulated, shows bipolar staining in fresh isolates and pleomorphism in older cultures
- Motile by peritrichous flagella (but it does not show darting motility)
- On TCBS, agar it produces green colonies (sucrose non-fermenter)
- **Kanagawa phenomenon:** It causes β hemolysis on Wagatsuma agar (a special type of high salt blood agar)
- **Swarming:** It swarms on blood agar
- Urease test is positive in few strains
- **Salt tolerance test:** It can resist maximum of 8% NaCl.

TREATMENT ***V. parahaemolyticus***

- Most of the gastroenteritis is self-limiting and treatment is same as that of cholera
- Indications for antibiotic use: Severe gastroenteritis or extraintestinal manifestations associated with underlying diseases, such as diabetes, pre-existing liver disease, iron-overload states, or immunosuppression.

Vibrio vulnificus

Though rare, *V. vulnificus* produces the most severe infection among the *Vibrio* species.

Clinical Manifestations

It can cause two distinct syndromes:

1. **Primary sepsis:** Usually occurs in patients with underlying liver disease and iron overload or rarely in renal insufficiency and immunosuppression.
2. **Primary wound infection:** It is characterized by painful erythematous swelling or cellulitis or even vesicular, bullous or necrotic lesions; generally affects people without underlying disease (*Vulnificus* is Latin word for "wound maker").

Laboratory Diagnosis

V. vulnificus can be cultured from blood or cutaneous lesions. Key biochemical reactions include:

- Ferments lactose [the only lactose fermenting (L⁺) *Vibrio*]
- Arginine is not dehydrolyzed.

TREATMENT ***Vibrio vulnificus***

Early antibiotic institution, wound debridement, and general supportive care are the keys to recovery. *V. vulnificus* is sensitive in vitro to a number of antibiotics, including tetracycline, fluoroquinolones, and third-generation cephalosporins.

Vibrio alginolyticus

V. alginolyticus can occasionally cause eye, ear and wound infections.

- Few cases of otitis externa, otitis media and conjunctivitis have been reported
- It rarely causes bacteremia in immunocompromised hosts
- It is the most salt-tolerant *Vibrio* and can grow at salt concentrations of more than 10%
- Disease is usually self-limiting. Severe infections respond well to antibiotics (tetracycline) and drainage.

AEROMONAS

Aeromonas was earlier placed in the family Vibrionaceae; however, it has now been assigned to a separate family, Aeromonadaceae. *A. hydrophila* causes **red leg disease** in frog.

Pathogenicity of *Aeromonas* in humans is mainly related to:

- Tissue adherence mediated by adhesions such as S-layer and fimbriae
- Capsular polysaccharide (prevents the bacilli from phagocytosis)
- Exotoxins, such as aerolysin, phospholipases, hemolysins, enterotoxin and cytotoxin similar to Shiga toxin
- Endotoxin or LPS.

Clinical manifestations: Over 85% of the human infections are caused by *A. hydrophila, A. caviae* and *A. veronii*. Most of the other species are mainly isolated from environmental sources and animals. Various manifestations include:

- Gastroenteritis (watery diarrhea, vomiting, fever and rarely dysentery) and peritonitis
- Musculoskeletal and wound infections
- Bacteremia in immunocompromised adults and infants
- Respiratory tract infections, such as epiglottitis, pharyngitis and pneumonia
- Hemolytic uremic syndrome (HUS) due to production of enterotoxin similar to Shiga-S toxin.

Laboratory diagnosis: The Key identification features are:

- Motile with single polar flagellum
- MacConkey agar—produce non-lactose fermenting pale colonies
- Oxidase and catalase positive
- Decarboxylase test—utilizes only arginine
- Growth is not stimulated by NaCl.

Genotypic classification: Recently, the taxonomy of *Aeromonas* has changed a lot. DNA hybridization studies have established 12 genomic species or hybridization groups (HG).

TREATMENT ***Aeromonas***

Aeromonas is susceptible to ciprofloxacin and levofloxacin. Alternatively, cotrimoxazole and cefepime can be given. However, the plasmid mediated drug resistance has been reported including β lactamase production.

EXPECTED QUESTIONS

I. Essay:

1. A 4-year-old boy developed severe watery diarrhea and vomiting. The stool collected has a rice water type of appearance. It was sent for bacteriological analysis.
   a. What is the probable etiological diagnosis of this condition?
   b. Describe in detail the pathogenesis of this condition.
   c. Add a note on its laboratory diagnosis.

II. Write short notes on:

1. Prophylaxis against cholera.
2. Halophilic vibrios.

III. Multiple Choice Questions (MCQs):

1. **Which of the following media can be used as transport medium for vibrios?**
   a. Selenite F broth
   b. Nutrient broth
   c. Tetrathionate broth
   d. Venkatraman-Ramakrishnan medium
2. **All of the following tests can differentiate between classical and El Tor biotypes of *V. cholerae, except:***
   a. β hemolysis on sheep blood agar
   b. Chick erythrocyte agglutination
   c. Growth on TCBS agar
   d. Polymyxin B (50 IU)
3. **Pathogenesis of *V. cholerae* involves one of the following second messenger systems:**
   a. cGMP b. cAMP
   c. Ca2+ d. IP3
4. **Selective media for *Vibrio cholerae*:**
   a. TCBS
   b. Mannitol salt agar
   c. Robertson cooked meat medium
   d. Modified Thayer Martin medium
5. **All of the following *Vibrio* species are halophilic, *except:***
   a. *V. cholerae*
   b. *V. parahaemolyticus*
   c. *V. alginolyticus*
   d. *V. vulnificus*
6. **Gardner and Venkatraman classification—which of the following is a biotype of *Vibrio*?**
   a. Ogawa b. Inaba
   c. Hikojima d. El Tor
7. **Cholera toxin (CT)—all are true, *except:***
   a. Phage coded
   b. Acts by ↑cAMP
   c. Ogawa and Inaba express CTX-1 and CTX-2 respectively
   d. Both O1 and O139 possess cholera toxin
8. **O139 (Bengal strain)—all are true, *except:***
   a. Capsulated
   b. Toxigenic
   c. Clinically similar to El Tor
   d. More common than El Tor
9. ***V. cholerae*—all are true, *except:***
   a. Acid-labile but stable to alkali
   b. Heat-labile
   c. Classical is more resistant than El Tor
   d. 'O' blood group are affected more frequently
10. **All are selective media for *V. cholerae, except:***
    a. Alkaline peptone water
    b. Alkaline bile salt agar
    c. TCBS agar
    d. Monsur's agar (GTTTA) medium
11. ***V. cholerae* gives the following test, *except:***
    a. Cholera red reaction positive
    b. String test positive
    c. Susceptible to O/129
    d. Salt tolerance test—can grow on medium with >6% salt
12. **Which of the following confirms the isolate of *V. cholerae* as Hikojima serotype?**
    a. If agglutinated with Ogawa antisera
    b. If agglutinated with Inaba antisera
    c. If agglutinated with Hikojima antisera
    d. If agglutinated with both Ogawa and Inaba antisera

Answers

1. d **2.** c **3.** b **4.** a **5.** a **6.** d **7.** c **8.** d **9.** c **10.** a **11.** d **12.** d

Pseudomonas and Other Non-fermenters

32 CHAPTER

Chapter Preview

- *Pseudomonas* species
- Other non-fermenters
    - *Burkholderia* species
    - *Acinetobacter* species
    - *Stenotrophomonas maltophilia*
    - *Elizabethkingia meningosepticum*
    - *Moraxella lacunata*
    - *Alcaligenes* species

Non-fermenters do not ferment any sugars, but they utilize the sugars oxidatively. Though the list is exhaustive, important human pathogens among non-fermenting gram-negative bacilli are *Pseudomonas*, *Burkholderia*, *Acinetobacter*, *Stenotrophomonas*, *Shewanella* and others.

PSEUDOMONAS

Pseudomonas is an oxidase positive, pigment producing, non-fermenting gram-negative bacilli. It is a major pathogen among the hospitalized patients and in patients with cystic fibrosis.

Virulence Factors and Pathogenesis

The pathogenesis of *Pseudomonas* is greatly attributed to its ability to develop widespread resistance to multiple antibiotics and disinfectants, and producing a number of virulence factors.

- **Colonization:** The first event to initiate the infection is to adhere and colonize the host surface. Various factors help in adhesion, such as pili or fimbria (the organ of attachment) and polar flagellum (mediates chemotactic motility to reach the host's surface)
- **Toxin-mediated immune evasion and tissue injury:** *Pseudomonas aeruginosa* produces probably the largest number of toxins and enzymes among the gram-negative bacteria. These can be grouped into:
    - **Nondiffusible toxins** (e.g. exotoxins S, U, T, and Y): Colonized *Pseudomonas* injects these toxins via a type III secretion system into the host cells, which allows the bacteria to evade the phagocytic cells and induce tissue injury by their cytotoxic activity
    - **Diffusible toxins:** e.g. exotoxin A, proteases, phospholipases, hemolysins, elastases, pyocyanin, etc. They are secreted by the organism's type II secretion system, can act freely and mediate tissue injury.

      Exotoxin A: This is the most important virulence factor of *P. aeruginosa*. It inhibits protein synthesis by inhibiting elongation factor-2 (mechanism of action is similar to diphtheria toxin).
- **Host's inflammatory response:** Host elicits inflammatory responses as a defense mechanism against various components of the bacilli, such as endotoxin and flagellin, mediated through the Toll-like receptors (TLR 4 and 5). However, florid and stronger inflammatory responses can lead to tissue injury and septic shock
- **Pigment production:** *Pseudomonas* produces a number of pigments which diffuse freely into the surroundings, inhibit other bacteria and mediate tissue injury
    - Pyocyanin: It is a blue green pigment, produced only by *P. aeruginosa*
    - Fluorescein (or pyoverdin): It gives greenish yellow color to the colony produced by most of the species
    - Pyorubin (imparts red color)
    - Pyomelanin (imparts brown black color).
- **Alginate coat**: Mucoid strains of *Pseudomonas* have a slime layer or alginate layer which facilitates biofilm formation, thus helps in adhesion to host cells and purulent mucus. Such strains can cause infections in patients with cystic fibrosis
- **Capsule**: Many strains of *Pseudomonas* possess polysaccharide capsule, which prevents the bacteria from phagocytosis
- **Multidrug resistance:** *Pseudomonas* is known to possess genes coding for resistance to several antimicrobial agents; thereby helping the bacilli to survive under antibiotic pressure especially in the hospital environment. **Biofilm formation** is another mechanism by which it prevents the entry of antibiotics into the bacterial cell

- **Multi-disinfectant resistance:** It helps the bacilli to grow in presence of various disinfectants; thus, spreading the infection in the hospitals
- **Wide temperature range**: *Pseudomonas* survives in extremes of temperatures (5–45°C), which allows the bacilli to be ubiquitous.

Clinical Manifestations

Psuedomonas aeruginosa is notorious to cause infections at almost all sites, most common being lungs, skin and soft tissues. Most of the infections are encountered in hospitalized patients who get colonized with the organisms either from heavily contaminated hospital environment or from the hospital staff (through contaminated hands). Colonized patients develop disease in the presence of underlying risk factors such as burn wounds, patients with immunosuppression and post surgeries. The manifestations are as follows:

- **VAP** (ventilator associated pneumonia): It develops among patients on ventilator in intensive care units
- **Chronic respiratory tract infections:** It occurs in patients with underlying conditions such as cystic fibrosis (in Caucasian populations), bronchiectasis or chronic panbronchiolitis (in Japan)
  - The mucoid strains (possessing alginate layer) of *Pseudomonas* commonly cause such infections
  - Structural abnormalities of the airways result in mucus stasis
  - Adherence to the mucus by the mucoid strains of *P. aeruginosa* initiates the infection. However, such strains lack most of the other virulence factors such as loss of O side chain of LPS.
- **Bacteremia** leading to sepsis and septic shock
- **Infective endocarditis (native valves):** It occurs among IV drug abusers
- **Ear infections:** The infections are either mild, such as **Swimmer's ear** (among children) or serious necrotizing form designated as **malignant otitis externa** (in elderly diabetic patients)
- **Eye infections** such as corneal ulcers (in contact lens wearers) and endophthalmitis secondary to bacteremia
- **Shanghai fever**: It is a mild febrile illness resembling typhoid fever
- **Skin and soft tissue infections**
  - **Burns patients**: *Pseudomonas* is the most common organism to infect the burn wounds
  - **Ecthyma gangrenosum:** It is an acute necrotizing condition resulting from bacteremia, occurs commonly in patients with febrile neutropenia and AIDS
  - **Dermatitis** (folliculitis and other papular or vesicular lesions): It cause outbreaks in spas and swimming pools
  - Toe-web infections (in the tropics)
  - **Green nail syndrome:** It is a 'paronychia' (inflammation of the tissues adjacent to the nail with green pus formation). It results from prolonged submersion of the hands in water
  - Cellulitis (characterized by **blue green pus**).
- **Other infections**
  - Bone and joint infections such as osteomyelitis and septic arthritis
  - Meningitis (in postoperative or post-traumatic patients)
  - UTI (urinary tract infection) in catheterized patients.

LABORATORY DIAGNOSIS — *Pseudomonas aeruginosa*

- **Sample collection:** Pus, wound swab, urine, etc.
- **Direct smear:** Gram-negative bacilli, and pus cells
- **Culture:**
  - Nutrient agar: opaque, irregular colonies with metallic sheen (iridescence) and blue green diffusible pigments
  - Blood agar: β-hemolytic grey moist colonies
  - MacConkey agar: NLF colonies
  - Selective media: e.g. cetrimide agar.
- **Culture smear and motility:** Motile, gram-negative bacilli
- **Biochemical identification:**
  - Catalase positive and oxidase positive
  - ICUT tests- Indole (–), Citrate (+), Urease (–), TSI:K/K, gas (–), H_2S(–)
  - OF test shows oxidative pattern (non-fermenter)
- **Antimicrobial susceptibility testing**

Laboratory Diagnosis

Specimen

Various specimens such as pus, wound swab, urine, sputum, blood or CSF are collected; depending up on the site infected.

Direct Smear

Gram staining of the specimen shows plenty of pus cells and slender gram-negative bacilli (1.5–3 × 0.5 µm), occasionally capsulated, but no spores.

Culture

Pseudomonas is non-fastidious, can grow in ordinary media. It is an obligate aerobe, hence specimens after being inoculated onto various media; should be incubated aerobically, at 37°C for 24 hours.

- **Peptone water**: *Pseudomonas* forms uniform turbidity with a **surface pellicle** formation, due to more oxygen tension at surfaces
- **Nutrient agar**: It produces large, opaque, irregular colonies with a metallic sheen (described as **iridescence**) (Fig. 32.1A)
  - **Pigments:** Most strains produce diffusible pigments which are either- blue green (pyocyanin), or yellow green (pyoverdin). Some strains are non-pigmented.

Pigment production can be enhanced in special media such as **King's media**
- Most colonies have a characteristic sweet ether or alcohol-like **fruity odor**
- **Morphotypes:** *Pseudomonas* colonies show various morphological appearances such as large spreading type, mucoid type, small round type, minute type, etc.

❖ **Blood agar:** It produces β hemolytic grey moist colonies on blood agar (Fig. 32.1B)
❖ **MacConkey agar:** It produces pale non-lactose fermenting colonies (Fig. 32.1C)
❖ Selective media such as **cetrimide agar** can be used to isolate the organism from mixed growth in purulent specimens.

Culture Smear and Motility Testing

Culture smear shows gram-negative bacilli. They are actively motile with single polar flagellum.

Biochemical Properties

Pseudomonas aeruginosa shows the following features:
❖ Oxidase and catalase positive
❖ Non-fermenter: Does not ferment any sugars, but utilize sugars oxidatively.
❖ **OF test** (Hugh and Leifson oxidative fermentative test): The test shows oxidative pattern.
❖ **ICUT test:**
- Indole test is negative
- Citrate test: positive
- Urease test: Negative
- Triple sugar iron (TSI) test: The test shows alkaline slant/alkaline butt (no change), with no gas and no H_2S.

Antimicrobial Susceptibility Testing (AST)

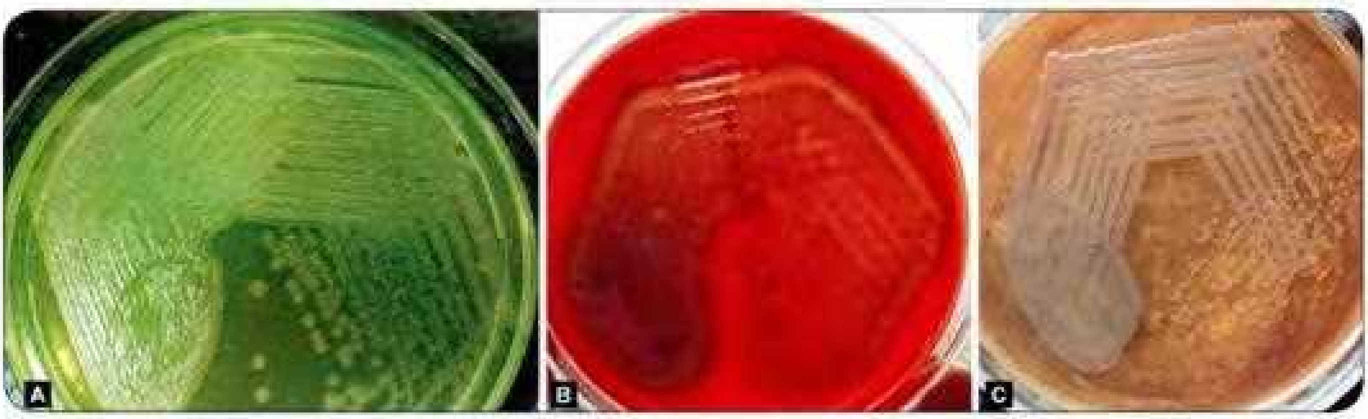
AST is essential to administer proper antibiotics. It is done on Mueller-Hinton agar by disk diffusion method.

Typing Methods

P. aeruginosa is an important cause of hospital-acquired infections, it is essential to type the isolates beyond the species level, to find out the relatedness between the isolates. This is useful during outbreaks, to trace the source of infection. For epidemiological studies, various typing methods are used such as:
❖ **Bacteriocin (pyocin) typing:** It is based on the ability of the strain producing distinct bacteriocin that inhibits certain indicator bacterial strains
❖ **Antibiogram typing:** It is based on the antimicrobial resistance pattern of the strains. It is the easiest and most commonly used method in hospitals
❖ **Serotyping:** It is based on O and H antigens, 17 serotypes of *P. aeruginosa* have been recognized
❖ **Molecular methods:** These methods, such as pulse field gel electrophoresis (PFGE) and sequence based typing method have highest discriminatory power to differentiate between the strains. However, their use is limited only to reference laboratories.

TREATMENT ***Pseudomonas***

Pseudomonas species are inherently resistant to most of the antibiotics. Only limited antimicrobial agents have antipseudomonial action, such as:
- **Penicillins:** Piperacillin, mezlocillin, ticarcillin
- **Cephalosporins:** Ceftazidime, cefoperazone, ceftolozane, and cefepime
- **β-lactam/β-lactamase inhibitor** combinations (piperacillin-tazobactam and cefoperazone-sulbactam)
- **Carbapenems:** Imipenem, meropenem and doripenem
- **Monobactam:** Aztreonam
- **Aminoglycosides:** Tobramycin, gentamicin, amikacin
- **Quinolones:** Ciprofloxacin, levofloxacin
- **Polymyxins:** Polymyxin B, colistin



Figs 32.1A and B: *Pseudomonas aeruginosa* showing: **A.** Nutrient agar with large irregular colonies with metallic sheen and green color pigmentation; **B.** Blood agar showing beta hemolytic moist colonies; **C.** MacConkey agar showing pale non-lactose fermenting colonies with metallic sheen

Source: Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (*with permission*).

Drug Resistance

Pseudomonas possesses a number of drug resistant plasmids which confer resistance to several antibiotics. Many strains are producers of β lactmases, such as ESBL (extended spectrum β lactamases), carbapenemases, and AmpC β lactamases. Many strains are resistant to aminoglycosides and quinolones.

Preventive Measures

Infection control measures (of contact precaution) such as hand hygiene are crucial to limit the spread of the infection (see Chapter 53).

OTHER NON-FERMENTERS

Burkholderia

Burkholderia species are also oxidase positive non-fermenters similar to *Pseudomonas*; however, they differ from the latter in being:
- Bipolar stained (safety pin appearance)
- Resistant to polymyxin B.

Burkholderia pseudomallei (Melioidosis)

B. pseudomallei is the causative agent of melioidosis.
- **Habitat:** *B. pseudomallei* is a saprophyte of soil and water and have large number of animal reservoirs. Melioidosis also occurs in rats, rabbits and guinea pigs
- **Mode of transmission:** Humans and animals are infected by various routes such as inoculation, inhalation, aspiration or ingestion. Man to man transmission is very rare
- **Virulence factors:** *B. pseudomallei* is perhaps the most virulent among the non-fermenters. Several virulence factors are described such as polysaccharide capsule, type III secretion system, LPS, toxins, enzymes and proteins (such as hemolysin, lipases and proteases), quorum sensing, type IV pili and siderophore for iron acquisition
- **Risk factors:** Melioidosis occurs mostly when underlying risk factors co-exist such as diabetes, renal failure in adults and traumatic inolcutaion in children, weather (rainy season) and occupation (rice farmers)
- **Incubation period** may range from 2 days to many years. Some cases may have long latency; presented long time after the exposure; hence melioidosis is also known as **'Vietnam time-bomb disease'**
- **Clinical feature:** Melioidosis can present with an array of manifestations (hence called as **'great mimicker'**); grouped into four types of clinical presentations:
  - *Acute, localized infection:* Presents as localized nodule, fever, general muscle aches, and may progress rapidly to infect the bloodstream
  - *Sub-acute (Pulmonary) infection:* Ranges from mild bronchitis to severe tuberculosis-like pneumonia with cellulitis and lymphangitis
  - *Acute bloodstream infection:* Seen in patients with underlying illness such as HIV, renal failure and diabetes and presents as septicemia (septic shock) with metastatic pus-filled skin lesions and disorientation
  - *Chronic suppurative infection* forming abscesses: Involves various organs such as joints, viscera, lymph nodes, skin, brain, liver, lung, bones, and spleen.
- **Bioterrorism:** *B. pseudomallei* can be used as a potential agent of biological warfare
- **Geographical distribution:** It is estimated that around 1.65 lakh new cases of melioidosis occur worldwide every year with mortality as high as 50%
  - **World:** Melioidosis has been endemic in Thailand, Australia, Singapore, Indian subcontinent and other Southeast Asian countries
  - **India:** It has been reported mainly from South India such as Tamil Nadu, Karnataka, Puducherry and Kerala.
- **Laboratory diagnosis**
  - **Specimen:** It depends on the site of infection, various specimens are collected such as sputum, purulent discharge from lesion, aspirated pus, etc.
  - **Direct microscopy:** They are gram-negative bacilli that typically exhibit a **bipolar or safety pin** appearance (Fig. 32.2A), which is better appreciated when stained with methylene blue
  - **Culture:** *B. pseudomallei* is an obligate aerobe, grows in various media, e.g. nutrient agar, blood agar and MacConkey agar. Colonies are typically rough and corrugated, similar to the colonies of *Pseudomonas stutzeri* (Fig. 32.2B). **Ashdown's medium** is used as a selective medium, where it produces wrinkled purple colonies (Fig. 32.2C)
  - **Biochemical reactions:** Important properties that differentiate it from *Pseudomonas stutzeri* include:
    - Gelatin liquefaction positive
    - Utilizes arginine
    - Positive for intracellular poly β hydroxy butyrate
    - Intrinsically resistant to polymyxin B.
  - **Latex agglutination test:** Cultures can be confirmed by latex agglutination test using specific antisera.

TREATMENT — Melioidosis

Treatment of melioidosis consists of:
- **Intensive phase** (2 weeks): Ceftazidime or a carbapenem is given followed by:
- **Maintenance phase** (12 weeks): Oral cotrimoxazole is given to eradicate the bacilli and to prevent relapse. Doxycycline or amoxicillin-clavulanate are the alternatives.

Contd...

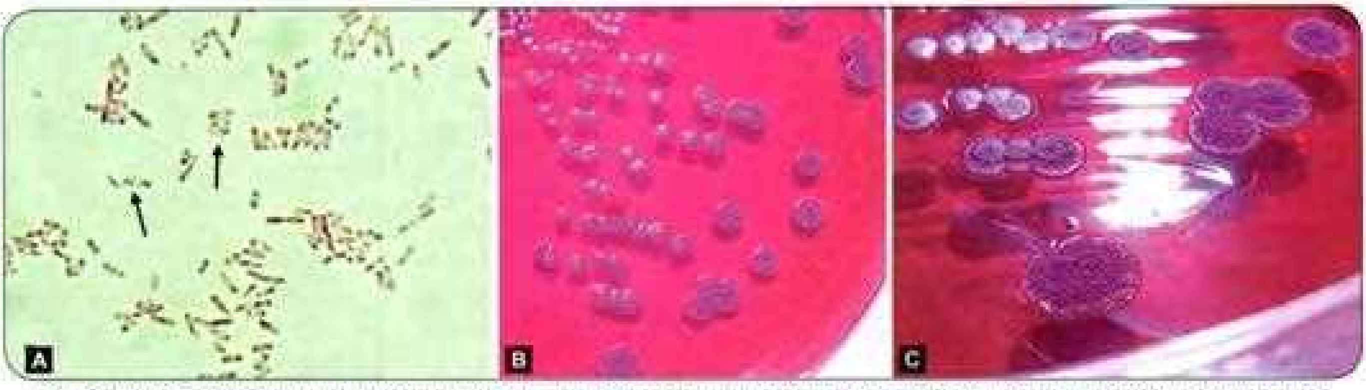


Fig. 32.2A to C: *Burkholderia pseudomallei*: A) Gram stained smear-arrows showing gram-negative bacilli with safety pin or bipolar appearance; B) rough and corrugated colonies on MacConkey agar, C) wrinkled purple colonies on Ashdown's medium

Source: Dr Isabella Princess, Apollo hospitals, Chennai (*with permission*).

Contd...

TREATMENT — **Melioidosis**

- *B. pseudomallei* is intrinsically resistant to many antibiotics including penicillin, first and second-generation cephalosporins, macrolides, rifamycins, colistin and aminoglycosides.
- **Post-exposure prophylaxis:** After exposure (particularly following a laboratory accident); combined treatment with cotrimoxazole and doxycycline is recommended.

Burkholderia mallei

B. mallei is a pathogen of horses; where it causes **glanders** (nasal discharge and ulcers in the nasal septum) and **farcy** (skin lesions and lymph node involvement).

- **Transmission:** Unlike other species, *B. mallei* is not an environmental organism. It is strictly zoonotic, transmitted from horses to man either by direct inoculation or inhalation
- **Human infection** is characterized by:
  - Local skin nodules and lymphadenitis (if transmitted by inoculation)
  - Pneumonia, ulceration of the trachea and sepsis (if transmitted by inhalation)
- **Laboratory diagnosis:** It is similar to that of *B. pseudomallei*. However, *B. mallei* differs from *B. pseudomallei* in being:
  - Non-motile
  - Oxidase negative
  - Inability to grow on MacConkey agar
  - Does not grow at 42°C

  Strauss reaction: It has been seen that intra-peritoneal inoculation of *B. mallei* into guinea pigs can cause testicular swelling.
- **Treatment:** It is same as that of *B. pseudomallei*.

Burkholderia cepacia Complex

B. cepacia complex comprises of 18 closed related genomic species; is currently the most commonly encountered *Burkholderia* species.

- It is an environmental organism that inhabits moist environments, detergents and IV fluids
- It has been recognized as a plant pathogen causing onion rot (cepia, Latin for onion)
- **Typing:** Based on DNA hybridization study, the organism has been typed into nine subgroups or genomovars, of which **type III and II** are associated with most of the cases of cystic fibrosis
- **Virulence factors:** It possesses multiple virulence factors, such as:
  - Cable pilus: A type of fimbriae which is capable of binding to lung mucus
  - Elastase
  - Possesses secretion system like that of *P. aeruginosa*
  - LPS of *B. cepacia* is among the most potent of all bacteria; stimulates inflammatory response in the lungs.
- **Various manifestations** include:
  - **Cepacia syndrome:** It is characterized by a rapidly fatal respiratory infection and septicemia in patients with cystic fibrosis
  - **Nosocomial pathogen:** It is resistant to multiple antibiotics, hence has been emerging as an important nosocomial pathogen in ICUs causing pneumonia, wound infections, etc.
- **Laboratory diagnosis:** Clinical and environmental specimens can be inoculated on selective media. Optimum growth occurs at 30°C. Biochemical reactions can be carried out to differentiate the genomospecies.

- **Prevention:** Implementation of infection control measures is crucial to prevent nosocomial spread of infection (refer contact precaution, Chapter 53).

TREATMENT ***Burkholderia cepacia***

B. cepacia is intrinsically resistant to many antibiotics. Therefore, treatment must be based on the sensitivity report.

- Cotrimoxazole, meropenem, and doxycycline are the most effective agents
- Levofloxacin, ceftazidime-avibactam, ceftolozane-tazobactam are the alternatives.

Acinetobacter

Acinetobacter are saprophytic bacilli, present in the environment (soil, water and phytosphere). However, during the last two decades, it has gained increasing attention as a nosocomial pathogen.

- **Genomospecies**: DNA hybridization studies have shown that *Acinetobacter* can be grouped into several genomospecies (currently, up to 25). The commonly isolated species are:
  - *A.baumannii* (genomospecies 2): It is the most pathogenic species.
  - *A.calcoaceticus* (genomospecies 1): It is a soil saprophyte.
  - *A. lwoffii*: It is a commensal (genomospecies 8).
- **Sources:** Hospital environment is heavily contaminated with these organisms. They are commensals in skin, oral cavity and intestine. The carriage rate is much higher among hospital staff than community
- **Promote colonization:** Unhygienic practices in hospitals (contaminated hands of staff) and warm hospital environment (summers) promote colonization. Patients with underlying diseases or immunosuppression are predisposed to invasion and pathogenesis

Fig. 32.3: Lactose non-fermenting colonies (with faint pink tint) of *Acinetobacter* on MacConkey agar

Source: Department of Microbiology, JIPMER, Puducherry (with permission).

- **Pathogenesis:** It is not fully understood
  - **Multidrug resistance:** Its ability to develop drug resistance rapidly to almost all available antibiotics makes it dangerous in hospital settings
  - **Various virulence factors** are also attributed to pathogenesis such as:
    - Outer membrane protein A (OmpA): It mediates adhesion, invasion and cytotoxicity through mitochondrial damage
    - LPS: It induces inflammatory responses that leads to tissue injury
    - Ability to form biofilm
    - Siderophores (help in iron acquisition).
- **Clinical manifestations:** *A. baumannii* causes widespread hospital infections such as:
  - Ventilator associated pneumonia
  - Central line associated bloodstream infection
  - Post-neurosurgical meningitis
  - Catheter- associated UTI
  - Wound and soft tissue infections
  - Infections in burn patients.
- **Epidemiology:** Several hospital outbreaks due to *Acinetobacter* have been reported throughout the world
- **Laboratory diagnosis:** It is an obligate aerobe, grows well on ordinary medium. Specimens can be inoculated onto blood agar (non-hemolytic colonies) and MacConkey agar (lactose non-fermenting colonies with faint pink tint) (Fig. 32.3). Important identification features are:
  - **Gram staining:** They are gram-negative coccobacilli, arranged singly or in pair, or chain. However, often they appear gram variable or even gram-positive
  - Oxidase negative and catalase positive
  - Non-motile
  - Non-fermenter, utilizes the sugars oxidatively
  - TSI (triple sugar iron agar test) shows alkaline slant/alkaline butt with no gas and no H_2S.
  - Negative for indole and urease test
  - *A. baumannii* can be differentiated from *A. lwoffii* by various properties (Table 32.1).
- **Prevention:** Infection control measures such as improved hand hygiene are essential to prevent nosocomial infections due to *Acinetobacter* (refer contact precaution, Chapter 53).

Table 32.1: Differences between *A. baumannii* and *A. lwoffii*

Properties	*A. baumannii*	*A. lwoffii*
Citrate	Positive	Negative
At 42°C	Grows	No growth
Oxidative-fermentation test (OF) glucose	Shows oxidative pattern	Asaccharolytic
10% lactose	Can ferment	Cannot ferment

TREATMENT ***Acinetobacter***

Acinetobacter is notorious to develop resistance to multiple drugs including β-lactams, aminoglycosides and quinolones.

- β lactam resistance can be attributed to production of β lactamases such as metallo β lactamases (MBL), AmpC β lactamases and OXA-type β lactamases. Choice of antibiotics should always be based on susceptibility reports.
- Most of the hospital acquired isolates are resistant to carbapenems.
- Common antibiotics indicated are β-lactam/β-lactamase inhibitor combinations (e.g. piperacillin-tazobactam), amikacin, tigecycline and colistin.

Stenotrophomonas maltophilia

S. maltophilia is a saprophyte found in the rhizosphere (soil surrounding the plant roots).

- **Colonization:** The organism is acquired from the environment which is favoured by:
  - Immunocompromised conditions
  - Patients on broad-spectrum antibiotics.
- **Clinical manifestations:** *S. maltophilia* can cause various hospital infections such as pneumonia in ventilated patients, blood stream infections and ecthyma gangrenosum in neutropenic patients.
- **Laboratory diagnosis:** It is a non-fermenter that is oxidase negative, motile and utilizes glucose, maltose and lactose oxidatively and decarboxylates lysine.

TREATMENT ***Stenotrophomonas maltophilia***

S. maltophilia is intrinsically resistant to most antibiotics such as all beta lactams including carbapenems. The recommended antibiotics are cotrimoxazole, ticarcillin/clavulanate, colistin, minocycline, tigecycline, and levofloxacin.

Elizabethkingia Meningosepticum

It was formerly called *Chryseobacterium* or *Flavobacterium*.

- **Manifestations:** It is saprophyte in soil, water and hospital environment. However, it causes nosocomial infections in patients with underlying immunosuppression such as:
  - Neonatal meningitis
  - Pneumonia, sepsis, endocarditis, bacteremia and soft tissue infections.
- **Laboratory diagnosis**
  - It is a non-motile, gram-negative bacillus
  - Nonfermenter: It utilizes sugar oxidatively
  - Produces **yellow non-diffusible pigment**
  - Does not grow on MacConkey agar, but grows on nutrient agar.
- **Treatment:** It is susceptible to fluoroquinolones and cotrimoxazole; however β lactams should be given with caution as it produces β lactamases.

Alcaligenes

Alcaligenes faecalis and *Alcaligenes xylosoxidans* (renamed as *Achromobacter xylosoxidans*) are commensals of human intestine and saprophytes in variety of water sources, including well water, and humidifiers; sometimes found as contaminants in IV fluids.

- They are asaccharolytic (however, *A. xylosoxidans* utilizes sugars oxidatively), motile and produce thin spreading irregular colonies.
- They occasionally cause nosocomial outbreaks and pseudo-outbreaks (may be a contaminant in culture) in immunocompromised hosts causing acute otitis, UTI and bacteremia.

EXPECTED QUESTIONS

I. Write short notes on:

1. Virulence factors of *Pseudomonas aeruginosa*.
2. Melioidosis.
3. Medically important nonfermenters.

II. Multiple Choice Questions (MCQs):

1. **Ecthyma gangrenosum is caused by:**
   a. *Pseudomonas* b. *Bordetella*
   c. *Brucella* d. *H. influenzae*
2. **Drug used in *Pseudomonas* treatment:**
   a. Cefixime b. Ceftazidime
   c. Ampicillin d. Cotrimoxazole
3. **Cause of melioidosis is:**
   a. *Burkholderia mallei*
   b. *Burkholderia pseudomallei*
   c. *Burkholderia cepacia*
   d. None
4. **Which of the following pigment is only produced by *Pseudomonas aeruginosa*?**
   a. Pyocyanin b. Pyoverdin
   c. Pyorubin d. Pyomelanin
5. ***Pseudomonas* is differentiated from *Acinetobacter* by all, *except:***
   a. Oxidase positive b. Motile
   c. Non-fermenter d. Pigmented
6. **Which of the following drug is not active against *Stenotrophomonas maltophilia*?**
   a. Cotrimoxazole b. Colistin
   c. Meropenem d. Levofloxacin
7. **Which of the following drug is not active against *Burkholderia*?**
   a. Cotrimoxazole b. Colistin
   c. Meropenem d. Ceftazidime

Answers

1. a **2.** b **3.** b **4.** a **5.** c **6.** c **7.** b

Haemophilus and HACEK Group

33
CHAPTER

Chapter Preview

- *Haemophilus*
  - *Haemophilus influenzae*
  - *Haemophilus ducreyi*
  - *Haemophilus aegyptius*
  - Other *Haemophilus* species
- HACEK group

HAEMOPHILUS

Haemophilus species are oxidase positive, capsulated pleomorphic gram-negative bacilli that require special growth factors present in blood, such as factor X and V (*Haemo* means blood, *philus* means loving). The important species are:

- *H. influenzae*: It is the most pathogenic species, which causes pneumonia and meningitis in children.
- Other species encountered are as follows (Table 33.1)
  - *H. ducreyi*: It causes a sexually transmitted disease called **chancroid,** which presents as genital ulcer
  - *H. aegyptius*: It causes conjunctivitis and rashes
  - *H. haemolyticus* and *H. parahaemolyticus* produce hemolysis on blood agar
  - *H. aphrophilus* and *H. paraphrophilus* (renamed as *Aggregatibacter aphrophilus* and *A. paraphrophilus*)
  - *H. parainfluenzae*.

Table 33.1: Growth characteristics of various *Haemophilus* species

Haemophilus species	Growth requirements			Hemolysis on blood agar**
	X	V	CO_2	
H. influenzae	+	+	–	–
H. aegyptius	+	+	–	–
H. haemolyticus	+	+	–	+
H. ducreyi	+	–	V*	V*
A. aphrophilus	+	–	+	–
H. parainfluenzae	–	+	–	–
H. parahaemolyticus	–	+	–	+
A. paraphrophilus	–	+	+	–

*V, variable; **, horse blood agar ; A., *Aggregatibacter*

HAEMOPHILUS INFLUENZAE

H. influenzae is also called **Pfeiffer's bacillus** as it was discovered by Pfeiffer (1892). The species name was coined wrongly, thinking that it would cause human influenza which is actually a viral disease caused by influenza virus.

Growth Requirements

H. influenzae requires two accessory growth factors present in blood.

1. **Factor X:** It consists of group of heat-stable compounds such as hemin or other porphyrins required for the synthesis of enzymes such as cytochrome, catalase and peroxidase; involved in the aerobic respiration. It is not required when *H. influenzae* grows anaerobically.
2. **Factor V:** It is a heat-labile, nicotinamide adenine dinucleotide (NAD), which is also produced by some animals, plant cells and other bacteria, such as *Staphylococcus aureus*. It was so named as it was thought to be a vitamin. It gets inactivated by NADase present in sheep blood.

The growth of *H.influenzae* vary in different media depending on the availability of X and V factors.

- **Does not grow on ordinary media:** Nutrient agar or peptone water lack X and V factors, hence does not support *Haemophilus* growth
- **Growth is scanty on blood agar:** It is because only factor X is available in this medium and V factor is largely intracellular, present only inside the RBCs. It is available in very minute quantities freely in the medium. More so, sheep blood contains NADase that destroys factor V
- **Grows well on chocolate agar:** While preparing chocolate agar, blood is poured into molten agar at 75°C which inactivates NADase and lyses RBCs releasing excess of factor V. Hence it supports the growth of *H. influenzae*

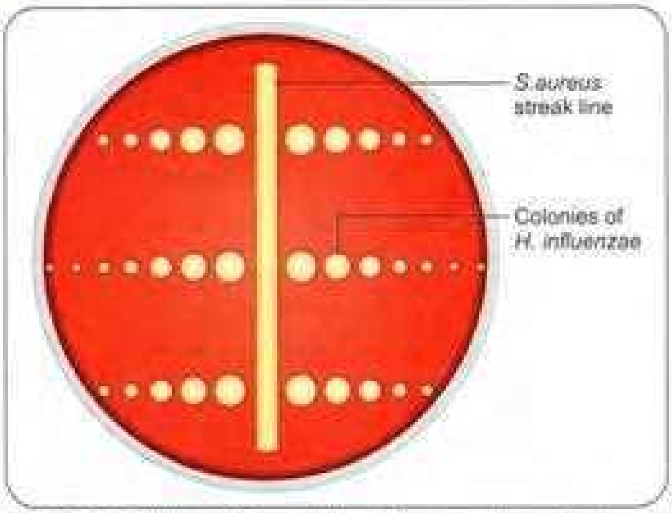


Fig. 33.1: Satellitism of *Haemophilus influenzae* (schematic diagram)

- **Satellitism:** It is observed that, *H. influenzae* can grow on blood agar if the source of V factor is provided (see the box below).

Satellitism

- When *S. aureus* is streaked across a blood agar plate perpendicular to the *H. influenzae* streak line, factor V is released from *S. aureus*. Hence, it forms larger colonies adjacent to *S. aureus* streak line and size of the colonies decreases gradually away from the *S. aureus* streak line
- This phenomenon is called **satellitism**, a property that is routinely employed for the isolation of *H. influenzae* (Figs 33.1 and 33.2B).

Serotyping

Based on the capsular polysaccharide of *H. influenzae*, it can be typed into six serotypes (a to f). However, some strains lack capsule and are referred to as nontypeable strains.

- *H. influenzae* serotype b (*Hib*) is the most virulent among all types and accounts for most of the invasive infections
- Hib capsule has a unique chemical structure, made up of polyribosyl ribitol phosphate (PRP) antigen. It is strongly immunogenic, induces IgG, IgM and IgA antibodies which are bactericidal, opsonic and protective. Hence, PRP antigen is used for vaccination
- Next to Hib, non-typeable strains are commonly isolated clinically. Other capsular serotypes are very rarely isolated
- *H. influenzae* was the first free-living organism whose entire genome was sequenced.

Virulence Factors

Various virulence factors of *H. influenzae* are:

- Capsular polysaccharide is the most important virulence factor, acts by inhibiting phagocytosis
- Endotoxin: It induces host's inflammatory response
- Outer member proteins
- IgA1 proteases: They inactivate IgA1 present on the mucosal surface
- Pili and other adhesion proteins: They are present in all strains including the non-typeable strain, which help in colonization on epithelial surface.

Clinical Manifestations

H. influenzae Type B (Hib)

Hib is the most common and most invasive serotype of *H. influenzae*, causes serious manifestations.

- **Central nervous system infections:**
  - Pyogenic meningitis: It mainly occurs in children less than 2 years of age; characterized by fever, neck rigidity, vomiting, headache and altered sensorium
  - Subdural effusion: It is a common complication following meningitis, characterized by seizures or hemiparesis
  - Mortality rate is high if untreated. Survivors develop neurologic sequelae, such as partial hearing loss and delayed language development.
- **Epiglottitis:** It is a cellulitis of the epiglottis and supraglottic tissues. It is life threatening as it can lead to acute airway obstruction. It typically affects older children (2–7 years old) and rarely adults
- **Pneumonia in infants:** It is clinically similar to other types of bacterial pneumonia except that, pleural involvement is more common in Hib infection
- **Less common** invasive conditions seen in children include:
  - Cellulitis of neck and head region
  - Osteomyelitis, septic arthritis
  - Pericarditis
  - Orbital cellulitis, endophthalmitis
  - Urinary tract infection
  - Bacteremia without an identifiable focus.

Nontypeable H. influenzae

Next to Hib, non-typeable strains are the most common group encountered clinically. They are noninvasive, spread by contagious spread and usually affect adults. The clinical manifestations include:

- Childhood otitis media
- Exacerbations of chronic obstructive pulmonary disease (COPD): They are the most common bacterial cause for this condition.
- Pneumonia in adults with underlying COPD or AIDS.
- Puerperal sepsis and neonatal bacteremia: These infections are caused mainly by non-typeable strains of *biotype IV* that usually colonize the female genital tract.

- Sinusitis in adults and children
- Rarely they cause invasive infections, especially in countries where Hib vaccines are used widely. The differences between Hib and non-typeable *Haemophilus* strains are tabulated in Table 33.2.

Table 33.2: Differences between type b and nontypeable *Haemophilus* strains

Features	Type b strains	Nontypeable strains
Capsule	Made up of poly ribosyl ribitol phosphate (PRP)	Noncapsulated
Manifestations	Invasive—meningitis, epiglottitis, pneumonia, bacteremia, endocarditis	Noninvasive—otitis media (in children) and pneumonia (adult)
Age	Affect children	Affect adult
Spread	Hematogenous spread	Contiguous spread
Vaccine	Hib vaccine is available	Not available

LABORATORY DIAGNOSIS ***Haemophilus influenzae***

- **Specimens:** CSF, blood, sputum
  - Processed immediately, should never be refrigerated
- **Direct examination:**
  - Pleomorphic gram-negative coccobacilli
  - Capsule detection: By Quellung reaction,
  - Antigen detection: By latex agglutination test, direct-IF
- **Culture:**
  - Blood agar with *S. aureus* streak line shows satellitism
  - Others: Chocolate agar, Fildes agar and Levinthal's agar
- Biochemical tests: Disk test for X and V factor requirement
- Biotyping (indole, urease and ornithine test)
- Serotyping (using specific antisera)
- Antimicrobial susceptibility testing

Laboratory Diagnosis

Specimen Collection and Transport

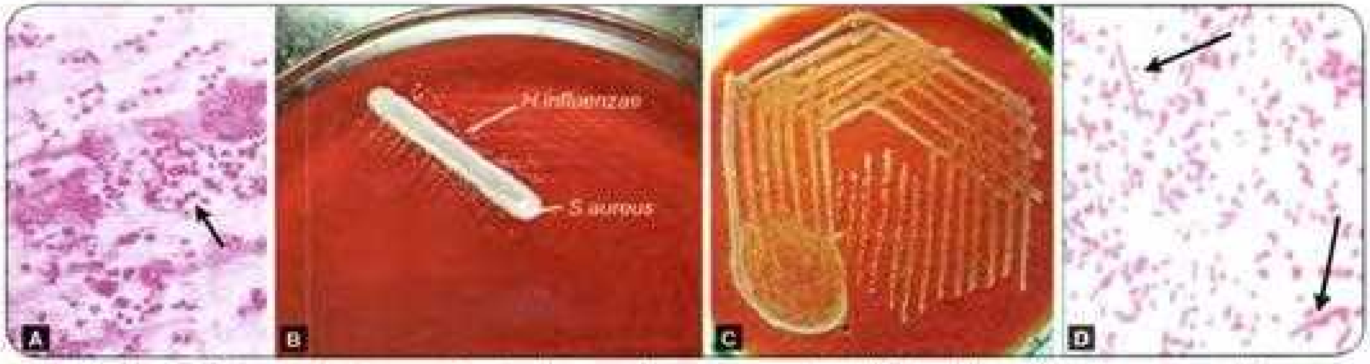
- Depending upon the site of infection, various specimens may be collected such as cerebrospinal fluid (CSF), blood, sputum, pus, aspirates from joints, middle ears or sinuses
- As *H. influenzae* is highly sensitive to low temperature, the specimens **should never be refrigerated**
- The specimens should be transported to the laboratory without any delay and processed immediately.

Direct Detection

- **Gram staining** of CSF and other specimen shows pleomorphic gram-negative coccobacilli (Fig. 33.2A)
- **Capsule detection** (Quellung reaction): Capsular swelling occurs when a drop of CSF is mixed with type b antiserum and methylene blue and observed under microscope
- **Antigen detection:** The type b capsular antigen can be detected in CSF, urine or other body fluids by—(1) latex agglutination test using latex particles coated with antibody to type b antigen or (2) by direct-IF test.

Culture

- **Cultural conditions:** *H. influenzae* is highly fastidious, requires the presence of factor X and V in blood. It is largely aerobic and growth is poor anaerobically. Growth is enhanced by 5–10% CO_2
- **Culture media used are as follows:**
  - Blood agar with *S. aureus* streak line: Colonies of *H. influenzae* grow adjacent to *S. aureus* streak line **(satellitism)** (Figs 33.1 and 33.2B)
  - Chocolate agar: It grows well on chocolate agar but sparsely on blood agar (Fig. 33.2C)



Figs 33.2A to D: A. Gram-stained smear showing pleomorphic gram-negtave bacilli; **B.** Satellitism of *H. influenzae* around *S. aueus* streak line; **C.** Colonies of *H. influenzae* on chocolate agar; **D.** Colony smear showing pleomorphic gram-negative bacilli

Source: A. Department of Microbiology JIPMER, Puducherry; B, C and D. Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (with permission).

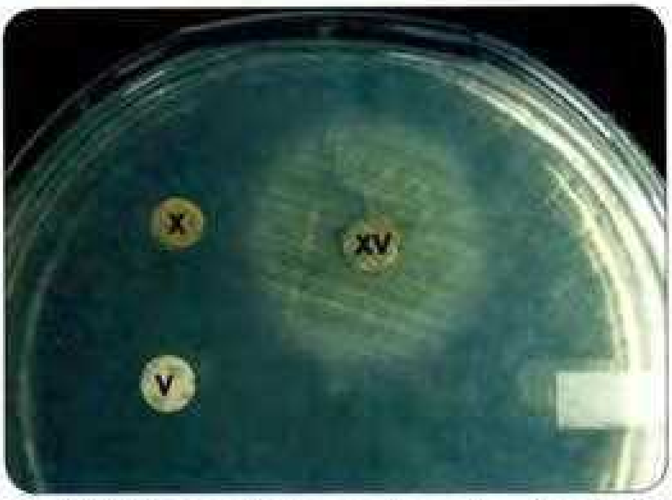


Fig. 33.3: *H. influenzae* showing growth around combined XV disk only, but not around X and V disks

Source: Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (with permission).

- **Fildes agar and Levinthal's agar:** These are transparent media used for *Haemophilus* (produces iridescent colonies). Here, the RBCs are lysed and NADase is inactivated by—(1) adding peptic digest (Fildes agar) or (2) by heat (Levinthal's agar)
- ***Haemophilus* selective medium:** It media contains bacitracin (selective only for *Haemophilus*) and sucrose (differentiates *H. influenzae* from *H. parainfluenzae*, the latter ferments sucrose).

Culture Smear and Motility Testing

Gram staining of culture isolates reveals pleomorphic gram-negative nonmotile bacilli (Fig. 33.2D).

Biochemical Tests

- Catalase positive and oxidase positive
- Reduces nitrate to nitrite
- Ferments glucose and xylose but not sucrose, lactose and mannitol
- **Disk Test for X and V Requirement:** *Haemophilus* species vary in their X and V requirement. This property can be exploited for speciation by inoculating the isolate onto medium lacking X and V factors and then placing the X, V, and combined XV disks on the medium for demonstrating the growth requirements (Table 33.1, Fig. 33.3)
  - Growth surrounding X and XV disks only: *H. ducreyi* and *A. aphrophilus*
  - Growth surrounding V and XV disks only: *H. parainfluenzae*, *H. parahaemolyticus* and *A. paraphrophilus*
  - Growth surrounding combined XV disk only: *H. influenzae*, *H. aegyptius* and *H. haemolyticus* (Fig. 33.3).

Typing Methods

- **Biotyping:** Strains are typed based on three biochemical properties (indole, ornithine decarboxylase and urease).
  - *H. influenzae* has eight biotypes (I-VIII)
  - Most of the clinical isolates belong to type I, II and III
  - Majority of invasive type b strains belong to biotype I.
- **Serotyping:** It is carried out by using type-specific antisera.

Molecular Method

Multiplex PCR has been developed for simultaneous detection of common agents of pyogenic meningitis such as pneumococcus, meningococcus, *Listeria* and *H. influenzae* (targeting conserved capsular gene *bexA*).

Antimicrobial Susceptibility Testing

It is done on *Haemophilus* Test Medium (HTM) or chocolate agar.

TREATMENT ***Haemophilus influenzae***

- Invasive infections due to *Haemophilus influenzae* type b cephalosporins, such as ceftriaxone, cefotaxime are the drug of choice
- Nontypeable strains of *H. influenzae* are often resistant to β lactams [due to β-lactamase production (20–35% of strains) or rarely by expressing altered penicillin binding protein-3]. Those strains are usually susceptible to quinolones (levofloxacin) and macrolides (azithromycin).

Prophylaxis

Hib Conjugate Vaccine

The polyribosyl ribitol phosphate (PRP) capsular antigen of *H. influenzae* type b is used for vaccination.

- As capsular antigens are poorly immunogenic to children, they are conjugated with adjuvants such as diphtheria toxoid, tetanus toxoid and *N. meningitidis* outer membrane proteins
- In addition to eliciting protective antibody, this vaccine can also reduce the rate of pharyngeal colonization with Hib
- **Schedule:** Under national immunization program, Hib vaccine is given in combination with DPT, hepatitis B (pentavalent vaccine) at 6, 10 and 14 weeks of birth. It is administered in IM route, at anterolateral side of mid-thigh
- The widespread use of conjugate vaccines has dramatically reduced the incidence of Hib disease in the developed countries.

Chemoprophylaxis

Oral rifampin is the drug of choice. It is indicated to:
- Household contacts or
- Health care workers (if two or more cases occur in the hospital within 60 days).

OTHER HAEMOPHILUS SPECIES

Haemophilus ducreyi

Haemophilus ducreyi is an etiologic agent of **chancroid (or soft chancre)**, a sexually transmitted infection (STI) characterized by:
- Painful genital ulceration (Fig. 33.4) that bleeds easily; no inflammation of the surrounding skin
- Enlarged, tender inguinal lymph nodes (bubo).

Incubation period of 4–7 days. There is no immunity following the infection; however, hypersensitivity may develop.

Epidemiology

Chancroid is a common cause of genital ulcers in developing countries.
- Transmission is predominantly heterosexual
- Males to females ratio is about 3:1 to 25:1
- Chancroid and HIV: Chancroid increases both the efficiency of transmission and the degree of susceptibility to HIV infection.

Laboratory Diagnosis

- **Specimens:** Exudate or swab from the edge of the ulcer and lymph node aspirate are the useful specimens

Fig. 33.4: Chancroid (painful ulcer)

Source: Public Health Image Library, ID# 15567/ Dr Pirozzi/Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*).

- **Direct microscopy:** *H. ducreyi* is a pleomorphic gram-negative coccobacillus; occurs in groups or in parallel chains
  - They frequently take bipolar staining
  - The arrangement has been described as **school of fish** or **rail road track** appearance.
- **Culture:** *H. ducreyi* requires factor X (hemin), but not factor V for its growth. Primary isolation is difficult. It can be grown on—
  - Rabbit blood agar or chocolate agar enriched with 1% isovitalex and made selective by adding vancomycin
  - It may also be grown on chorioallantoic membrane of the chick embryo.
- **Optimum conditions** required for isolation are 10% CO_2, high humidity and incubation at 35°C for 2–8 days
- **Colony morphology:** Colonies are small, grey, translucent, 1–2 mm in size in 2–3 days
- **Biochemical reactions:** *H. ducreyi* is biochemically inert. Growth surrounding X disk can be used for presumptive diagnosis
- **Slide agglutination test:** *H. ducreyi* is antigenically homogeneous and cultures can be confirmed by agglutination with the antiserum
- A **multiplex PCR** assay has been developed for simultaneous detection of common agents of STIs such as *H. ducreyi* (targeting 16S rRNA), *Treponema pallidum* and herpes simplex virus.

TREATMENT ***Haemophilus ducreyi***

- Drug of choice: Azithromycin (1g oral; single dose)
- Alternative drugs: Ceftriaxone, ciprofloxacin or erythromycin
- Treatment of all the sexual partners is essential

Haemophilus aegyptius

It is also called **Koch-Weeks bacillus**; closely resembles *H. influenzae* biotype III. However, it differs from the latter in having more predilection for conjunctiva and not occurring as pharyngeal carrier.
- *Haemophilus aegyptius* causes:
  - Purulent contagious conjunctivitis (Egyptian ophthalmia)
  - **Brazilian purpuric fever:** A fulminant condition, characterized by fever, purpura, hypotension and shock
- It requires both factors X and V, similar to *H. influenzae*, but differs from the latter by—
  - Fails to ferment xylose
  - Shows hemagglutination with guinea pig RBC at 4°C
  - Slower growth than *H. influenzae*.

Haemophilus parainfluenzae

It is a commensal in mouth and throat.

- Occasionally, it can be an opportunistic pathogen causing endocarditis, conjunctivitis, abscesses, genital tract infections and bronchopulmonary infections in patients with cystic fibrosis
- It differs from *H. influenzae* by:
  - Requires only factor V, but not X
  - Ferments sucrose, but not xylose.

H. haemolyticus and H. parahaemolyticus

They are also commensals in throat (both) or mouth (*H. parahaemolyticus* only).

- They differ from *H. influenzae* in being β hemolytic, which is best produced in sheep or ox blood agar and when incubated aerobically
- *H. parahaemolyticus* is a rare cause of endocarditis
- *H. haemolyticus* requires both factors X and V, where as *H. parahaemolyticus* requires only factor V.

Aggregatibacter aphrophilus and A. paraphrophilus

They are capnophilic and require 5–10% of CO_2 for optimum growth.

- *A. aphrophilus* requires only factor X, whereas *A. paraphrophilus* requires only factor V
- They are commensals of mouth and occasionally cause endocarditis, head and neck infections, invasive bone and joint infections.

HACEK GROUP

HACEK is an abbreviation used to represent a group of highly fastidious, slow-growing, capnophilic, gram-negative bacteria, that normally reside in the oral cavity as commensal, but occasionally have been associated with local infections of the mouth and systemic infections such as bacterial endocarditis.

Species belonging to this group include:

- ***Haemophilus parainfluenzae***
- ***Aggregatibacter species:*** *A.actinomycetemcomitans, A.aphrophilus and A.paraphrophilus*
- ***Cardiobacterium hominis***
- ***Eikenella corrodens***
- ***Kingella kingae.***

HACEK ENDOCARDITIS

It accounts for 3% of total endocarditis cases.

- Typically has a subacute course
- Occurs in patients with preexisting valvular defects or those undergoing dental procedures
- The aortic and mitral valves are most commonly affected.

Laboratory Diagnosis

The laboratory diagnosis of HACEK endocarditis is as follows:

- They are highly fastidious, require special media
- They are capnophilic, growth is optimum in presence of 5–10% of CO_2
- Incubation up to 30 days may be required. However, the detection time may be reduced to 1 week if automated culture systems such as BacT/ALERT are used
- **Molecular methods:** Simultaneous detection of HACEK members from clinical specimen is possible by performing (i) broad-range bacterial PCR targeting 16S rRNA gene followed by sequencing; (ii) multiplex PCR or multiplex real-time PCR
- The *Haemophilus* species of HACEK group are described in the preceding text. Other agents are described below.

Aggregatibacter actinomycetemcomitans

(Former name: *Actinobacillus actinomycetemcomitans*).

It is the most common member of HACEK to cause endocarditis.

- It can also be isolated from soft tissue infections and abscesses associated with *Actinomyces israelii*
- Rarely, it causes periodontitis, brain abscess, meningitis and endophthalmitis
- **Laboratory diagnosis:**
  - It is small non-motile gram-negative coccobacillus
  - It grows on blood agar supplemented with 5% defibrinated horse blood
  - Catalase and nitrate positive
  - Ferments only glucose, galactose and maltose
  - Negative for indole, citrate, urease and decarboxylase tests.

Cardiobacterium hominis

It frequently affects the aortic valve. It is also associated with arterial embolization, immune complex glomerulonephritis or arthritis.

Laboratory diagnosis:

- It is non-motile, non-capsulated pleomorphic and gram-negative bacillus
- It grows on blood agar under 3–5% CO_2 and high humidity
- It ferments a wide range of sugars and forms indole
- Oxidase positive, but catalase and nitrate negative.

Eikenella corrodens

It is small slender non-capsulated gram-negative bacillus. Apart from endocarditis, it also occasionally causes skin and soft tissue infections.

Laboratory diagnosis:

- It lacks flagella, but shows **twitching or jerky** motility which is due to contraction of fimbria.

- The name 'corrodens' refers to the characteristic **pitting or corroded colonies** on blood agar.
- It is oxidase-positive, catalase negative
- Positive for lysine and ornithine decarboxylase tests
- Indole, citrate and urease tests: negative.

Kingella kingae

It also causes infections of bones, joints and tendons.

- Non-motile, and gram-negative; exists as as coccobacillary and diplococcal forms
- Oxidase-positive, catalase negative

TREATMENT **HACEK endocarditis**

The prognosis of HACEK endocarditis is good.

- Ceftriaxone (2 g/day) is the drug of choice for most of the HACEK organisms except *Eikenella corrodens* where ampicillin is indicated.
- Quinolones are given if the strain is a β-lactamase producer.
- Duration of treatment: Antibiotics are given for 4 weeks for native valve endocarditis and 6 weeks for prosthetic-valve endocarditis.

EXPECTED QUESTIONS

I. Write short notes on:

1. Satellitism.
2. Chancroid.
3. HACEK group.

II. Multiple Choice Questions (MCQs):

1. **Which of the following agent of meningitis can grow on chocolate agar but not on blood agar?**
   a. *Neisseria meningitides*
   b. *Haemophilus influenzae*
   c. *Moraxella catarrhalis*
   d. *Escherichia coli*
2. ***Haemophilus influenzae* grows on all of the following media, *except*:**
   a. Chocolate agar
   b. Fildes' agar
   c. Nutrient agar
   d. Blood agar with *S.aureus* streak line
3. **HACEK group includes all, *except*:**
   a. *Kingella kingae*
   b. *Haemophilus influenzae*
   c. *Eikenella corrodens*
   d. *Cardiobacterium hominis*
4. **All the following species of *Haemophilus* show satellitism, *except*?**
   a. *H. influenzae* b. *H. aegyptius*
   c. *H. haemolyticus* d. *H. ducreyi*
5. **Agent of chancroid:**
   a. *H. influenzae*
   b. *H. aegyptius*
   c. *H. ducreyi*
   d. *H. haemolyticus*
6. **Polyribosyl ribitol phosphate (PRP) antigen is present in the capsule of *H. influenzae*:**
   a. Serotype a b. Serotype b
   c. Serotype c d. Serotype d
7. **Next to Hib, which strains are the most common group of *H. influenza* encountered clinically?**
   a. Serotype a strains b. Serotype c strains
   c. Serotype d strains d. Non-typeable strains
8. **Which specimen should never be refrigerated?**
   a. Urine b. Sputum
   c. CSF d. Pus
9. **The drug of choice for chemoprophylaxis for *H. influenzae* is:**
   a. Rifampin b. Ceftriaxone
   c. Penicillin d. Erythromycin
10. **Characteristic pitting or corroded colonies on blood agar is produced by:**
    a. *Aggregatibacter* b. *Cardiobacterium*
    c. *Eikenella* d. *Kingella*

Answers

1. b **2.** c **3.** b **4.** d **5.** c **6.** b **7.** d **8.** c **9.** a **10.** c

Bordetella

34
CHAPTER

Chapter Preview

- *Bordetella pertussis*
  - Virulence factors
- Clinical manifestations
- Laboratory diagnosis
- Treatment and prevention

Bordetella is highly fastidious, very small, gram-negative coccobacillus, described first by Bordet and Gengou in 1906. It is a non-fermenter, belongs to family Alcaligenaceae. It comprises of several species (Table 34.1).

- *Bordetella pertussis*: It causes whooping cough in children, a highly contagious vaccine preventable bacterial disease, characterized by paroxysmal cough ending in a high-pitched inspiratory sound described as "whoop"
- *B. parapertussis*: It causes milder form of whooping cough
- *B. bronchiseptica*: It is a pathogen of domestic animals that causes kennel cough in dogs, atrophic rhinitis and pneumonia in pigs, and pneumonia in cats. Rarely, respiratory infections in humans have been reported

Table 34.1: Characteristics of *Bordetella* species

Features	*B. pertussis*	*B. parapertussis*	*B. bronchiseptica*
Host	Humans	Humans, sheep	Mammals
Disease	Whooping cough	Whooping cough (mild)	Various respiratory diseases
Growth on			
Blood agar	–	+	+
MacConkey	–	+/–	+
Motility	–	–	+
Oxidase	+	–	+
Urease	–	+ (24 hours)	+ (4 hours)
Nitrate reduction	–	–	+
Toxin			
Pertussis toxin	+	–	–
Others*	+	+	+

*Other toxins include filamentous hemagglutinin, fimbriae, pertactin, adenylate cyclase, lipopolysaccharide, tracheal cytotoxin, *brkA* protein

- *B. avium*: It causes respiratory disease in turkeys
- *B. hinzii* and *B. holmesii*: They occasionally cause bacteremia in immunocompromised people.

BORDETELLA PERTUSSIS

B. pertussis causes a violent paroxysmal productive cough in children called **whooping cough** or **100 days fever**.

Virulence Factors

B. pertussis produces a wide array of toxins and biologically active products that are important in its pathogenesis and in immunity. Most of these virulence factors are under the control of a single genetic locus that regulates their production.

Toxins

- **Tracheal cytotoxin:** It is a part of cell wall peptidoglycan, which causes damage to the cilia of respiratory epithelial cells by producing interleukin-1 and nitric oxide intracellularly
- **Pertussis toxin:** (See the box below)
- **Adenylate cyclase toxin**: It activates cyclic AMP, which impairs the host immune function
- **Dermonecrotic toxin**: It may contribute to the respiratory mucosal damage
- **Endotoxin:** It has properties similar to those of other gram-negative bacterial LPS
- **Adhesins:** They play a role in bacterial attachment. Examples include:
  - Filamentous hemagglutinin (FHA)
  - Pertactin, an outer-membrane protein
  - Fimbriae or pili or agglutinogens
  - **BrkA** (*Bordetella* resistance to killing) protein: It mediates the serum resistance and adhesion.

Pertussis Toxin (PT)

It is the most important virulence factor, and is expressed only by *B. pertussis*. *B. parapertussis* and *B. bronchiseptica* possess the genes coding for PT, but due to mutation in the promoter region of the genes, they do not express PT.

Mechanism: PT is similar to cholera toxin in its structure and is composed of A and B subunits.

- **B subunit:** It is pentameric, responsible for binding to target cells and inserting A-subunit into the cytoplasm
- **A subunit:** It is the active subunit, which causes ADP ribosylation of G protein, which activates adenylyl cyclase, leading to ↑ concentrations of cAMP; which is responsible for producing a variety of biologic effects, such as:
  - T cell mitogenicity
  - Hemagglutination
  - Adhesion to respiratory ciliated cells
  - Inhibition of neutrophil oxidative burst, monocyte migration, histamine release from mast cells
  - Induction of leukocytosis
  - Enhancement of insulin secretion leading to hypoglycemia.

Clinical Manifestations

The clinical course of whooping cough (or pertussis) passes through three stages following an incubation period of 7–10 days.

- **Catarrhal phase:** It lasts for 1–2 weeks, and is characterized by common cold like nonspecific symptoms, such as coryza, lacrimation, mild cough, low-grade fever and malaise. It is highly infectious stage. In this stage, both smear and cultures are likely to be positive.
- **Paroxysmal phase:** In this stage, patients are less infectious; smear and culture may become negative. It is characterized by specific symptoms, such as whooping cough and post-tussive vomiting (see the below box).
- **Convalescent stage:** It occurs following the paroxysmal stage, during which the frequency and severity of coughing gradually decreases. Antibodies may appear in serum.

Whooping Cough

Each paroxysm consists of bursts of 5–10 repetitive violent spasmodic coughs, often within a single expiration which ends with an audible sound or whoop. Whoop occurs due to rapid inspiration against a closed glottis at the end of the paroxysm (Fig. 34.1A).

- Paroxysms may be precipitated by noise, eating or physical contact. In between the paroxysms, patient may appear to be normal
- The frequency of paroxysms varies widely, from several per hour to 5–10 per day
- Episodes are often worse at night
- During a spasm, there may be visible neck vein distension, bulging of eyes, tongue protrusion and cyanosis
- Weight loss may be seen, but fever is uncommon.

Complications

Complications are more common among infants than among older children or adults.

- **Pressure effects** during the violent spasms of coughing results in subconjunctival hemorrhage, hernias, pneumothorax, rib fracture and petechiae on the face and body.
- **Pneumonia** may develop especially in old age, due to secondary infection due to encapsulated bacteria. If occurs in infants, it is usually due to *B. pertussis*.
- **Neurological complications**, such as convulsions, encephalopathy and coma may also occur.

Differential Diagnosis

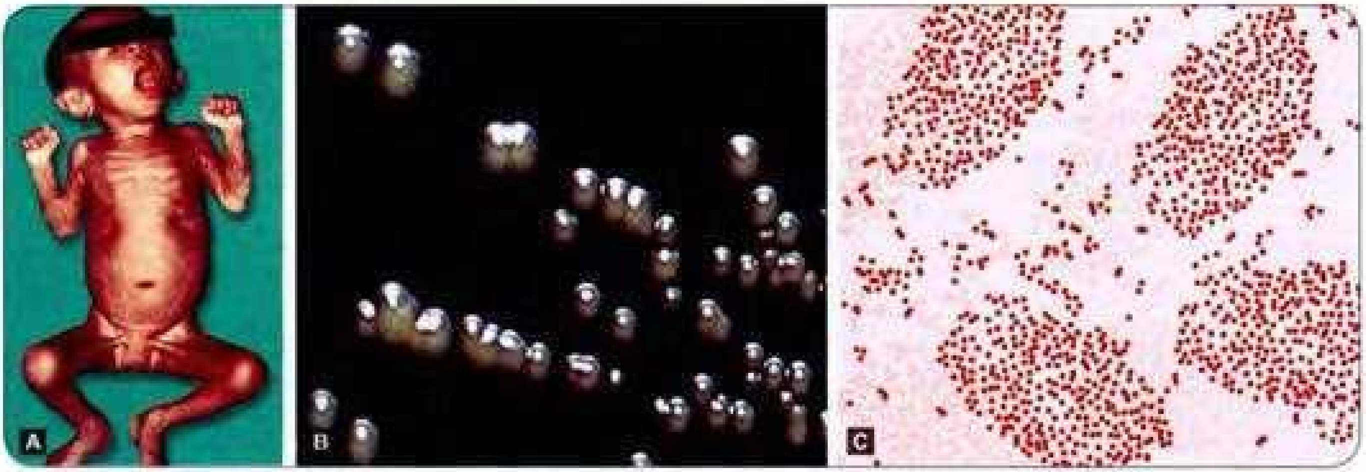
Whooping cough like symptoms may be seen with:

- *Mycoplasma pneumoniae*
- *Chlamydophila pneumoniae*
- Adenovirus
- Influenza and other respiratory viruses
- Use of angiotensin-converting enzyme (ACE) inhibitors
- Reactive airway disease
- Gastroesophageal reflux disease

Epidemiology

Whooping cough is an exclusively human disease. There is no animal reservoir.

- **Source:** Early cases (catarrhal stage) are the main source of infection. Previously, it was believed that there is no carrier state. However, recent molecular studies proved that a transient carrier state in nasopharynx occurs among children following the disease, which may contribute to the spread of the infection. However, there is no evidence of a long-term carrier state.
- **Age:** Whooping cough is predominantly a disease of preschool children below 5 years. As the maternal antibodies are not protective, infants remain the most vulnerable group, accounting for highest morbidity and mortality
- **Shift of median age:** Pertussis has shifted from infants to older children and adolescence in countries with high vaccination coverage. This indicates that pertussis immunizations or natural infection do not provide lifelong immunity
- **Mode of transmission:** It is via inhalation of droplets (by coughing or sneezing or even talking) or rarely through direct contact
- **Recent outbreaks:** Several outbreaks have been reported recently; which includes the Washington epidemic in 2012 and California epidemic in 2014
- **Worldwide,** the incidence of pertussis is declining. WHO estimated around 1,39,535 cases of pertussis in 2016 and 1,42,512 in 2015 with 89,000 deaths. Most deaths occurred in unvaccinated children; although WHO reported an estimated global vaccine coverage of 86% in 2016



Figs 34.1A to C: A. Female infant suffering from pertussis; **B.** Colonies of *Bordetella pertussis* on Regan-Lowe agar (mercury drops appearance); **C.** Gram-stained smear (schematic) of *Bordetella pertussis* (thumb print appearance)

Source: A. Public Health Image Library, ID# 6379/Centers for Disease Control and Prevention (CDC), Atlanta; B. American Type Culture Collection (ATCC)® 9797 (with permission).

- **India:** There is marked decline of the disease after launch of the vaccine under universal immunization programme in India
- There is no cross protection to *B. parapertussis* infection.

LABORATORY DIAGNOSIS	*Bordetella pertussis*

- **Specimen:** Nasopharyngeal secretions, collected by alginate swabs
- **Direct smear:** Gram-negative coccobacilli and pus cells
- **Culture:**
  - Regan-Lowe medium and Bordet-Gengou agar
  - Produces mercury drops or bisected pearls colony.
- **Culture smear:** Reveals small, ovoid gram-negative coccobacilli arranged in 'thumb print' appearance
- **Detection of serum antibodies:** By enzyme immunoassays
- **PCR:** Detecting IS481 and PT promoter region genes
- **Typing of *B. pertussis*:** By serotyping and genotyping.

Laboratory Diagnosis

- **Specimen collection**
  - Nasopharyngeal secretions are the best specimens which may be obtained by—
    - Nasopharyngeal aspiration (best method)
    - Pernasal swab (by using a sterile swab on a flexible wire).
  - Throat and/or sputum specimens are unacceptable
  - **Type of swabs used:** For culture, alginate swabs are the best followed by dacron swabs. However, for PCR, only dacron or rayon swabs are recommended
    - Cotton swabs are not satisfactory as fatty acids present in cotton may inhibit the growth of the bacilli. However charcoal-impregnated cotton swabs (Stuart's) may be useful
    - It is recommended to collect **six swabs,** at 1–2 days intervals to achieve maximum yield.
  - Cough plate method and post-nasal swabs used before are no longer recommended
  - **Transport:** Specimens should be processed immediately. If delay is expected, then suitable charcoal-based medium (Amies, Regan-Lowe, Jones-Kendrick transport media) can be used.
- **Direct detection:** *B. pertussis* may be directly detected from nasopharyngeal secretions by direct immunofluorescence test using fluorescein labelled polyclonal or monoclonal antibodies. Because of poor sensitivity (18-75%) and specificity (65-99%), it is not widely used
- **Culture:** Nasopharyngeal culture remains the gold standard method of diagnosis
  - *B. pertussis* is a strict aerobe, grows best at 35-37°C. It is fastidious, requires special complex media for primary isolation, such as—
    - Charcoal agar supplemented with 10% horse blood and cephalexin **(Regan and Lowe medium).** It is currently the medium of choice
    - Bordet-Gengou glycerine-potato-blood agar was a traditional medium used before
    - Other media: Jones Kendrick agar and Stainer-Scholte synthetic medium.
  - Colonies are greyish white, convex with a shiny surface appear after 3–5 days, described as **mercury drops** or **bisected pearls appearance** (Fig. 34.1B)
  - Culture remains positive only during the first three weeks of infection (i.e. mainly in catarrhal stage) where the symptoms are nonspecific
  - Culture becomes negative within 5 days of starting antibiotics.
- **Culture smear:** Gram-staining of culture reveals small, ovoid coccobacilli (0.5 µm), tend to arrange in loose clumps, with clear spaces in between giving a **thumb print** appearance (Fig. 34.1C)

- Capsules may be demonstrated in fresh cultures, but are often lost on sub-culturing
- Bipolar metachromatic granules may be demonstrated on staining with toluidine blue.

❖ **Detection of serum antibodies:** Enzyme immunoassays (EIAs) using purified antigens of *B. pertussis*, such as PT, FHA and pertactin are the methods of choice
- Demonstration of a rise of IgG antibodies in paired sera or detection IgA or IgM antibodies provides definite diagnosis
- However, antibodies are also elevated in immunized people.

❖ **Molecular methods:** PCR is being increasingly used in many laboratories replacing culture, because of increased sensitivity, specificity and quicker results. The most common targeted genes are IS481 and the PT promoter region genes

❖ **Typing of *B. pertussis*:** It is important during outbreak investigation to find out the epidemiological link between the isolates
- **Serotyping:** It is based on two fimbrial antigens (type 2 and 3) and one lipooligosaccharide antigen (type 1), of *Bordetella pertussis*. Out of the four recognized serotypes (1,2,3; 1,2; 1,3; and 1) of *B. pertussis*, only the first three types (possessing fimbriae) infect man
- **Genotyping:** It can be carried out by gene sequencing, and pulsed-field gel electrophoresis (PFGE).

❖ **Others:** Lymphocytosis is common among young children but not among adolescents.

TREATMENT **Pertussis**

As pertussis is mainly toxin mediated, antibiotics are less useful once the infection is established. However, they play a vital role to eliminate the bacteria from nasopharynx.

- Macrolides are the drugs of choice (azithromycin for 5 days or erythromycin for 7–14 days)
- Cotrimoxazole is recommended as an alternative in macrolide resistance.

Isolation in a quiet environment may inhibit the stimulation of paroxysms. Cough suppressants are not much effective.

Prevention

Chemoprophylaxis

Erythromycin is widely recommended as chemoprophylaxis for household contacts of pertussis cases.

Vaccine

Whole-cell Pertussis Vaccines

It is prepared by heating followed by chemical inactivation and purification of whole *B. pertussis* bacilli.

❖ Efficacy is good, average being 85%

❖ **DPT vaccine:** In India and many other countries, whole cell (WC) pertussis vaccine is given under national immunization programme, along with diphtheria toxoid and tetanus toxoid. Three doses of pentavalent vaccine (DPT, hepatitis B and *H. influenzae*) are given at 6, 10 and 14 weeks, followed by two boosters of DPT at 1½ years and 5 years. *Pertussis* component acts as an adjuvant and increases immunogenicity of DT and TT

❖ **Adverse effects:** WC vaccine is associated with the following adverse effects, such as:
- Common: Fever, injection-site pain, erythema, swelling, and irritability
- Uncommon: *Bordetella pertussis* is encephalitogenic. It is associated with neurological complications (encephalitis, prolonged convulsion) and **hypotonic hyporesponsive syndrome**. The estimated risk is 1:1,70,000 doses administered.

❖ **Contraindication:** Because of the adverse effects, the WC vaccine is contraindicated in—
- Children more than 5-6 years age
- Any associated progressive neurological conditions
- Children with strong family history of epilepsy
- Hypersensitivity to previous dose.

Acellular Pertussis Vaccine

It is composed of pertussis toxoid and 2 or more other bacterial components such as FHA, pertactin or fimbriae

❖ Though the efficacy is same as WC vaccine, it is associated with fewer side effects as compared with the latter and can be safely given after 5-6 years

❖ It is available as DapT (along with diphtheria and tetanus toxoid).

EXPECTED QUESTIONS

I. Write short notes on:
1. Virulence factors of *Bordetella pertussis*.
2. Laboratory diagnosis of pertussis.
3. Vaccination against pertussis.

II. Multiple Choice Questions (MCQs):
1. **Mercury drop appearance colony of *B. pertussis* is seen on which of the following culture media?**
   a. Blood agar b. Chocolate agar
   c. Regan-Lowe agar d. Nutrient agar
2. **Pertussis toxin is produced by:**
   a. *B. pertussis* b. *B. parapertussis*
   c. *B. avium* d. *B. bronchiseptica*
3. **Which is the highly infective stage in whooping cough?**
   a. Catarrhal stage b. Paroxysmal stage
   c. Convalescent stage d. All of the above

Answers

1. c **2.** a **3.** a

Brucella

35
CHAPTER

Chapter Preview

- Introduction
- Pathogenesis
- Clinical manifestations
- Epidemiology
- Laboratory diagnosis
- Treatment and prevention

INTRODUCTION

Brucella is an obligate aerobic, fastidious, nonsporing, nonmotile, noncapsulated small gram-negative coccobacillus, responsible for a highly contagious febrile illness called **brucellosis**.

- Brucellosis (also called **undulant fever**) is primarily a zoonotic disease affecting various domestic animals, such as sheep, goat or cattle
- Human infection is usually associated with occupational or domestic exposure to infected animals or their products.

History

- *Brucella* was named after British army physician Sir David Bruce (1886), who isolated the first recognized species, *Brucella melitensis* (*melita* is Roman name for Malta) from Malta Island (Europe); hence the disease was called Malta fever
- Danish physician Bernhard Bang (1897) isolated *Brucella abortus* from cattle (Bang's disease)
- *Brucella suis* was isolated from aborted swine in 1914 by Jacob Traum from USA.

Nomen System of Classification

Brucella belongs to the family Brucellaceae.

- DNA hybridization studies reveal that the members of the genus *Brucella* are very closely related and probably represent variants of a single species
- However, for the sake of convenience, these have been classified into **nomen species**, based on various properties, as given in Table 35.1
- **Nomen species:** Currently there are six nomen species identified, among which few are further classified into several biovars (Table 35.1)
  1. *B. melitensis:* It has 3 biovars. It is usually pathogenic to sheep, goat and camel. Man is also a susceptible host.
  2. *B. abortus:* It has 9 biovars and infection is acquired from cattle and buffalo.
  3. *B. suis:* It has 5 biovars, and they infect most often pigs, but some biovars may infect reindeers and rodents.
  4. *B. canis:* It causes abortion in dogs. Occasional cases of human infection have been reported.
  5. *B. ovis:* It causes reproductive disease in sheep.
  6. *B. neotomae:* It infects desert rodents.

Antigenic Structure

Brucellae have two major types of lipopolysaccharide (LPS) antigens designated as **M** and **A**.

- They are present in varying proportion in the three major species of *Brucella*; however, one of them is predominant in each species
  - In most of the biovars of *B. melitensis*, M antigen is predominant
  - In most of the *B. abortus* biovars, A antigen is predominant
  - *B. suis* biovars contain either M or A antigens.
- The virulent colonies on primary isolation are smooth and possess the LPS antigen. However, on repeat subcultures, strains may lose LPS and become rough strains which may not agglutinate with anti-M or anti-A antisera
- *B. canis* produces rough (R) strains even on primary isolation, which do not agglutinate with antisera to M or A antigen but agglutinate with antiserum prepared against R strains.

PATHOGENESIS

B. melitensis is the most pathogenic species, followed by *B. abortus* and *B. suis*. Human infection with other species is extremely rare.

Table 35.1: Differentiating characters of *Brucella* species

Nomen species	Biovars	CO_2 requirement	H_2S production	Urease	Growth on media containing dyes[a]		Lysis by bacteriophage at RTD			Agglutination by antiserum[b]		Common animal reservoir
					Thionin	Basic fuchsin	Tbilisi	Weybridge	Berkeley	A	M	
B. melitensis	1	–	–		+	+				–	+	
	2	–	–	Variable	+	+	NL	NL	L	+	–	Sheep, goats
	3	–	–		+	+				+	+	
B. abortus	1	(+)	+		–	+				+	–	
	2	(+)	+		–	–	L	L	L	+	–	
	3	(+)	+		+	+				+	–	
	4	(+)	+	Slow	–	(+)				–	+	Cattle
	5	–	–		+	+				–	+	
	6	–	(+)		–	+				+	–	
	9	–	+		+	+				–	+	
B. suis	1	–	+		+	(–)				+	–	Pig
	2	–	–		+	–	NL	L	L	+	–	Pig, hare
	3	–	–	Rapid	+	+				+	–	Pig
	4	–	–		+	(–)				+	+	Reindeer
	5	–	–		+	–				–	+	Rodents
B. canis[c]		–	–	Rapid	+	–	NL	NL	NL	–	–	Dogs
B. ovis		+	–	–	+	(–)	NL	NL	NL	–	–	Sheep
B. neotomae		–	+	Rapid	–	–	NL	L	L	+	–	Desert wood rat

+, Positive; –, negative; (+), most strains positive; (–), most strains negative. [a]Dye concentration, 20 µg/mL.

[b]A, monospecific antisera to *B.abortus* A antigen, M, monospecific antisera to *B. melitensis* M antigen; [c]*B.canis* can agglutinate with antiserum prepared against rough (R) strains; L, lysis; NL, no lysis at routine test dilution (RTD) by *Brucella* phages

Transmission

Brucellosis is usually transmitted from infected animals to man. There is no evidence of man-to-man transmission. The various modes of transmission are:

- **Direct contact:** The most common route is direct contact with the infected animal tissue, blood, urine, vaginal discharge or placenta with abraded skin or mucosa of men, who are involved in animal handling
- **Food-borne:** By ingestion of raw milk or dairy products from infected animals or rarely vegetables or water contaminated with animal excreta
- **Air-borne:** By inhalation of dust or aerosols in the infected cowshed or slaughter houses.

Spread

From the initial site of infection, the organisms spread via lymphatic vessels → infect the local lymph glands → organisms spillover to bloodstream results in bacteremia → disseminate throughout to involve various organs.

Organs Involved

Brucellae are facultative intracellular pathogens, primarily infecting organs of the **reticuloendothelial system**, such as lymph nodes, spleen, liver and bone marrow which are rich in mononuclear phagocytic cells (macrophages and monocytes).

- They also have a special predilection for placenta as their growth is enhanced in the presence of erythritol present in placenta
- Musculoskeletal tissues and genitourinary systems are also frequently targeted
- Local tissue response: Initially, an acute neutrophilic infiltration occurs. Later on, it is replaced by chronic inflammatory cells leading to granuloma formation with or without necrosis and caseation.

Intracellular Survival and Virulence Factors

The **cell-wall LPS** appears to be the major virulence factor. It has a distinct O-chain and core-lipid composition. Though the endotoxin activity is relatively low, it plays a key role in:

- Providing resistance to phagocytosis and serum complement-mediated killing of bacteria
- Suppressing phagosome-lysosome fusion
- Diverting the internalized bacteria into vacuoles located in endoplasmic reticulum, where intracellular replication takes place
- Development of pyrogenicity.

Other virulence factors are as follows:

- Type IV secretion system (VirB): It is a type of secretory system present in brucellae, that regulates intracellular survival in phagosomes and trafficking
- Cu-Zn superoxide dismutase: It is expressed by *B. abortus* that inhibits reactive oxygen radicals
- Nucleotide-like substances have been recovered from *B. abortus* that inhibit phagolysosome fusion and reactive oxygen radicals produced from neutrophils.

Host Immune Response

Cell-mediated immunity (CMI) is the key to control the infection.

- Activation of T helper-1 (T_H1) cells provides protective immunity by production of interferon γ, which leads to macrophage activation and killing of intracellular *Brucella*
- On the other hand, T_H2 activation leads to secretion of IL-4, IL-6 and IL-10, that downregulates the protective T_H1 immune response and stimulates humoral immunity
- However, antibodies play only a minor role as they are active only in extracellular milieu. Once the brucellae are internalized, antibodies are ineffective.

CLINICAL MANIFESTATIONS

The incubation period varies from 1 week to several months and the onset is either abrupt or more often insidious.

- **Classic triad:** Though the manifestations vary, the classic triad of fever with profuse night sweats, arthralgia/arthritis and hepatosplenomegaly are present in most patients
- **Typhoid-like illness:** Overall brucellosis resembles typhoid-like illness except that it is less acute, less severe with undulating pattern of fever and more musculoskeletal symptoms
- **Undulating fever**: Fever has a typical remittent course, i.e. in between febrile periods (which last for weeks), there will be afebrile periods. It is also called Malta fever or Mediterranean fever
- **Musculoskeletal symptoms** are present in about one-half of all patients, which may mimic skeletal tuberculosis
  - Vertebral osteomyelitis involves lumbar and low thoracic vertebrae commonly
  - Septic arthritis: Most commonly affected joints are knee, hip, sacroiliac and shoulder joints.
- **Other nonspecific symptoms:** These include abdominal pain, headache, diarrhea, rash, weakness/fatigue, weight loss, vomiting, cough, pharyngitis, and refusal to eat (children)
- **CNS:** Depression and lethargy with meningitis or lymphocytic meningoencephalitis are the most common neurological manifestations
- **CVS:** Endocarditis may occur rarely, affecting the aortic valve
- **Genitourinary manifestations:** These include acute epididymo-orchitis, prostatitis, salpingitis and pyelonephritis.

All the nomen species produce similar manifestations; but exhibit slight variations between each other, e.g.

- *B. melitensis* tends to produce more acute and aggressive presentation
- *B. suis* produces focal abscess
- *B. abortus* tends to be more insidious and chronic
- *B. canis* produces acute gastrointestinal symptoms.

EPIDEMIOLOGY

Brucellosis is a worldwide zoonotic disease. The occurrence in humans is closely related to their prevalence in various domestic animals.

- **Endemic area:** Human brucellosis is endemic in areas where animals are raised in large numbers, such as countries of Mediterranean zone, Eastern Europe, Central Asia, Mexico and South America
- The disease is rare in most European countries, Australia and North America
- **Prevalence:** The true prevalence of human brucellosis is difficult to estimate. Many cases are under-reported either because they are inapparent or due to difficulty in diagnosis
- **Sources of infection** are:
  - Infected animals excreting the organisms in urine, milk, placenta or vaginal discharge
  - Contaminated animal food products, such as dairy products, especially soft cheeses, milk, icecream and rarely raw meats and bone marrows.
- **People at higher risk** are farmers, shepherds, goatherds, butchers and abattoir workers in endemic areas (occupationally exposed to infection).

LABORATORY DIAGNOSIS *Brucella melitensis*

- **Specimens:** Blood, bone marrow, etc.
- **Culture medium:**
  - Castaneda's biphasic media (BHI broth/agar)
  - Automated techniques such as BACTEC or BacT/ALERT.
- **Culture smear and motility testing:** Reveals non-motile gram-negative coccobacilli
- **Biochemical tests (see text):** Catalase, oxidase and urease test positive
- **Nomen species identification (see text)**

Contd...

Contd...

LABORATORY DIAGNOSIS	*Brucella melitensis*

- **Serological tests (antibody detection)**
  - Standard agglutination test (SAT)- detects IgM
  - Tests to detect IgG antibody -2ME test, CFT, ELISA.
- **Molecular method:** PCR detecting *rrs-rrl* gene, *Omp2* gene and IS711 insertion sequence
- Brucellin skin test
- Guinea pig inoculation
- **Diagnosis of brucellosis in animals**
  - Isolation of brucellae from milk and dairy products
  - Antibody detection in milk: By Milk ring test and Rose Bengal card test, whey agglutination test.

LABORATORY DIAGNOSIS

Culture and Identification

Sample

Brucellae are recovered from blood, bone marrow, CSF, joint fluid or other tissues.

- Blood should be collected during the febrile period before starting antibiotics. Multiple blood cultures (5-10 mL, 2-3 times a day) over 3 consecutive days yield better result
- Bone marrow culture remains positive even after starting antibiotics and gives a higher yield than blood culture.

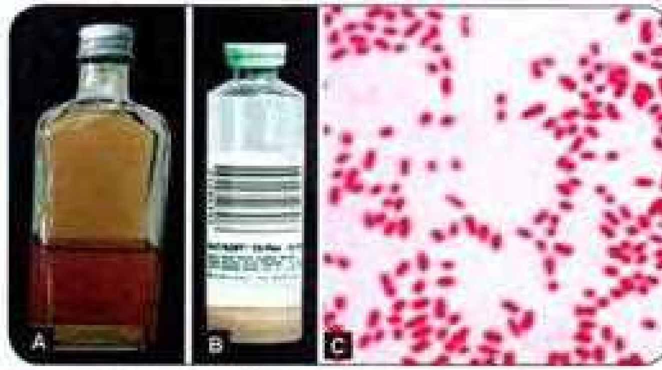
Culture Conditions

Brucellae are highly fastidious and the growth is enhanced by the addition of blood or serum.

- They are obligate aerobes, but growth is promoted in the presence of 5-10% CO_2
- Primary isolation requires prolonged (several weeks) incubation at 37°C.

Culture Media

- Blood culture bottles are the recommended media. Biphasic media (Castaneda's) are superior to monophasic media (Fig. 35.1A)
  - Brain heart infusion (BHI) broth/agar or serum dextrose broth/agar are the appropriate media used
  - Repeat subcultures are made onto blood agar and chocolate agar
  - Biphasic media have less contamination rate as the colonies can be obtained on the solid phase just by tilting the bottles so that the broth runs over the agar slope, thereby avoiding contamination during subculture
  - Blood culture bottles should be incubated at least for 2-4 weeks or more.
- Automated techniques, such as BACTEC and BacT/ALERT systems can be used which take less time for isolation (7-10 days) and have better recovery rates (Fig. 35.1B).



Figs 35.1A to C: A. Blood cuture bottles (Biphasic medium); **B.** BacT/Alert; **C.** Gram-stained smear of *Brucella* species showing small gram-negative coccobacilli

Source: A and B: Department of Microbiology, JIPMER, Puducherry; C: Public Health Image Library, ID# /15243, Dr WA Clark/Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

Culture Smear and Motility Testing

On blood agar and chocolate agar, colonies are small, smooth, transparent, low convex with entire edge and non-hemolytic.

- Gram-staining of colonies reveals non-capsulated, non-sporing, small, gram-negative coccobacilli (Fig. 35.1C)
- Brucellae are nonmotile.

Biochemical Tests

Brucellae show the following biochemical properties:

- Do not ferment carbohydrates
- Catalase positive
- Oxidase positive (except some strains of *B. abortus, B. neotomae* and *B. ovis*)
- Urease test is variable but often rapidly positive, especially with strains of *B. suis* and *B. canis*
- Nitrate is reduced to nitrite (except *B. ovis*)
- Citrate, indole, MR and VP tests are negative.

Nomen Species Identification

The nomen species are identified based on the following tests:

- Preference of animal host
- CO_2 requirement
- H_2S production
- Tolerance to bacteriostatic dyes, such as basic fuchsin and thionine
- Agglutination with monospecific antisera: Predominant A or M antigen of LPS in a strain can be determined in slide or tube agglutination tests by testing a killed suspension of organisms against absorbed monospecific antisera to A or M strain
- Lysis by bacteriophages: A standard reference strain of Tbilisi phage is used for phage typing.

Serological Tests (Antibody Detection)

As isolation is difficult, serological tests are of paramount importance in diagnosis of brucellosis. Several tests have been developed, such as standard agglutination test, complement fixation and ELISA.

Standard Agglutination Test (SAT)

It remains the gold standard test against which other serological tests are compared.

- **Procedure:** It is a tube agglutination test. The equal volumes of serial dilutions of patient's sera are mixed with the killed smooth suspension of a standard strain of *B. abortus* and incubated at 37°C for 48 hours
- **Result:** Significant titer is:
  - Titer of more than 1:160 is considered as significant in nonendemic areas
  - In an endemic area, demonstration of rising titer by repeating the test after 2–4 weeks is more reliable.
- **Interpretation:**
  - SAT detects antibodies against antigens of smooth LPS. Use of *B. abortus* can detect antibodies against *B. melitensis, B. abortus* and *B. suis* but not *B. canis* (as it lacks smooth LPS)
  - In acute infection, IgM antibodies appear early and are followed by IgG and IgA antibodies. The SAT measures the total quantity of agglutinating antibodies but does not differentiate between immunoglobulin isotypes
  - As the disease progresses, IgM levels decline, and the avidity and subclass distribution of IgG and IgA change. Thus, SAT may become negative.
- **False-negative SAT** may occur due to:
  - Prozone phenomenon (due to excess of antibodies in patient's sera): This may be obviated by performing the test in serially diluted patient's sera
  - Presence of 'blocking' or nonagglutinating IgG or IgA antibodies: This can be removed by:
    - Prior heating of serum at 55°C for 30 min
    - Using 4% saline to dilute patient's sera
    - Detecting 'incomplete' antibodies by Coombs antiglobulin test (most reliable).
- **False-positive SAT** may occur due to antigenic cross-reactions with some other gram-negative bacteria having similar O chains, such as *Escherichia coli* O157, *Francisella tularensis, Salmonella enterica* group N, *Stenotrophomonas maltophilia* and *Vibrio cholerae*.

Serological Tests to Detect IgG Antibody

- **2-mercaptoethanol (2ME) agglutination test:** Here, the serum is treated with disulfide reducing agents such as 2-ME so that it destroys the agglutinability of IgM, but does not alter IgG. SAT performed in 2ME treated serum, would detect only IgG
- Complement fixation test (CFT) detects both IgM and IgG antibodies
- **ELISA** detecting antibodies to LPS antigen is more sensitive and can detect IgM and IgG antibodies separately.

Molecular Method

PCR using primers for *rrs-rrl* gene, *Omp2* gene (outer-membrane protein), the insertion sequence *IS711* are available. PCR is rapid, sensitive and specific and can also differentiate between the species and biovars.

Older methods such as brucellin skin test and guinea pig inoculation are obsolete now.

Diagnosis of Brucellosis in Animals

Brucellosis is diagnosed in animals by various methods such as:

- **Isolation from milk and dairy products:** Culture of the overnight cream of the cow's milk has shown better isolation rate
- **Antibody detection in milk:**
  - **Milk ring test:** Pooled milk is mixed with a drop of the stained *Brucella* antigen in a narrow test tube and incubated at 70°C for 40–50 minutes
    - If antibodies are present in the milk, bacilli are agglutinated and rise to form a blue ring at the top, leaving the milk unstained
    - If antibodies are absent, no coloured ring is formed and milk remains uniformly blue.
  - **Rose Bengal card test** and the **whey agglutination test** are the other useful methods.

TREATMENT — Brucellosis

Various regimens are recommended for the treatment of brucellosis.

- **Gold-standard regimen** in adults: Streptomycin for 14–21 days plus doxycycline for 6 weeks
- **WHO regimen** in adults: Rifampin for 6 weeks plus doxycycline for 6 weeks
- Relapse or treatment failure occurs in 5–10% of cases
- **For CNS involvement:** Ceftriaxone is added to the regimen and treatment is prolonged for 3–6 months.

PREVENTION

Prevention in Animals

The most rational approach to control human brucellosis is to control and eradicate infection from its animal reservoirs.

- **Test and slaughter:** Active case finding is done in animals by skin test or CFT. Infected animals are slaughtered

❖ **Vaccine:** Live attenuated vaccine using *B. abortus* 19 strain for cattle and *B. melitensis* rev-1 strain for sheep and goat are available.

Prevention in Humans

General precautions such as:

❖ Use of pasteurized milk or properly cooked food

❖ Use of protective measures to prevent direct contact with animals.

Vaccine:

Live attenuated *B. abortus* 19-BA is available for human use but provides short term protection and had shown high reactogenicity.

EXPECTED QUESTIONS

I. Write short notes on:

1. Pathogenesis of brucellosis.
2. Laboratory diagnosis of brucellosis.
3. Castaneda's method of blood culture.

II. Multiple Choice Questions (MCQs):

1. ***Brucella melitensis* is commonly found in which animal?**
   a. Pig
   b. Dog
   c. Cattle
   d. Goat
2. **Malta fever is also called as:**
   a. Undulant fever
   b. Relapsing fever
   c. Hemorrhagic fever
   d. Rat bite fever
3. **All of the following serological tests would be helpful in the diagnosis of chronic brucellosis except:**
   a. Standard agglutination test
   b. Mercaptoethanol test
   c. Complement fixation test
   d. ELISA detecting IgG
4. **The following tests are used for detection of brucellae antibodies in milk, *except:***
   a. Standard agglutination test
   b. Milk ring test
   c. Rose Bengal card test
   d. Whey agglutination
5. **The following drugs are indicated in brucellosis, *except:***
   a. Streptomycin b. Doxycycline
   c. Meropenem d. Rifampin
6. **Fever in *Brucella* infection is described as-**
   a. Breakbone fever b. Step ladder fever
   c. Undulating fever d. Pontiac fever
7. **The most common mode of transmission of *Brucella* is:**
   a. Direct contact
   b. Ingestion of raw milk
   c. Air-borne
   d. Man to man
8. **The classic triad of brucellosis include all, *except:***
   a. Fever with profuse night sweats
   b. Meningoencephalitis
   c. Arthralgia/arthritis
   d. Hepatosplenomegaly
9. **Brucellosis is common in all the following region, *except:***
   a. Mediterranean zone
   b. Central Asia
   c. North America
   d. South America
10. ***Brucella* can be transmitted by all, *except:***
    a. Direct contact
    b. Ingestion of raw milk
    c. Air-borne
    d. Man to man

Answers

1. d **2.** a **3.** a **4.** a **5.** c **6.** c **7.** a **8.** b **9.** c **10.** d

Miscellaneous Gram-negative Bacilli

36 CHAPTER

Chapter Preview

- *Campylobacter* species
- *Helicobacter* species
- *Legionella* species
- *Pasteurella* species
- *Francisella* species
- *Chromobacterium* species
- *Capnocytophaga* species
- Agent causing donovanosis
- Agents causing rat-bite fever
- Agents causing bacterial vaginosis

CAMPYLOBACTER

Campylobacter species cause both diarrheal and systemic diseases. They are motile, nonsporing, microaerophilic, curved gram-negative rods.

Human pathogens fall into two major groups:

1. Primarily diarrheal disease: It is caused by *C. jejuni* (accounting for 80–90% of total cases), and others such as *C. coli, C. upsaliensis, C. lari, C. hyointestinalis, C. fetus.*
2. Extraintestinal infection: Caused by *C. fetus.*

Epidemiology

- **Source:** *Campylobacter* species are zoonotic, found in the intestine of many animals (poultry, cattle, sheep and swine) and household pets (including birds, dogs and cats). However, animals are asymptomatic
- **Mode of transmission:** *Campylobacter* is transmitted by the following routes
  - By raw or undercooked food products: ingestion of contaminated poultry (most common), raw (unpasteurized) milk or untreated water
  - Through direct contact with the infected household pets
  - Travel to developing countries (can cause traveller's diarrhea)
  - Oral-anal sexual contact.
- **Age:** Persons of all ages are affected; however:
  - *C. jejuni* infection is common among children
  - In contrast, *C. fetus* infection is the highest in extremes of age.
- **Developing versus developed countries:**
  - In the developing countries, *C. jejuni* infections are hyperendemic, mostly present as asymptomatic infection except children less than 2 years (usually symptomatic)
  - In developed countries, *Campylobacter* is the leading bacterial cause of diarrheal disease, more common than *Shigella* and *Salmonella.*
- **Seasonality:** Incidence peaks during summer and early autumn.

Pathogenesis

Pathogenesis of *C. jejuni* is due to expression of the following virulence factors:

- Motility of the strain (possesses single polar flagellum and exhibits darting motility)
- Capacity to adhere to host tissues
- The following toxins play a minor role:
  - Enterotoxin (Heat-labile, similar to cholera toxin)
  - Cytotoxins (cytolethal distending toxin, or CDT).
- Proteinaceous capsule-like structure (S-layer) expressed by *C. fetus*: It prevents the bacilli from complement-mediated killing and opsonisation and may contribute to the chronicity and high rate of recurrence of *C. fetus* infections in immunocompromised hosts.

Clinical Manifestations

The clinical manifestations seen in campylobacteriosis are as follows:

- **Intestinal infection:** It is characterized by inflammatory diarrhea, abdominal pain and fever. Degree of diarrhea varies from several loose stools to grossly bloody stools. It is self-limiting; however, relapse is seen in 5–10% of untreated cases
- **Extraintestinal infection:** It is mainly due to *C. fetus* developing mostly in immunocompromised hosts and at the extremes of age. Common manifestations include bacteremia, sepsis, meningitis, vascular infections (endocarditis, aneurysm, and thrombophlebitis)

- **In persons with the HLA-B27 phenotype:** Reactive arthritis and other rheumatologic manifestations may develop several weeks after infection with *Campylobacter*
- *Campylobacter* triggers the pathogenesis of various other diseases such as:
  - Guillain-Barré syndrome (mainly by *C. jejuni* serotype O19)
  - Alpha chain disease, a form of lymphoma that originates in small intestinal mucosa-associated lymphoid tissue.

Laboratory Diagnosis

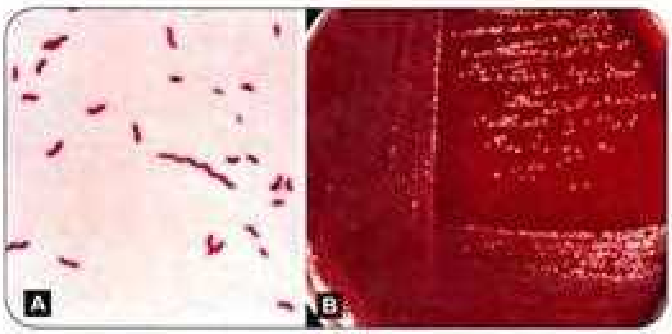
Direct Microscopy

- Gram-staining of smear of feces may show curved gram-negative bacilli, 0.2–0.5 × 1.5–5 µm, appearing comma (resembling *Vibrio*), S-shaped or spiral (*gull wing*-shaped) (Fig. 36.1A)
- Dark ground microscopy demonstrates the darting motility of the bacilli.

Culture

The culture media for *Campylobacter* are as follows:

- **Transport medium:** If delay is expected, transport medium such as Cary-Blair medium can be used. It survives for 1–2 weeks at 4°C
- **Selective media:** Feces or rectal swabs are plated onto selective media such as:
  - Skirrow's selective medium (Fig. 36.1B)
  - Butzler's selective medium
  - Campy BAP selective medium (contains lysed blood agar, vancomycin, polymyxin B, trimethoprim, cephalothin and amphotericin B).
- **Culture conditions:** Inoculated plates are incubated at:
  - Microaerophilic condition (5% O_2, 10% CO_2 and 85% nitrogen)
  - **Growth at 42°C:** Thermophilic *Campylobacter* species (*C. jejuni, C. coli and C. lari*) can be differentiated from *C. fetus*, which is nonthermophilic



Figs 36.1A and B: *Campylobacter*—**A.** Gram-negative spiral rods; **B.** Growth on Skirrow's media

Source: Public Health Image Library: A. ID#: 6657; B. D#: 3918/Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*)

  - After 2–5 days of incubation, characteristic **effuse droplet-like colonies** are produced which can be further subjected to biochemical tests for species identification.

Biochemical Identification

Campylobacters are oxidase positive, catalase positive, nitrate reducers and do not ferment sugars.

- *C. jejuni* has two subspecies: *jejuni* and *doylei*. The subspecies *jejuni* can be differentiated from the latter by:
  - Nitrate positive
  - Hippurate hydrolysis positive
  - Growth at 42°C
- *C. upsaliensis* is catalase negative
- *C. hyointestinalis* is H_2S positive.

TREATMENT — Campylobacteriosis

Fluid and electrolyte replacement is the mainstay of treatment. Antibiotics can be given, such as:

- **Diarrheal disease:** Oral macrolides are the drug of choice (erythromycin or azithromycin). Ciprofloxacin can be used as an alternative drug for adults
- **Systemic infection:** Parenteral gentamicin (or imipenem or chloramphenicol) should be started empirically, but susceptibility testing should then be performed.

HELICOBACTER

Helicobacter pylori is curved gram-negative rod that colonizes stomach and is associated with peptic ulcer disease and gastric carcinoma. Two other *Helicobacter* species; *H. cinaedi* and *H. fennelliae* are intestinal rather than gastric organisms and cause diarrheal disease.

Pathogenesis

Colonization of the Gastric Mucosa

H. pylori colonizes the stomach of 50% of the world's human population (30% in developed countries to nearly 80% in developing countries). The colonization is favored by the following factors:

- **Motility:** *H. pylori* is highly motile (conferred by 4 to 8 unipolar flagella), which allows it to remain in the viscous environment of the mucus layer overlying the gastric mucosa
- **Acid-resistance:** It may be due to:
  - **Urease enzyme:** It produces abundant urease that catalyzes urea hydrolysis to produce ammonia which in turn buffers the gastric acid
  - **Amidase and arginase:** May contribute to the production of ammonia
  - **Ure-I** protein: It regulates the passage of urea across the cell membrane into cytoplasm.
- **Adhesins:** Though most *H. pylori* remain within the mucus layer, a few (~2%) may bind to mucosal epithelium by expressing adhesion molecules such as:

- Blood group antigen-binding adhesin: Binds to Lewis blood group antigen
- Adherence-associated lipoprotein

❖ **Resistance to oxidative stress:** *H. pylori* produces many detoxifying enzymes that protect the organism against the effects of oxygen-derived free radicals generated from the bacterium's own metabolism and the inflammatory defences of the host.

Induces Pathological Changes

❖ **Vacuolating cytotoxin (VacA):** *H. pylori* secretes VacA that induces the formation of vacuoles in the cytoplasm of epithelial cells

❖ **Cytotoxin-associated Gene A (CagA):** It is a pathogenicity island that encodes a type IV secretion system. It is a syringe-like structure that gets translocated on host cell surface which helps to inject effector molecules into the host cell. This allows the bacterium to modulate certain aspects of the host cell's metabolism including:
- Cytoskeletal rearrangements
- Host-cell morphological changes
- Expression of proto-oncogenes
- Release of proinflammatory cytokines from gastric epithelial cells.

❖ **Molecular mimicry:** Lipopolysaccharide of *H. pylori* (glycoprotein moiety) is identical to the Lewis blood group antigen expressed on gastric parietal cells which may result in:
- Immune tolerance by downregulating T cells
- Induction of autoantibodies that cross-react with mucosal epitopes and contribute to the development of chronic active gastritis.

❖ **Alteration in gastric mucus:** LPS also inhibits glycosylation and sulfation of gastric mucus, which may impede its protective function and increase the vulnerability of the epithelial surface to gastric acidity

❖ **Host factors:** People with polymorphisms in cytokine genes (e.g. interleukin-1) or genes coding Toll-like receptors are at increased risk of gastric adenocarcinoma

❖ **Environmental risk factors**
- Smoking increases the risk of ulcers and cancer in *H. pylori*-colonized individuals
- Diets high in salt and preserved foods increase cancer risk, whereas diets high in antioxidants and vitamin C are protective.

Clinical Manifestations

❖ **Acute gastritis** (Antrum is the most common site involved, cardiac end is not involved)
- Antral gastritis: It predisposes to duodenal ulcers
- Pangastritis: It predisposes to adenocarcinoma of stomach.

❖ **Peptic ulcer disease:** 80% of duodenal ulcers and 60% of gastric ulcers are due to *H. pylori*
- Mechanism of duodenal ulcer: *H. pylori*-induced inflammation inhibits somatostatin producing D cells → ↑ gastrin release → ↑ meal-stimulated acid secretion → induces duodenal ulcer and gastric metaplasia of duodenal mucosa
- Mechanism of gastric ulcer: Though not clear, however, it is believed that there is hypochlorhydria despite increased gastrin release
- Epigastric pain with burning sensation: It is the most common presentation
  - In duodenal ulcer: Pain occurs usually following a meal
  - In gastric ulcer: Pain occurs in empty stomach.

❖ Chronic atrophic gastritis

❖ Autoimmune gastritis

❖ Pernicious anemia

❖ Adenocarcinoma of stomach

❖ Non-Hodgkin's gastric lymphoma.

Protective Role for H. pylori

Colonization of *H. pylori* has an inverse relation with the occurrence of:

❖ Gastroesophageal reflux disease (GERD)

❖ Barrett's esophagus

❖ Adenocarcinoma of esophagus

❖ Allergic disorders including asthma.

Laboratory Diagnosis

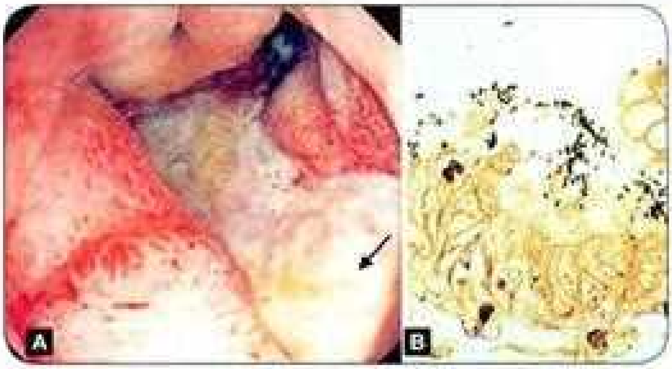
Diagnosis of *H. pylori* infection may be established by invasive and noninvasive methods.

Invasive Test

Endoscopy-guided multiple biopsies can be taken from gastric mucosa (antrum and corpus) (Fig. 36.2A) and are subjected to:

❖ **Histopathology** with Warthin Starry silver staining (Fig. 36.2B). Sensitivity can be improved by the use of immunostaining with anti-*H. pylori* antibody

❖ **Microbiological methods**
- **Gram-staining:** Curved gram-negative bacilli with seagull-shaped morphology
- **Culture media** for *H. pylori*:
  - Media for *Campylobacter* can be used, such as Skirrow's media
  - Chocolate agar can be used
  - Plates are incubated at 37°C under microaerophilic condition (5% O_2, 10% CO_2 and 85% nitrogen)
  - Culture is the most specific test; however, it is not sensitive.
- **Biochemical tests:** Oxidase, catalase and urease tests are positive.



Figs 36.2A and B: A. Endoscopy shows duodenal ulcer due to *H. pylori* (arrow) **B.** *H. pylori* (black curved rods) colonized on the gastric mucosa, Warthin-Starry staining

Source: A. Wikipedia; B. Yutaka Tsutsumi, MD, Professor, Department of Pathology, Fujita Health University School of Medicine (with permission)

- **Biopsy urease test** (also called **rapid urease test**): It detects the presence of urease activity in gastric biopsies by using a broth that contains urea and a pH indicator. It is rapid, sensitive and cheap.

Noninvasive Tests

- **Urea breath test:** Patient drinks a solution of urea labeled with the nonradioactive ^{13}C and then blows into a tube. If *H. pylori* urease is present, the urea is hydrolyzed and labeled CO_2 is detected in breath samples by mass spectroscopy. Urea breath test is very popular nowadays as it is noninvasive and is:
  - Most consistent and accurate test
  - Most sensitive, quick and simple
  - Used to monitor treatment response (becomes negative after improvement).
- **Stool antigen (coproantigen) assay:**
  - Used to monitor treatment response
  - Useful for screening of children.
- **Antibody (IgG) detection** by ELISA: It is used for:
  - Screening before endoscopy
  - Seroepidemiological study.

TREATMENT — ***H. pylori* infection**

Treatment for *H. pylori* infection

Treatment in *H. pylori* infection is indicated for: (i) duodenal or gastric ulceration; (ii) low-grade gastric B-cell lymphoma.

- However, treatment is not recommended for asymptomatic colonizers or primary prophylaxis for gastric cancer because of risk of adverse side effects and development of antibiotic resistance
- Usually multidrug regimens are used. Monotherapy is not useful because of inadequate antibiotic delivery to the colonization niche
- Success of treatment depends on: (i) Patient's close compliance with the regimen; and (ii) Use of susceptible antibiotics

Contd...

Contd...

TREATMENT — ***H. pylori* infection**

- Treatment guideline recommended is as follows:

Treatment regimen for *H. pylori* infections:

1st line triple drug therapy (OCM or OCA regimen):

Omeprazole + Clarithromycin + Metronidazole or Amoxicillin given for 7–14 days.

↓

Urea breath test is done after 1 month's gap

↓

If 1st line regimen fails (Urea breath test is +ve)

↓

2nd line quadruple drug therapy (OBMT regimen):
Omeprazole + Bismuth subsalicylate + Metronidazole + Tetracycline given for 14 days

↓

If 2nd line quadruple drug therapy fails, then:
Culture of endoscopy-guided biopsy is done and treatment is given based on antimicrobial susceptibility test

LEGIONELLA

Legionellae are fastidious, pleomorphic gram-negative, short rods, associated with two clinical syndromes:

- Pontiac fever is an acute, milder flu like self-limited illness
- Legionnaires' disease—severe interstitial pneumonia.

History

Legionella was first recognized in 1976 when an outbreak of pneumonia took place at a Philadelphia hotel during an American Legion convention.

Classification

The family Legionellaceae comprises more than 50 species (with >70 serogroups), out of which 19 species have been associated with human infections.

- *L. pneumophila* is the most important species, associated with 80–90% of human infections. It consists of 15 serogroups. Majority of cases are associated with **serogroup 1** followed by 4 and 6
- Other species are rarely associated with human infection particularly in immunocompromised state, such as *L. micdadei* (Pittsburgh pneumonia agent), *L. bozemanii, L. dumoffii,* and *L. longbeachae.*

Epidemiology

- **Reservoir:** *Legionella* inhabits on aquatic bodies which could be either:
  - Natural water sources, such as rivers, streams or even inside amoeba
  - Artificial aquatic sources, such as air conditioners, water coolers

- *L. longbeachae* has been isolated from natural soil and commercial potting soil
- There is no animal reservoir
- There is no carrier stage.

❖ **Transmission:** Multiple routes have been proposed
- Aspiration (predominant mode): It occurs either via oropharyngeal colonization or directly via drinking of contaminated water
- Aerosols from contaminated air conditioners, nebulizers, and humidifiers
- Direct instillation into the lungs during respiratory tract manipulations
- There is no man-to-man transmission.

❖ **Predisposing factors for *Legionella* infections include:**
- Smoking, alcoholism and chronic lung disease—impair mucociliary clearance
- Advanced age
- Immunosuppression-transplantation, HIV infection, steroid therapy
- Prior hospitalization
- Patients with nasogastric tubes or those undergoing surgery with general anesthesia promotes aspiration.

Pathogenesis

After legionellae enter the lungs through aspiration or direct inhalation, the following events take place:

❖ Attachment to the respiratory mucosa is mediated by bacterial type IV pili, heat-shock proteins, a major outer-membrane protein, and complement

❖ Both macrophages and neutrophils are recruited to the local sites

❖ **Coiling phagocytosis:** Alveolar macrophages phagocytose legionellae by a coiling mechanism

❖ Evades intracellular killing by inhibiting phagosome-lysosome fusion

❖ Because of their intracellular location, humoral immunity plays a minor role. Cellular immunity is responsible for the recovery.

Clinical Manifestations

Pontiac Fever

It is an acute, flu-like illness characterized by malaise, fever, and headache. Incubation period is about 24–48 hours. It is self-limiting, never develops into pneumonia.

Legionnaires' Disease (Pneumonia)

It is an interstitial atypical pneumonia with incubation period about 2–10 days.

❖ It is characterized by non-productive cough (with or without blood tinged), dyspnea, chest pain, high fever and diarrhea

❖ Chest X-ray shows pulmonary infiltrates

❖ Most common neurologic abnormality is confusion or changes in mental status

❖ *Legionella* is among the leading causes of pneumonia both in the community and hospital settings
- It is the fourth common cause of community-acquired pneumonia, accounting for 2–9% of cases
- It is responsible for 10–50% of cases of nosocomial pneumonia when a hospital's water system is colonized with the organisms. Serogroup 6 is more commonly involved in hospital outbreaks.

Extrapulmonary Legionellosis

Usually it results from blood-borne dissemination from the lung.

❖ The most common extrapulmonary site is heart (myocarditis, pericarditis and prosthetic valve endocarditis)

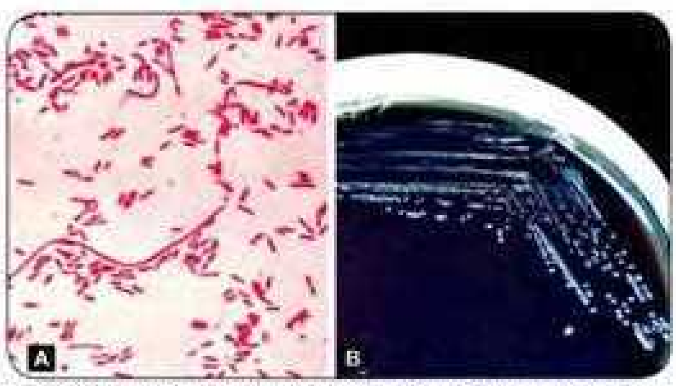
❖ Other manifestations include sinusitis, peritonitis, pyelonephritis, skin and soft tissue infection.

Laboratory Diagnosis

Useful specimens for Legionnaires' disease include sputum, bronchoalveolar lavage fluid, bronchial wash and pleural fluid.

❖ **Direct Microscopy**
- **Gram stain** reveals numerous neutrophils but no organisms (as legionellae are poorly stained, often missed or sometimes appear as faint pleomorphic gram-negative rods or coccobacilli) (Fig. 36.3A)
- Silver impregnation and Giemsa stains can be used
- Direct immunofluorescence test using monoclonal or polyclonal sera is more specific but sensitivity is poor than culture. It is more useful in advanced stage of disease
- Acid-fast staining: *L. micdadei* is weakly acid fast.

❖ **Culture:** Culture is highly sensitive (80–90%) and specific (100%) and provides definite diagnosis.



Figs 36.3A and B: *Legionella*: **A.** Gram-staining; **B.** Growth on BCYE agar

Source: Public Health Image Library, A. ID#: 15328/ Dr. Gilda Jones, B. ID#: 11766/ Megan Mathias and J. Todd Parker/Centers for Disease Control and Prevention (CDC), Atlanta *(with permission)*

Buffered charcoal, yeast extract (BCYE) agar: Legionellae are highly fastidious and grow on complex media, such as **BCYE agar** (pH 6.8–6.9) (Fig. 36.3B)

- Plates are incubated at 37°C in 5% CO_2 for 3–5 days
- Colonies are round with an entire edge, glistening, convex, green or pink iridescent and have granular or speckled opalescence resembling ground glass.

- **Biochemical tests:** They are motile, catalase positive and oxidase negative. Hippurate hydrolysis test is positive for only *L. pneumophila*. Autofluorescence of colonies under long-wavelength UV can be used for speciation
- **Antibody detection:** Primarily, serology is used for epidemiologic purpose
    - Indirect immunofluorescent antibody test and enzyme immunoassays are available
    - A single titer of more than 1:128 or fourfold rise in titer is considered as significant
    - Antibodies usually appear late after 12 weeks
    - Cross-reactivity has been observed with other *Legionella* species.
- **Urinary antigen:** Enzyme immunoassays are available to detect *L. pneumophila* serogroup 1 specific soluble antigens in urine. Advantages include as follows:
    - It is rapid, cheaper, easy to perform
    - Next to culture, it is highly sensitive, and specific
    - Antigen in urine is detectable 3 days after the onset of symptoms and disappears over 2 months
    - The test is not affected by prior antibiotic administration.
- **Molecular methods:** Polymerase chain reaction (PCR) has been proven useful in the identification of *Legionella* from environmental water. Most common targets are *mip* (macrophage inhibitor protein) gene, 5S and 16S rRNA and 16S-23S spacer region genes.

TREATMENT **Legionnaires' disease**

Macrolides (especially azithromycin) and the respiratory quinolones are now the antibiotics of choice.

Prevention

Routine environmental culture of hospital water supplies is recommended.

- In aquatic environment, *L. pneumophila* can form microcolonies within biofilms; its eradication from drinking-water systems requires disinfectants that can penetrate the biofilm
- Disinfection of the drinking water by: (1) superheat-and-flush method; (2) commercial copper and silver ionization systems.

DONOVANOSIS

Donovanosis, also called *granuloma inguinale*, is a sexually transmitted disease caused by *Klebsiella granulomatis* (it shows 98% gene homology with *Klebsiella*). However, many authors still recommend to use the old name—*Calymmatobacterium granulomatis*.

- Disease was first described in Calcutta (now Kolkata) by McLeod in 1882, and the characteristic pathological finding "Donovan bodies" in the genital lesion was recognized by Charles Donovan in Madras (now Chennai) in 1905
- Donovanosis is prevalent in India, Brazil, Papua New Guinea and parts of South Africa
- Risk factors include poor hygiene, lower socioeconomic status and multiple sex partners
- Globally, the incidence of donovanosis has greatly decreased.

Clinical Features

- Incubation period is about 1–4 weeks (may be up to 6 months). It runs a chronic course
- Lesion starts as a painless papule which subsequently becomes a **beefy red ulcer** that bleeds readily when touched (Fig. 36.4A)
- Most common sites, genitals are affected in 90% of patients affecting prepuce, frenum and glans in men and the labia minora in women
- Lymph node involvement is rare however, *pseudobubos* may be seen in the inguinal region in 10% of cases due to subcutaneous abscess.

Laboratory Diagnosis

- **Clinical diagnosis** is made by the appearance of characteristic lesion
- **Specimen collection:** A swab should be rolled firmly over an ulcer previously cleaned with a dry swab to remove debris. Alternatively, a piece of granulation tissue crushed and spread between two slides can be used

Figs 36.4A and B: Donovanosis. **A.** Beefy red ulcer; **B.** Donovan bodies: Cyst-like macrophages filled with deeply stained capsulated bacilli having a safety-pin appearance (Giemsa stain)

Source: Public Health Image Library: A. /ID#5363/ Dr. Tabua; B. ID#: 18899, Susan Lindsley/Centers for Disease Control and Prevention (CDC), Atlanta (with permission)

- **Direct microscopy:** Smears can be examined after a rapid Giemsa or Wright's stain
  - Donovan bodies can be seen as large cyst like macrophages filled with deeply stained capsulated bacilli having a *safety-pin* (bipolar) appearance (Fig. 36.4B). These cysts eventually rupture releasing the bacilli
  - They are non-motile, capsulated and gram-negative bacilli.
- **Culture:** They can be grown on egg yolk medium and on HEp-2 cell lines
- **Molecular Method:**
  - PCR has been developed to differentiate *Klebsiella granulomatis* from other *Klebsiella* species by detecting unique base changes in the *phoE* gene
  - A genital ulcer multiplex PCR has been developed for simultaneous detection of agents of common genital ulcers such as donovanosis, syphilis, chancroid, lymphogranuloma venereum and genital herpes.

TREATMENT **Donovanosis**

- Macrolides are drug of choice. Azithromycin is given 1 g on day 1, then 500 mg daily for 7 days
- Alternatively, doxycycline (100 mg twice a day) or co-trimoxazole (900 mg bd for 14 days) is given for 14 days
- Both the sexual partners should be treated.

GARDNERELLA VAGINALIS

Gardnerella vaginalis (formerly known as *Haemophilus vaginalis* or *Corynebacterium vaginale*) is isolated from the normal female genital tract in low numbers. When outnumbered, causes a condition called **bacterial vaginosis**.

- It is gram-negative (appears gram variable in smears), nonmotile, small pleomorphic rod, which shows metachromatic granules
- It produces minute hemolytic colonies on blood agar, incubated aerobically under 5% CO_2 for 24–48 hours
- It is catalase, oxidase, indole and urease negative.

Bacterial Vaginosis

Organisms Associated

Bacterial vaginosis affects women of reproductive age. This condition is associated with an alteration of the normal vaginal flora, which is as follows:

- Increase in the concentrations of:
  - *Gardnerella vaginalis*
  - *Mobiluncus* (motile, curved, gram-variable or gram-negative, anaerobic rods)
  - Several other anaerobes [*Prevotella* and some *Peptostreptococcus*]
  - *Mycoplasma hominis*
- Decrease in the concentrations of lactobacilli (lactobacilli usually maintain the acidic pH of the vagina, thereby inhibiting the growth of pathogenic organisms).

Risk Factors

Bacterial vaginosis is associated with the following risk factors:

- Coexisting other infections such as HIV, *Chlamydia trachomatis*, and *Neisseria gonorrhoeae*
- Recent unprotected vaginal intercourse
- Vaginal douching
- Premature rupture of membranes and preterm labor.

Laboratory Diagnosis

Bacterial vaginosis is so named because there is no associated inflammation. It is clinically diagnosed by *Amsel's criteria* (see below in box).

Amsel's Criteria

Bacterial vaginosis is diagnosed if any 3 of the following 4 findings are present:

1. Profuse thin (low viscous), white homogeneous vaginal discharge uniformly coated on vaginal wall
2. pH of vaginal discharge more than 4.5
3. Accentuation of distinct fishy odor (attributable to volatile amines such as trimethylamine) immediately after vaginal secretions are mixed with 10% solution of KOH (*Whiff test*)
4. **Clue cells:** They are vaginal epithelial cells coated with coccobacilli, which have a granular appearance and indistinct borders observed on a wet mount (Fig. 36.5).

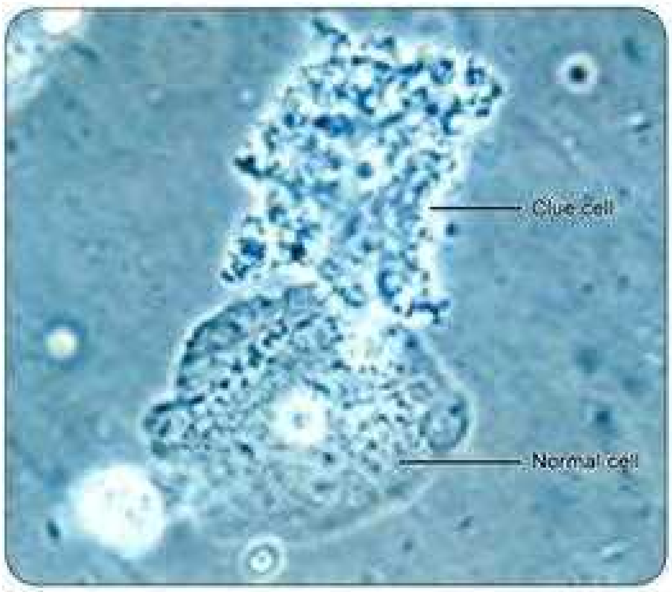


Fig. 36.5: Wet mount of vaginal secretion depicting clue cell

Source: Public Health Image Library/ID#: 14574/ M. Rein /Centers for Disease Control and Prevention (CDC), Atlanta (with permission)

Nugent's score: It is a scoring system followed for the diagnosis of bacterial vaginosis; done by counting the number of *Gardnerella vaginalis*, *Mobiluncus* and lactobacilli present in the Gram-stained smear of vaginal discharge. A score of more than or equal to 7 is diagnostic.

TREATMENT — **Bacterial vaginosis**

Drug of choice is oral metronidazole, given 500 mg twice daily for 7 days.

RAT-BITE FEVER

Rat-bite fever is characterized by septic fever, petechial rashes, and very painful polyarthritis with frequent relapses. It is caused by either of these two pathogens:
- *Streptobacillus moniliformis*
- *Spirillum minus*.

Streptobacillus moniliformis

S. moniliformis is present as normal flora in the throat of rats; however, it can be a pathogen of many rodents including guinea pigs and mice.

Clinical Manifestations

- Humans can be infected by rat bites. Ingestion of raw milk or water contaminated with rats can also transmit the infection
- The disease has occurred in epidemics in Haverhill (USA) and, hence, called *Haverhill fever* or *erythema arthriticum epidemicum*.

Laboratory Diagnosis

Useful specimens are blood, joint fluid or pus.
- **Microscopy:**
  - It is gram-negative, highly pleomorphic nonmotile organism
  - Forms irregular chains of gram-negative bacilli with beaded or fusiform swellings readily developing into L-forms.
- **Culture:**
  - It grows best at 37°C aerobically, in media containing serum protein, egg yolk, or starch
  - L-forms are frequently formed in fresh cultures, which revert back to streptobacilli when subcultured in liquid media.
- All strains of streptobacilli appear to be antigenically identical
- Other methods include: Mouse inoculation and serum agglutination tests.

Spirillum minus

Rat-bite fever is caused by *Spirillum minus*. It is called **sodoku** in Japan. It was first observed in a rat by Carter (1888) in India.

Clinical Features

Clinical features are similar to that of streptobacillary rat-bite fever except (Table 36.1):
- Longer incubation period of 1–4 weeks
- Enlarged lymph nodes.

Laboratory Diagnosis

- **Microscopy:** These are very small (3-5 µm) and rigid, spirally coiled bacilli
  - Motile with 1-7 amphitrichous flagella
  - Gram-negative but is better visualized by Giemsa or Fontana stains or by dark field microscopy.
- **Culture:** *Spirillum* cannot be cultured in artificial culture media. However, it can be isolated by inoculation into guinea pigs or mice with material from enlarged lymph nodes or blood.

TREATMENT — **Rat-bite fever**

- **Drug of choice:** Penicillin is considered to be the treatment of choice for both the agents
- **Alternative drugs** that can be used are: streptomycin, tetracycline, doxycycline, cephalosporin
- Penicillin resistance, though rare, has been reported for *S. moniliformis*.

FRANCISELLA TULARENSIS

Francisella tularensis is the causative agent of 'tularemia' primarily a plague-like disease of rodents and other small animals.

Epidemiology

- **Source:** It persists in contaminated environments, insects, and animal carriers

Table 36.1: Types of rat-bite fever

	Streptobacillary rat-bite fever	Spirillary rat-bite fever
Agent	*Streptobacillus moniliformis*	*Spirillum minus*
Disease also known as	Haverhill fever (USA)	Sodoku (Japan)
Incubation period	7–10 days	1–3 weeks
Clinical features	Septic fever Skin rashes Painful polyarthritis Frequent relapses	Similar features with additional lymph node enlargement
Gram-staining	Gram-negative, highly pleomorphic bacilli in chain	Gram-negative, spirally coiled bacilli
Motility	Nonmotile	Motile
Artificial media	Cultivable	Noncultivable
Drug of choice	Penicillin	Penicillin

- **Transmission:** Human infection is zoonotic and usually results from:
  - Interaction with biting or blood-sucking insects (especially ticks and tabanid flies)
  - Contact with wild or domestic animals
  - Ingestion of contaminated water or food
  - Inhalation of infective aerosols.

Prevalence

F. tularensis has four subspecies: *tularensis, holarctica, novicida*, and *mediasiatica*.

- The first three subspecies are found in North America, whereas subspecies *mediasiatica* is found in central Asia
- Subspecies *tularensis* is the most common and the most virulent among all. It has been isolated only from North America, where it accounts for more than 70% of cases
- Increasing number of cases due to other subspecies have been reported from the Scandinavian countries, Eastern Europe, and Siberia.

Clinical Manifestations

Tularemia is characterized by various clinical syndromes:

- **Ulceroglandular tularemia:** It is the most common form, accounting for 75–85% of total cases, characterized by ulcerative lesion at the site of inoculation, with regional lymphadenopathy
- **Pulmonary tularemia:** It can result from aerosol inhalation (laboratory workers) or can spread to the lungs following bacteremia. Patients present with atypical pneumonia
- **Oropharyngeal tularemia:** It occurs following ingestion of contaminated undercooked meat. It is characterized by membranous pharyngitis with cervical lymphadenopathy. ***Lemming fever*** in Norway results from consumption of water contaminated with excreta of infected lemmings
- **Oculoglandular tularemia:** It is characterized by purulent conjunctivitis with preauricular lymphadenopathy
- **Typhoid-like** illness
- **Agent of bioterrorism:** Because of the highly infectious nature, *F. tularensis* is currently classified as category A agent of bioterrorism.

Laboratory Diagnosis

- **Culture:** Isolation is very difficult as *F. tularensis* is highly fastidious
  - It needs special media, such as:
    - BCG agar (blood cysteine glucose agar)
    - CHAB agar (cysteine heart agar supplemented with 9% heated sheep blood).
  - **Specimen:** Ulcer scrapings, lymph node biopsy, gastric washings, sputum, and blood are inoculated onto the media and incubated at 37°C for 2–4 days aerobically as *F. tularensis* is an obligate aerobe
  - Colonies are blue-gray, round, smooth, and slightly mucoid with small zone of α-hemolysis
  - Safety precautions such as biosafety level III must be used to handle clinical specimens to avoid the risk of laboratory-acquired infection.
- **Identification:**
  - *F. tularensis* is a small gram-negative coccobacillus with bipolar appearance, nonmotile and capsulated
  - It is weakly catalase positive, oxidase negative and H_2S positive
  - It produces acid but not gas from glucose, maltose and mannose
  - Direct fluorescent antibody tests can be done using commercially available antisera, directly from the culture colonies for subspecies identification.
- **Antibody detection** is the mainstay of diagnosis as isolation is difficult. Agglutination tests (latex and tube agglutination) and ELISA formats are available
- **PCR assay** has been used to detect *F. tularensis* specific genes encoding the outer-membrane proteins. It can also differentiate subspecies. FT-1 targeting *Tul4* (lipoprotein gene) is another gene target.

TREATMENT **Tularensis**

Gentamicin is considered as the drug of choice; given 5 mg/kg for 7–10 days. Doxycycline or ciprofloxacin can be given as alternatives.

PASTEURELLA

Pasteurella species are primarily harbored in respiratory tracts of many animals and some time cause fatal diseases including hemorrhagic septicemia in animals.

- *Pasteurella multocida* is probably the most common organism in human wounds inflicted by the bites of cats and dogs
- Other species such as *P. haemolytica* and *P. pneumotropica* rarely infect humans. *P. aviseptica* is the chicken cholera bacillus, used by Louis Pasteur for the development of first attenuated bacterial vaccine hence the name *Pasteurella* was coined.

Clinical Findings

Following animal bite (most common mode of transmission), the affected area becomes red, swollen and painful with variable regional lymphadenopathy and low grade fever.

Pasteurella is present as a commensal in human respiratory tract and infection may occur following trauma or surgeries that leads to bacteremia or systemic manifestations, such as:

- Meningitis (following head injury)
- Appendicitis
- Chronic respiratory infection.

Laboratory Diagnosis

- *Direct microscopy* of wound swabs shows nonmotile gram-negative coccobacilli with a bipolar staining
- *Culture*: They are aerobes or facultative anaerobes that grow readily on ordinary media like nutrient agar at 37°C
- They resemble yersiniae; however, they differ from the latter in being:
  - Oxidase-positive
  - Indole-positive
  - Failure to grow on MacConkey agar.

TREATMENT ***Pasteurella***

Penicillin G or amoxicillin-clavulanate is considered as the drug of choice for *P. multocida* infections.

CHROMOBACTERIUM VIOLACEUM

It is a saprophyte of water and soil in tropics. It occasionally causes skin lesions, sepsis, and liver abscesses.

- It is a motile, gram-negative, facultative anaerobe, non-sporing, coccobacillus
- It produces characteristic violet color nondiffusible pigment (called **violacein**) (Fig. 36.6).

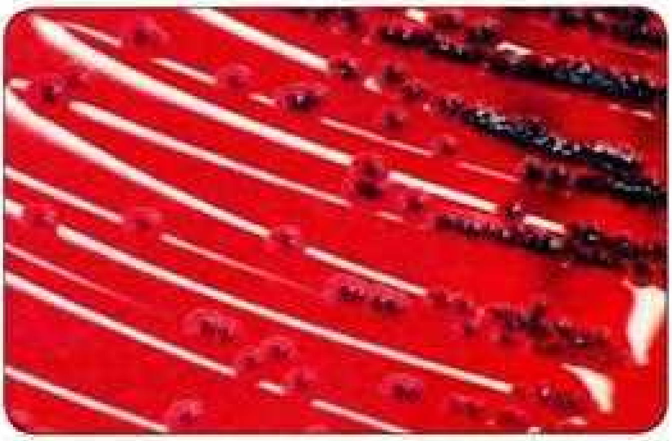


Fig. 36.6: Violet-colored colonies of *Chromobacterium violaceum* on blood agar

Source: Public Health Image Library, ID#:12434/Amanda Moore/Centers for Disease Control and Prevention (CDC), Atlanta (with permission)

CAPNOCYTOPHAGA SPECIES

Several species such as *C. ochracea, C. gingivalis* and *C. sputigena* have been a part of human mouth flora.

- They occasionally cause periodontal diseases; and sepsis in immunocompromised hosts
- Certain species such as *C. canimorsus* and *C. cynodegmi* are commensals in mouth of dogs and are transmitted by dog bites.

Laboratory Diagnosis

- They are fusiform or filamentous gram-negative coccobacilli
- Highly fastidious, require carbon dioxide for optimal growth
- They produce yellow orange pigment
- They lack flagella but exhibit *gliding motility* on agar surface.

TREATMENT ***Capnocytophaga***

Due to their ability to produce β lactamases, ampicillin-sulbactam combination is the drug of choice.

EXPECTED QUESTIONS

I. Write short notes on:

1. Laboratory diagnosis of *Helicobacter pylori*.
2. Donovanosis.
3. Pathogenesis of *Legionella pneumophila*.
4. Bacterial vaginosis.
5. Rat bite fever.

II. Multiple Choice Questions (MCQs):

1. **Most common mode of transmission of *Legionella pneumophila* is:**
   a. Aspiration b. Ingestion
   c. Insect bite d. Blood
2. **Most sensitive method of diagnosis of *Helicobacter pylori* is:**
   a. Culture b. Biopsy urease test
   c. Histopathology d. Urea breath test
3. **Rat bite fever is caused by:**
   a. *Borrelia recurrentis*
   b. *Streptobacillus moniliformis*
   c. *Yersinia pestis* d. *Leptospira*
4. **Wrong about Bacterial vaginosis is:**
   a. Discharge has offensive smell
   b. pH >4.5
   c. Causative agent is *Chlamydia trachomatis*
   d. Clue cell is diagnostic
5. **Amsel's criteria used for diagnosis of bacterial vaginosis includes all, *except*:**
   a. Vaginal discharge
   b. pH <4.5
   c. Whiff test positive
   d. Clue cells seen
6. **All the subspecies of *F. tularensis* are found in North America, *except*:**
   a. *F. tularensis* b. *F. holarctica*
   c. *F. novicida* d. *F. mediasiatica*

Answers

1. a **2.** d **3.** b **4.** c **5.** b **6.** d

Spirochetes

37
CHAPTER

Chapter Preview

- Classification and morphology
- *Treponema*
  - *T. pallidum* (agent of Syphilis)
  - Non-venereal treponematoses
- *Borrelia*
  - *B. recurrentis* (relapsing fever)
  - *B. burgdorferi* (lyme disease)
  - *B. vincentii* (vincent's angina)
- *Leptospira* (Weil's disease)

CLASSIFICATION AND MORPHOLOGY

Spirochetes are thin, flexible, elongated spirally coiled helical bacilli (*speira*, meaning coil; and *chaite*, meaning hair). They belong to the order Spirochetales that comprises of two classes:

1. Class Spirochaetaceae: It consists of four genera: *Spirocheta, Cristispira, Treponema, Borrelia.*
2. Class Leptospiraceae: It consists of the following two genera: *Leptospira, Leptonema.*

Most of the spirochetes are saprophytes. Only three of them are major human pathogens—*Treponema, Borrelia* and *Leptospira*.

Ultrastructure of Spirochetes (Figs 37.1A to E)

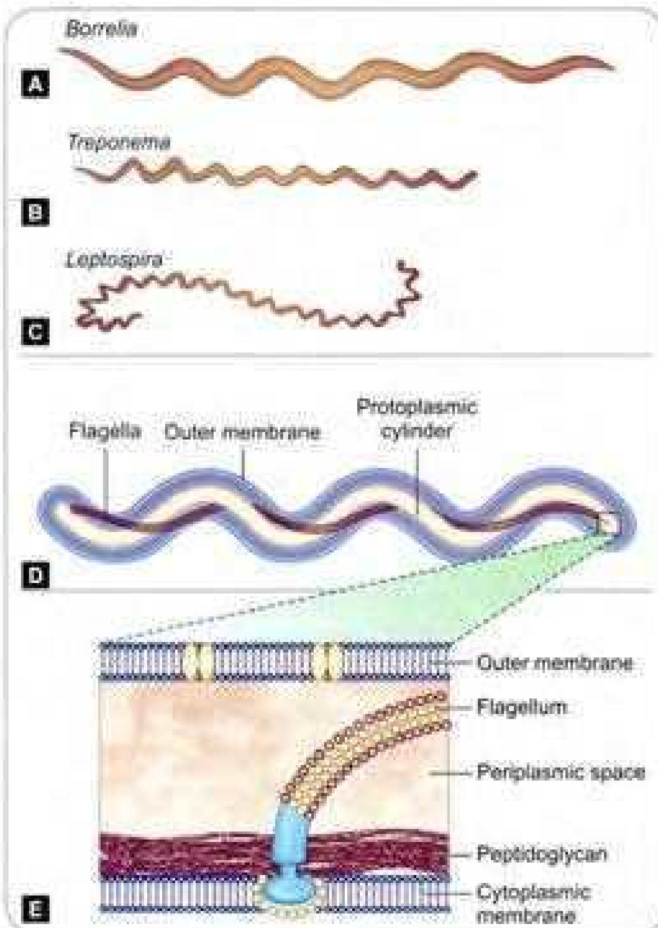
The cell wall of spirochetes is similar to that of gram-negative bacteria but differs by bearing endoflagella. It is more complex, consisting of:

- Outer membrane
- Periplasmic space containing flagella
- Peptidoglycan layer
- Inner (cytoplasmic) membrane.

Endoflagella and Motility

The characteristic feature of spirochetes is that the flagella are internal, present in the periplasmic space between the outer membrane and the peptidoglycan layer.

- They attach to the membrane only at the pole
- The number of periplasmic flagella varies from species to species
- Endoflagella are responsible for the motility of spirochetes. Motility may be of various types, such as:
  - Flexion-extension type
  - Corkscrew type rotatory movement
  - Translatory type.
- Spirochetes can swim even in highly viscous, gel-like medium, such as that found in connective tissues, where the motility of most other bacteria is inhibited.



Figs 37.1A to E: Ultra structure of Spirochetes. **A. to C.** General shape; **D.** Magnified view; **E.** Cross-sectional view

Pathogenic spirochetes, such as *Treponema*, *Borrelia* and *Leptospira* can vary from each other in various aspects (Tables 37.1 and 37.2):

- **Relative size:** Spirochetes vary widely in size from 5 µm to 500 µm in length
- **Spirals:** Number of spirals, spiral width (wavelength) and amplitude (or depth) of spirals
- **Staining property:** Larger spirochetes like *Borrelia* are gram-negative but other spirochetes cannot be stained by routine staining methods. However, they can be seen under dark ground microscopy, silver impregnation method and immunofluorescence
- Number of endoflagella at each pole
- Disease potential
- Mode of transmission.

Table 37.1: Morphological differences between *Treponema*, *Borrelia* and *Leptospira*

	Treponema	*Borrelia*	*Leptospira*
Size	6–14 µm × 0.2 µm	10–30 µm × 0.2–0.5 µm	6–20 µm × 0.1 µm
Spirals (in number)	6–12	3–10	Numerous, tightly coiled with hooked ends
Wavelength	1 µm	3 µm	0.5 µm
Amplitude of spiral	1–1.5 µm	Up to 2 µm	0.1 µm
Endoflagella at each pole	3–4	7–11	1

Table 37.2: Pathogenic spirochetes

Spirochetes	Disease	Transmission
Treponema		
T. pallidum	Syphilis	Sexual
T. pertenue	Yaws	
T. endemicum	Endemic syphilis	Direct contact
T. carateum	Pinta	
Borrelia		
B. recurrentis	Relapsing fever (epidemic)	Louse borne
B. duttonii, *B. hermsii*	Relapsing fever (endemic)	Tick borne
B. burgdorferi	Lyme disease	Tick borne
B. vincentii	Vincent's angina	Direct contact
Leptospira		
L. interrogans	Leptospirosis • Milder form • Severe form (Weil's disease)	Contact with rodent urine

TREPONEMA

Treponemes are slender spirochetes with fine spirals having pointed ends (*trepos*, meaning 'turn' and *nema*, meaning 'thread').

Most of them are commensals in mouth and genitalia; while few are pathogenic to men, such as:

- *T. pallidum* subspecies *pallidum*
- *T. pallidum* subspecies *pertenue*
- *T. pallidum* subspecies *endemicum*
- *T. carateum*.

These pathogenic treponemes are almost identical in their morphology, antigenic structure and in genetic composition. It has been well accepted that the subspecies *pallidum*, *pertenue* and *endemicum* are the evolutionary variations of a single species *T. pallidum*. For the sake of convenience, they are referred hereafter as *T. pallidum*, *T. pertenue* and *T. endemicum*.

TREPONEMA PALLIDUM (AGENT OF SYPHILIS)

Treponema pallidum is the causative agent of an ancient sexually transmitted infection 'syphilis.' The name *pallidum* refers to its pale-staining property. It was discovered by Schaudinn and Hoffmann in 1905.

- **Morphology:** Treponemes are extremely thin and delicate with tapering ends
- **Size:** They vary in size (6–14 µm × 0.2 µm)
- **Spirals:** They are flexible, spirally coiled around the long axis; possess 6–12 spirals spaced at intervals of 1 µm with amplitude of 1–1.5 µm
- **Motility:** They are actively motile exhibiting flexion extension, translatory, and corkscrew motility. They have a typical tendency to bend at right angle at the midpoint
- **Endoflagella:** About 3–4 flagella are present in periplasmic space. They provide motility to the bacteria, thus helping in tissue invasion and dissemination. They are also highly antigenic, stimulating a strong early antibody response
- **Microscopy:** Treponemes cannot be visualized by light microscope but can be seen under dark ground or phase contrast microscope
- **Staining:** They do not take up the ordinary stain but can be stained by fluorescence staining and sliver impregnation methods (which increase the thickness of the bacilli)
- **Cultivation:** Pathogenic treponemes cannot be grown in artificial culture media but are maintained by subcultures in susceptible animals such as rabbit testes
  - Nichols strain, a virulent *T. pallidum* strain was isolated from a case of tertiary syphilis (1912) and has been maintained thereafter in rabbit testes for several decades

- However, non-pathogenic *Treponemes* such as *T. phagedenis* (Reiter's treponemes) and *T. refringens* (Noguchi strain) can be grown in various media, such as **Smith Noguchi medium** under strict anerobic conditions.

❖ **Antigens:** *T. pallidum* is antigenically complex and poorly understood. Based on the type of antibody response, three antigens are identified:
1. **Group-specific antigen:** It is protein antigen present in all treponemes (pathogenic and nonpathogenic). Antibodies to this antigen can be detected in sera of syphilitic patients by using antigens of Reiter treponemes.
2. **Species-specific antigen:** It appears to be polysaccharide in nature. Treponemal antibodies induced by this antigen in a syphilitic patient can be detected by using specific *T. pallidum* antigens.
3. **Non-specific antigen:** It is a heterophile antigen. Antibody against this antigen is detected (by using beef heart antigen) by various non-treponemal tests described later in the text.

PATHOGENESIS OF SYPHILIS

Syphilis is one of the ancient sexually transmitted infection known since fifteenth century. Name was derived from a famous poem in the year 1530 which described a legend of a shepherd boy named Syphilus, who had suffered from the disease.

❖ **Mode of transmission:** Venereal syphilis is acquired by sexual contact. However, it can also be transmitted by non-venereal modes such as direct contact, blood transfusion or transplacental transmission

❖ **Spread:** *T. pallidum* rapidly penetrates through the minute abrasions on the skin or mucosa and, within a few hours, enters the lymphatics and blood to produce systemic infection and metastatic foci long before the appearance of a primary lesion. Blood is infectious even during the incubation period or in the early stage of syphilis

❖ **Incubation period** is variable (9-90 days) and is inversely proportional to the number of organisms inoculated. The median incubation period in humans is about 21 days which corresponds to an average inoculum of 500-1000 infectious organisms.

CLINICAL MANIFESTATIONS OF SYPHILIS

Approximately, 30% of persons who have sexual exposure with an infected partner develop syphilis. Clinically, patients suffering from syphilis pass through four stages if left untreated: primary, secondary, latent and tertiary (or late) stages. Apart from this, if transmitted vertically, the newborn babies develop a congenital form of syphilis.

Fig. 37.2: Primary syphilis (hard chancre)

Source: Public Health Image Library, ID# 6803, Dr/M. Rein/Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

Primary Syphilis

Primary syphilis is characterized by:

❖ **Primary (or hard) chancre:** It usually begins as a single painless papule that rapidly becomes ulcerated, hard, and indurated. It is covered by thick exudate, very rich in spirochetes. The most common sites are penis (in males), cervix or labia (in females), and anal canal, rectum or mouth (in homosexuals) (Fig. 37.2)

❖ **Regional (usually inguinal) lymphadenopathy** appears within 1 week of onset of skin lesions. Lymph nodes are painless firm, non-suppurative, and often bilateral

❖ The chancre generally heals within 4-6 weeks (range 2-12 weeks), but lymphadenopathy may persist for months

❖ If acquired by non-venereal mode, then the primary syphilis is presented as follows:
- If transmitted by direct contact→the primary chancre is extragenital, usually on the fingers
- If transmitted by blood transfusion→the primary chancre does not occur.

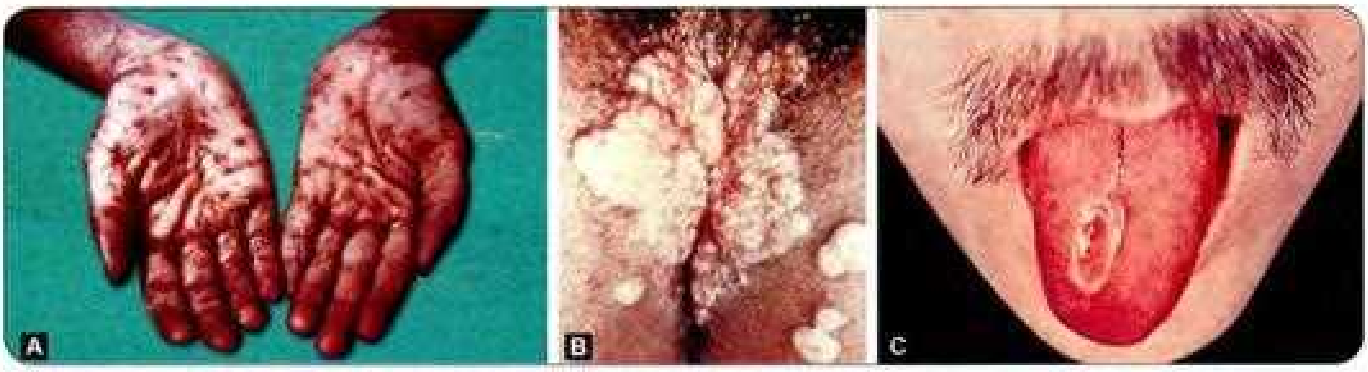
Secondary Syphilis

Secondary syphilis usually develops 4-8 weeks after the healing of primary lesion. Skin and mucous membranes are commonly affected and characterized by:

❖ **Skin rashes** (palms and soles Fig. 37.3A)

❖ **Condylomata** lata (mucocutaneous papules which coalesce to form large pink to grey lesion in warm moist intertriginous areas (such as perianal region, vulva, and scrotum) (Fig. 37.3B)

❖ **Mucous** patches (superficial mucosal erosions Fig. 37.3C)



Figs 37.3A to C: Clinical manifestations of secondary syphilis. **A.** Skin rashes; **B.** Condylomata lata and **C.** Mucosal patch

Source: Public Health Image Library/A-ID# 6808, B- ID# 4098 and C- ID# 4816/Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

- Generalized lymphadenopathy is seen. Chancre may also persist in up to 1/3rd cases.

Latent Syphilis

Latent syphilis is characterized by absence of clinical manifestations of syphilis with positive serological tests for syphilis and normal CSF findings.

- Latent syphilis may be early latent syphilis (occurs within first year after infection) and late latent syphilis (occurs after the first year of infection)
- Patients are still infectious transmitting the infection either by bloodstream or *in utero*
- Latent syphilis may have one of the following fates:
  - Persistent lifelong infection (common)
  - Development of late syphilis (rare)
  - Spontaneous cure.

Late or Tertiary Syphilis

Several decades after the initial infection, about one-third of untreated patients develop tertiary syphilis, of which 15% develop gummatous lesions, about 10% develop cardiovascular lesions and remaining 10% develop neurosyphilis. The latter two stages are sometimes classified as *quaternary syphilis*.

- **Gumma (late benign syphilis):** Gummas are locally destructive granulomatous lesions. They can occur in any organ but most commonly seen in bone and skin
- **Neurosyphilis:** Though neurosyphilis is generally considered as a part of late syphilis, invasion of CNS occurs early within first few weeks of infection, which is followed by years of asymptomatic period. Common manifestations include:
  - Meningeal syphilis (meningitis)
  - Meningovascular syphilis (vasculitis of arteries leading to embolic stroke)
  - General paresis of insane
  - Tabes dorsalis (demyelination of the posterior columns).
- **Cardiovascular syphilis:** Characterized by aneurysm of ascending aorta and aortic regurgitation.

Congenital Syphilis

Though transmission of infection across the placenta may occur at any stage of pregnancy, but fetal damage occurs only after fourth month of gestation. Untreated cases of early maternal syphilis are at higher risk. Antenatal screening and treatment of positive cases during pregnancy may prevent congenital syphilis.

Manifestations of congenital syphilis include:

- **Earliest manifestations** occur within 2 years of age. Affected children are infectious and they suffer from rhinitis (or snuffles), mucocutaneous lesions, bone changes, hepatosplenomegaly and lymphadenopathy
- **Late congenital syphilis** occurs after 2 years and is noninfectious. It is characterized by interstitial keratitis, eighth-nerve deafness, bilateral knee effusions (Clutton's joints). **Residual stigmata** may remain for long time such as:
  - Hutchinson's teeth (notched central incisors)
  - Mulberry molars
  - Saddle nose, and saber shins.

LABORATORY DIAGNOSIS OF SYPHILIS

Laboratory diagnosis of syphilis consists of demonstration of treponemes, detection of antibodies and PCR.

LABORATORY DIAGNOSIS — Syphilis

Microscopy
- Dark ground microscopy
- Direct IF staining for *T. pallidum* (DFA-TP)
- Silver impregnation method
  - Levaditi stain (for tissue section)
  - Fontana stain (smear).

Culture: Not cultivable, maintained in rabbit testes

Serology (antibody detection)
- ***Non-treponemal or STS (standard tests for syphilis):*** (reagin antibodies are detected by using cardiolipin antigen)
  - Old methods: Wassermann test (CFT) and Kahn Test

Contd...

Contd...

LABORATORY DIAGNOSIS	Syphilis
➢ Newer methods (slide flocculation tests): ♦ VDRL (Venereal disease research laboratory) test ♦ RPR (Rapid plasma reagin) ♦ TRUST (toluidine red unheated serum test) ♦ USR (Unheated serum reagin test). ❑ ***Specific/Treponemal test:*** Specific antibodies are detected by using *T. pallidum* antigens ➢ TPI (*Treponema pallidum* immobilization test) ➢ FTA-ABS (Fluorescent treponemal antibody absorption test) ➢ TPA (*T. pallidum* agglutination test) ➢ TPIA (*T. pallidum* immune adherence test) ➢ TPHA (*T. pallidum* hemagglutination test) ➢ TPPA (*T. pallidum* particle agglutination test). ❑ ***Group specific:*** RP CFT (Reiter protein Complement fixation test)-uses Reiter strain **Polymerase chain reaction (PCR)**	

Direct Microscopy (Demonstration of Treponemes)

Treponemes can be demonstrated from the superficial lesions of primary, secondary and congenital syphilis.

Surface of the chancre is cleaned with saline, gentle pressure is applied at the base of the lesion, and a drop of exudate is collected on a slide.

Dark Ground Microscopy (DGM)

Treponemes cannot be visualized by light microscope but can be seen by examining the wet film of specimen under dark ground (DGM) or phase contrast microscope.

- **Under DGM:** *T. pallidum* appears as slender, flexible, spirally coiled bacilli with tapering ends, measuring 6–20 µm in length and contains 6–20 spirals spaced at an interval of 1–1.5 µm wavelength (Fig. 37.4A)
- **Motility:** *T. pallidum* shows typical: (i) slow to rapid *flexion-extension type* of movement with (ii) rotation around its longitudinal axis **(corkscrew motility)**, (iii) rotation may be accompanied by a **soft bending** at right angle to the midpoint
- The **sensitivity** of DGM approaches 80% with a detection limit of 10^4 bacilli/mL.
- **Multiple specimens** should be examined on three consecutive days before declaring DGM to be negative
- **Saprophytic spirochetes:** It is difficult to differentiate *T. pallidum* from other saprophytic spirochetes of the genital area, such as *T. refringens* (shows very active serpentine-like movement), and *T. phagedenis* (shows jerky movement). Differentiation is based on size, spiral character and motility.

Direct Fluorescent Antibody Staining for T. pallidum (DFA-TP)

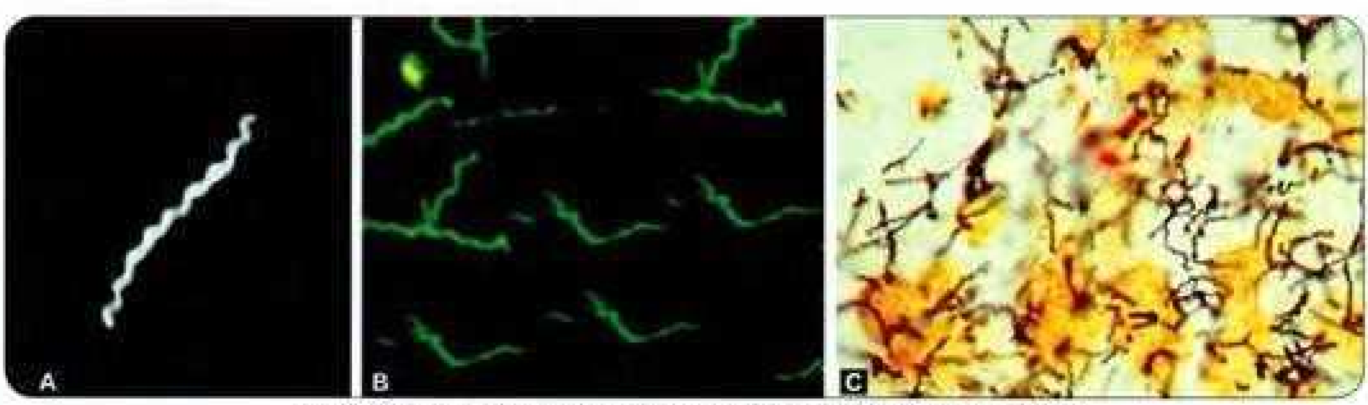
Smear made from the exudate or tissue sections is stained with fluorescent-labelled monoclonal antibody targetted against *T. pallidum* surface antigens.

- *T. pallidum* appears as distinct, sharply outlined, apple green fluorescent colored bacilli (Fig. 37.4B)
- Sensitivity of DFA-TP test approaches 100% when smear made from fresh lesions are examined.

Silver Impregnation Staining

Treponema do not take up ordinary stains as they are extremely thin and delicate (Fig. 37.4C).

- However, silver impregnation methods can be used to increase their thickness
- Treponemes reduce silver nitrate to metallic silver that is deposited on the surface, making them thicker
- Levaditi stain is used for staining tissue section and Fontana stain is used for staining smears made from exudates.



Figs 37.4A to C: Direct microscopy of *T. pallidum*. **A.** Dark ground microscope; **B.** Direct fluorescent antibody staining (DFA-TP) and **C.** Silver impregnation method

Source: Public Health Image Library, A. ID# 2043, B. ID# 14967/Dr Russell, C. ID# 836, Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

Cultivation

Pathogenic treponemes including *T. pallidum* cannot be grown in artificial culture media but are maintained by subcultures in susceptible animals such as rabbit testes.

Serology (Antibody Detection)

As microscopy is difficult and culture methods are not available, antibody detection methods are of paramount importance in the diagnosis of syphilis.

Depending upon the type of antigen used, three types of tests are available to detect antibodies in patient's sera:

- **Non-treponemal tests:** Detect non-specific reagin antibody by using cardiolipin antigen derived from bovine heart
- **Treponemal tests:** Detect species-specific antibody by using *T. pallidum* specific antigen; which is polysaccharide in nature
- **Group-specific tests:** Detect group or genus-specific antibody by using Reiter treponemal strains possessing protein antigen, which is present in all treponemes.

Non-treponemal or Non-specific tests or STS (Standard Tests for Syphilis)

Non-treponemal tests detect a characteristic non-specific antibody (called reagin antibody) in the sera of syphilitic patients by using cardiolipin antigen extracted from beef heart.

- Cardiolipin antigen is chemically a diphosphatidyl glycerol. Similar lipid haptens have been detected on the surface of *T. pallidum*
- However, it is not clear whether the reagin antibodies are induced against the lipid haptens present in *T. pallidum* or to the similar antigens released from the damaged host tissues
- Such reagin antibodies are IgG or rarely IgM type and are distinct from the IgE class of reagin antibodies seen in type I hypersensitivity reactions.

Various tests have been described, such as:

- Wassermann test (e.g. of complement fixation test) and Kahn test (e.g. of tube flocculation test)—both are no longer in use
- Slide flocculation tests such as: Venereal Disease Research Laboratory (VDRL), RPR (Rapid Plasma Reagin), USR (Unheated Serum Reagin) TRUST (Toluidine Red Unheated Serum Test).

Venereal Disease Research Laboratory (VDRL)

- This test was named after Venereal Disease Research Laboratory (VDRL), New York, where the test was developed. It is widely used, simple and rapid serological test. VDRL antigen is a cardiolipin antigen to which cholesterol and

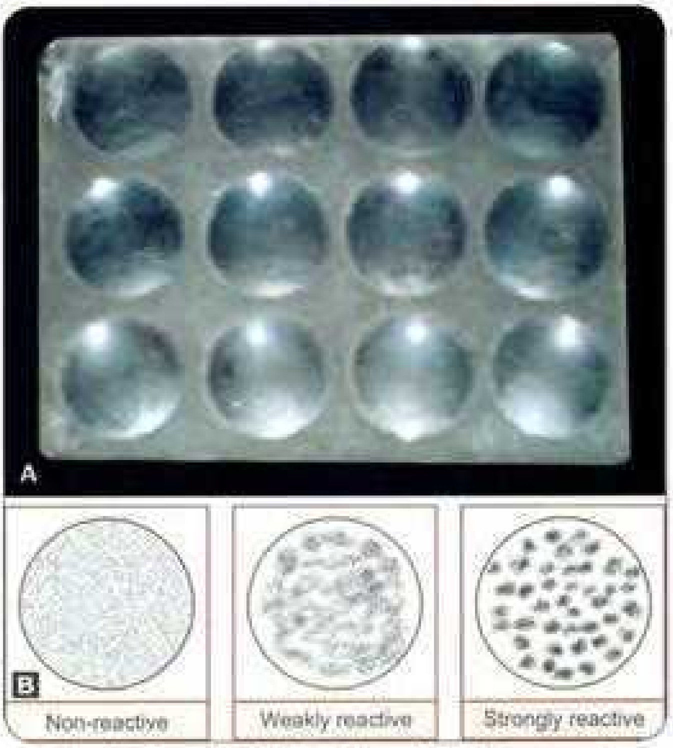
Contd...

Contd...

lecithin are added (standardized by Pangborn, 1945). In India, it is prepared at Institute of Serology, Kolkata.

Procedure

- **Antigen preparation:** VDRL antigen has to be reconstituted with a buffer present in the kit and has to be used within 24 hours. This step is needed for maturation and polymerization of the antigen
- Patient's serum is inactivated by heating at 56°C for 30 minutes to remove the non-specific inhibitors
- **VDRL slide** containing 12 concave rings are used (Fig. 37.5A)
- **Qualitative test:** 50 µL of inactivated serum is mixed with a drop of VDRL antigen and the slide is rotated at 180 revolutions per minute for 4 minutes in a VDRL rotator and examined under microscope (10x). The results are read as follows:
    - **Non-reactive:** Uniformly distributed fusiform crystals represent the presence of VDRL antigen only, which indicates a negative result
    - **Reactive:** Presence of medium to large clumps signifies antigen antibody complexes; hence, it indicates a positive result (Fig. 37.5B).
- **Quantitative test:** If the test is found reactive, antibody titer is determined by performing the test with serial dilutions (1:2, 1:4, 1:8 and so on) of serum done with 0.9% saline
- **VDRL-CSF:** VDRL test can also be performed to detect CSF antibodies. However, no preheating of CSF is needed.



Figs 37.5A and B: A. VDRL slide and **B.** VDRL test results

Source: Department of Microbiology, JIPMER, Puducherry (with permission).

Rapid Plasma Reagin (RPR)

RPR is another slide flocculation test using disposable plastic cards having 10 clearly defined circles. It is similar to VDRL test with some differences (Tables 37.3 and 37.4).

Unheated Serum Reagin Test (USR)

USR is similar to VDRL except for:

- EDTA is used as antigen stabilizer; hence, daily preparation of antigen is eliminated
- Choline chloride is used to inhibit the non-specific inhibitors in serum; hence pre-heating of serum is not needed.

Toluidine Red Unheated Serum Test (TRUST)

TRUST is a modified RPR test where toluidine red pigment particles are used instead of carbon particles. Like RPR test, it does not require microscope for examination.

Advantages of Non-treponemal Tests

- Non-treponemal tests are the most recommended tests to monitor the response to treatment. Reagin tests usually become negative 6–18 months after the effective treatment for syphilis, depending on the stage at which treatment is given
- Neurosyphilis: VDRL can also be used to detect CSF antibodies
- Reagin antibody becomes detectable 7–10 days after the appearance of primary chancre (or 3–5 weeks after acquiring the infection)
- Sensitivity: The sensitivity of nontreponemal tests varies from 78 to 85% in primary stage, 100% in secondary stage and 95–98% in latent stage.

Disadvantages of Non-treponemal Tests

Biological false-positive (BFP) reactions: BFP reactions are defined as positive results in non-treponemal tests, with negative results in treponemal tests, in the absence of syphilis and not caused by technical faults.

- Cardiolipin antigen being non-specific may react with the sera of patients suffering from unrelated diseases but not having syphilis
- Reagin antibodies are induced against the cardiolipin antigen present in *T. pallidum* or to similar lipid haptens released from the damaged host tissues
- **Frequency:** These BFP reactions may occur in about 1% of normal sera
- BFP antibody is usually of IgM type, while reagin antibody in syphilis is mainly IgG
- BFP reactions may be seen in the conditions such as lepromatous leprosy, relapsing fever, malaria, tropical pulmonary eosinophilia, viral hepatitis, infectious mononucleosis, HIV, pregnancy and IV drug abusers

Other disadvantages include:

- **Prozone phenomena:** If antibody titer in patient's sera is high, it may lead to false negative result hence it is essential to test sera in dilutions
- **Sensitivity** of non-treponemal tests is low in late stage of syphilis. VDRL-CSF is more reliable for neurosyphilis than VDRL test of serum
- **Screening tests:** Non-treponemal tests are used as screening tests which should always be confirmed by treponemal tests
  - If treponemal tests are found positive, this confirms syphilis

Table 37.3: Differences between VDRL and RPR tests

VDRL	RPR
Results read microscopically (low power) as clumps are smaller in size	Results read macroscopically: Finely divided **carbon particles** coated with cardiolipin antigens are used so that larger visible clumps are formed
Antigen, once reconstituted, should be used within 24 hours	**EDTA** is used as stabilizer; hence, RPR antigen can be stored longer (up to 6 months at 4–10°C)
Pre-heating of serum is required to remove nonspecific inhibitors	Preheating of serum is not required as **choline chloride** is used to remove inhibitors
Blood, plasma, serum, and CSF can be tested	Blood, plasma and serum can be tested **but not CSF**
Rotation of slide is done for 4 minutes	Rotation of card is done for 8 minutes
Sensitivity in primary syphilis is 78%	Sensitivity in primary syphilis is 86%
It is cheaper; hence preferred when sample load is high and for antenatal screening	RPR is expensive than VDRL. It is preferred when sample load is less.

Abbreviations: VDRL—Veneral disease research laboratory; RPR—Rapid plasma reagin; EDTA—Ethylenediaminetetraacetic acid; CSF—Cerebrospinal fluid

Table 37.4: Sensitivity and specificity of various tests for syphilis

	Sensitivity (%)				Specificity (%)
Treponemal	**Primary**	**Secondary**	**Latent**	**Late**	
FTA-ABS	84	100	100	96	97
TPPA	88	100	100	*	98
TPHA	76	100	97	94	99
EIA	90	100	100	*	99
Western blot	90	100	100	*	98
Non-treponemal					
VDRL	78	100	95	71	98
RPR	86	100	98	73	98
USR	80	100	95	*	99
TRUST	85	100	98	*	99

* sensitivity not reported

Abbreviations: FTA-ABS—Fluorescent treponemal antibody absorption; TPPA—Treponema pallidum particle agglutination; TPHA—*T. Pallidum* hemagglutination assay; EIA—Enzyme immuno assay; VDRL—Venereal Disease Research Laboratory; RPR—Rapid Plasma Reagin; USR—Unheated Serum Reagin; TRUST—Toluidine Red Unheated Serum Test

- If treponemal tests are found negative, this indicates false-positive non-treponemal tests.

Treponemal or Specific Tests

All reactive non-treponemal tests must be confirmed by treponemal tests using specific *T. pallidum* antigens to rule out the biological false positive reactions (Table 37.4). Treponemal tests include:

T. pallidum Immobilization (TPI) test (Uses live *T. pallidum*)

TPI test is based on the ability of patient's antibody and complement to immobilize the live actively motile *T. pallidum* (Nichols strain), observed under dark ground microscope. It was one of the widely used test for syphilis in the past, now not in use.

Fluorescent Treponemal Antibody-Absorption Test (FTA-ABS) (Uses killed *T. pallidum*)

It is an indirect fluorescent antibody technique.

- The patient's serum is first diluted with an extract of nonpathogenic Reiter treponemes to remove group-specific treponemal antibodies
- Patient's serum is layered on a slide previously coated with killed *T. pallidum*
- Serum antibodies bound to *T. pallidum* can be detected by addition of fluorescent labeled anti-human immunoglobulin and then slide is examined under fluorescent microscope
- **IgM-FTA-ABS test** is another modification used for congenital syphilis. It detects IgM antibodies in fetal serum
- **Advantages:** FTA-ABS is highly sensitive and specific in all the stages of syphilis and it is the first serological test to be positive following infection. It can also be used to detect CSF antibodies
- **Disadvantage:** False positive results may occur in Lyme disease (FTA-ABS being positive and VDRL test negative).

Test Using an extract of *T. pallidum*

T. pallidum Hemagglutination Assay (TPHA)

TPHA is usually performed in microtiter plates; hence also called microtiter hemagglutination.

T. pallidum (MHA-TP) test

Patient's serum (pretreated with Reiter treponemes) is added to a drop of tanned sheep RBCs coated with *T. pallidum* antigens.

- **Reactive result:** Smooth mat of agglutinated cells is formed in the wells of microtiter plate
- **Nonreactive result:** It is reported when compact button is formed in the center of the well
- **Quantitation** of treponemal antibody can be done by serial dilution of patient's sera
- **Advantages:** TPHA is affordable, easy to perform, available as commercial kit and no special equipment is needed. It can also be used to detect CSF antibodies. Thus, TPHA has been used as standard confirmatory test worldwide
- Sensitivity and specificity of TPHA are excellent in all the stages, except for primary syphilis where the sensitivity is low
- **TPPA:** *T. pallidum* particle agglutination test is a modification of TPHA where gelatin particles are used for sensitizing with *T. pallidum* instead of *tanned* RBCs.

Enzyme Immunoassays

ELISA specific to IgG and IgM have been developed for the diagnosis of syphilis.

- They have excellent sensitivity and specificity
- IgM ELISA is more sensitive than IgM FTA-ABS for diagnosis of congenital syphilis.

Western Blot

Western blot is available for detecting IgG and IgM antibodies separately. It is highly sensitive and specific.

Group-specific Test

Reiter's protein complement fixation test (RP-CFT): It works on the principle of CFT, detects group- or genus-specific treponemal antibodies against the protein antigen prepared from cultivable Reiter strain. Its sensitivity is low and is obsolete nowadays.

Molecular Methods

PCR-based techniques are available to amplify *T. pallidum* specific genes, such as gene coding for 47-kDa surface antigen (lipoprotein) and 39-kDa basic membrane protein. PCR is of paramount importance in the diagnosis of congenital and neurosyphilis.

Diagnosis of congenital syphilis

Definitive diagnosis:

Demonstration of *T. pallidum* by DGM of umbilical cord, placenta, nasal discharge, or skin lesion material.

Presumptive diagnosis:

- Infant born to a mother who had syphilis at the time of delivery regardless of findings in the infant
- Reactive treponemal test in infant
- One of the following additional criteria:
  - Clinical signs/symptoms of congenital syphilis
  - Abnormal CSF findings without other cause
  - Reactive VDRL-CSF test
  - Reactive IgM antibody test specific for syphilis (IgM FTA ABS or IgM ELISA).

Note: As IgM does not cross the placenta, its presence in neonatal serum confirms the diagnosis of congenital syphilis.

Syphilis and HIV

Both syphilis and HIV affect each other's pathogenesis.

- Genital syphilis facilitates the transmission of HIV through the abraded mucosa
- Patient with HIV, if develops syphilis later→there is rapid progression to late stages of syphilis and neurological involvement even after treatment of primary or secondary syphilis.

Problems in the diagnosis of syphilis in HIV infected people are:

- Confusing clinical signs and symptoms
- Lack of serologic response in a patient with a clinically confirmed case of active syphilis
- Unusually high titers in non-treponemal tests perhaps as a result of B-cell activation
- Failure of non-treponemal test titers to decline even after treatment with standard regimens
- Disappearance of treponemal test reactivity over time.

TREATMENT **Syphilis**

- **Penicillin** is the drug of choice for all the stages of syphilis:
  - Primary, secondary, or early latent syphilis: single dose of Penicillin G is given.
  - Late latent CVS or benign tertiary stage: penicillin G is given single dose weekly for 3 weeks.
  - Neurosyphilis or abnormal CSF in any stage or associated HIV-aqueous crystalline or procaine penicillin G is given for 10–14 days.
- **Alternative drug is used** in patients with penicillin allergy:
  - Primary, secondary, latent, CVS or benign tertiary syphilis—tetracycline is recommended.
  - Neurosyphilis or pregnancy or associated HIV—desensitization to penicillin has to be done, following which penicillin is administered.

Evaluation after Treatment

Non-treponemal tests, such as VDRL and RPR are preferred over treponemal tests for monitoring response to treatment. Antibody titers of treponemal tests remain elevated even after clinical improvement. VDRL has to be done at 3 months' intervals for at least 1 year.

- **For primary and secondary syphilis:** Following clinical improvement, there should be at least fourfold decline in the titer by the third or fourth month and an eightfold decline in the titer by sixth to eighth month
- **Latent or late syphilis, or patients with multiple episodes of syphilis:** It may show a gradual decline in titer, low titers may persist for years.

Prevention

Prevention of syphilis includes:

- Treatment of cases and contacts (sexual partners)
- Education about safe sex practices
- Prophylactic use of barrier contraceptive methods.

NON-VENEREAL TREPONEMATOSES

Endemic or nonvenereal treponematoses are caused by three close relatives of *T. pallidum*, out of which the first two are considered as subspecies of *T. pallidum*:

- *T. pertenue* (causes yaws)
- *T. endemicum* (causes endemic syphilis)
- *T. carateum* (causes pinta).

Endemic treponematoses can traditionally be distinguished from venereal syphilis by (Table 37.5):

- Mode of transmission (direct contact, not sexual)
- Age of acquisition (childhood)
- Geographic distribution (rural areas of developing nations of tropics, travelers in developed nations)
- Associated with poor hygiene
- Clinical features (described in the text).

Yaws

Yaws (also known as pian, framboesia, or bouba) is an endemic disease caused by *T. pallidum* subspecies *pertenue*.

Table 37.5: Comparison of venereal syphilis vs non-venereal treponematoses

Feature	Venereal syphilis	Yaws	Endemic syphilis	Pinta
Agent	*T. pallidum*	*T. pertenue*	*T. endemicum*	*T. carateum*
Mode of transmission	Sexual, transplacental blood	Skin-to-skin	Household contacts: kissing, sharing utensils	Skin-to-skin
Age	Adulthood	Early childhood	Early childhood	Late childhood
Primary lesion	Chancre—painless, non-indurated Lymphadenopathy	Papilloma, often ulcerative Lymphadenopathy	Rarely seen	Nonulcerating pruritic papule
Site of lesion	Genital, oral, and anal	Extremities	Oral	Extremities, face
Secondary lesions	Skin rashes Mucosal patches, condylomata lata	Skin lesions—macular or papular periostitis	Oral mucous patches, periostitis, lymphadenopathy	Pintides, pigmented and pruritic
Relapses	~25%	Common	Unknown	None
Late complications	Gummas, CVS and CNS lesions seen	Destructive gummas of skin, bone, cartilage Destruction of nose, maxilla and palate is termed as **gangosa**, No CNS or CVS lesion		Non-destructive, **dyschromic macule** No CNS or CVS lesion

- **Epidemiology:** Yaws is endemic in the tropical areas of Africa, South-east Asia, and Central America. Of the 13 currently endemic countries, 8 of them reported >46,000 cases in 2015. There was a recent outbreak in Cameroon in 2017.
  - In India, cases were found from tribal hilly areas of Odisha, Chhattisgarh, Assam, Andhra Pradesh and Madhya Pradesh
  - However, India actively participated in yaws eradication programme in 1996 and has reported no new cases since 2003. India has achieved the yaws free status in the year 2016 (as declared by WHO).
- **Transmission** is by direct skin-to-skin contact
- **Clinical manifestation:** Incubation period is about 3–4 weeks. Yaws is characterized by:
  - Primary lesions ("mother yaw"): Extragenital papule on extremities that enlarges in moist warm weather to become papillomatous or raspberry-like (thus the name 'framboesia')
  - Regional lymphadenopathy may be developed
  - Secondary eruptions are more generalized.
  - Skin lesions may take several forms such as macular, papular, or papillomatous. Painful lesions on the feet result in a crab-like gait ("crab yaws")
  - Periostitis may result in nocturnal bone pain
  - All early skin lesions are infectious
  - **Late yaws** occurs in 10% of untreated persons, and is manifested by destructive lesions (gumma) of skin, bone, and joints. Destruction of the nose, maxilla, palate and pharynx may be developed, termed as **gangosa** (Table 37.5)
  - Relapses are common during the first 5 years.

Yaws eradication

In 2012, WHO initiated the Yaws Eradication Strategy, also referred to as "the Morges strategy", aiming for global eradication by 2020.

- Criteria for Eradication include (i) absence of new indigenous case for three years and (ii) absence of evidence of transmission for 3 years, measured with sero-surveys (RPR test) among children.
- Mass treatment of azithromycin to at least 90% of the targeted at-risk population is carried out.

TREATMENT — Yaws

- Azithromycin (single oral dose) is the preferred choice for treatment (WHO). Patients should be examined 4 weeks after treatment for clinical recovery and for detection of azithromycin resistance.
- Benzathine penicillin (single intramuscular dose) is reserved for patients who clinically fail on azithromycin, or are allergic to azithromycin.

Endemic Syphilis

Endemic Syphilis (also called by local names: *bejel, siti, dichuchwa, njovera, skerljevo*) is caused by *T. pallidum* subspecies *endemicum*.

- **Epidemiology:** It is endemic in arid areas of Asia (Syria, Saudi, Iraq, and Iran), Africa (Ghana, Mali, Niger, and Senegal) and Australia, but not in Americas
- **Transmission:** Bejel is transmitted by direct contact, by kissing or by sharing drinking and eating utensils.

Clinical Manifestation

- **Early manifestations:** It starts as oral papule which progresses to mucosal patches on the oral mucosa and mucocutaneous lesions resembling the condylomata lata of secondary syphilis. Periostitis and regional lymphadenopathy are common
- **Late manifestations:** It occur in the form of destructive gummas, osteitis, and gangosa which are more common than in late yaws.

Pinta

Pinta (also known as *mal del pinto, carate, azul, purupuru*) is the most benign of all treponemal infections, caused by *T. carateum*.

- **Epidemiology:** Pinta is limited to Central America and northern South America, where it is found rarely and only in remote villages
- **Transmission** is by direct skin-to-skin contact
- **Clinical manifestations:** Pinta is characterized by marked changes in skin color without causing destructive lesions. It is manifested as:
  - **Pruritic papules:** They occur as primary lesions on the extremities or face
  - **Pintides** are disseminated secondary lesions, characterized by deeply pigmented, pruritic lesions. They are infectious and may persist for years
  - **Dyschromic macules** are the late pigmented lesions which may contain treponemes (Table 37.5)
  - Over time, most lesions become depigmented to form white achromic lesions.

Diagnosis of Non-venereal Treponematoses

Diagnosis is based on the clinical manifestations, dark ground microscopy and serological tests.

- As they are virtually indistinguishable from *T. pallidum* antigenically, the serological tests used for syphilis can also be used for diagnosis of non-venereal treponematoses
- Till date, there is no test available that can differentiate between various treponemes.

TREATMENT — Pinta and Endemic syphilis

Both patients and their contacts can be treated by benzathine penicillin.

BORRELIA

The ultrastructure of *Borrelia* is similar to *Treponema* and *Leptospira* with minor differences (refer to Table 37.1 and Fig. 37.1A).

- **Size:** Larger, 10–30 µm in length and 0.2–0.5 µm breadth
- **Spirals:** They are less in number (3–10) with wider spirals (3 µm) and longer amplitude (2 µm)
- **Endoflagella:** More in number (7–11), attached subterminally at the pole
- **Microscopy:** *Borrelia* is poorly Gram-stained (other spirochetes do not take up Gram stain). It is better viewed under dark ground microscope or by sliver impregnation staining.

Most of the species of *Borrelia* occur as commensals on the buccal and genital mucosa. Few are pathogenic to men, such as:

- *B. recurrentis* causes epidemic relapsing fever
- *B. burgdorferi* is the agent of Lyme disease
- *B. vincentii* causes Vincent's angina in association with fusiform bacilli.

RELAPSING FEVER

Relapsing fever (RF) is characterized by recurrent episodes of fever and nonspecific symptoms following exposure to insect vector carrying *Borrelia* species. Relapsing fever is of two types:

1. **Epidemic RF**: It is caused by *B. recurrentis* and transmitted by louse.
2. **Endemic RF** is caused by *Borrelia* species other than *B. recurrentis* such as *B. duttoni*, *B. hermsii* and *B. turicatae*. It is transmitted by tick.

Pathogenesis

- **Mode of transmission**
  - **Epidemic RF**: It is transmitted by human body louse (*Pediculus humanus*). Borreliae are introduced by crushing of the louse (e.g. by scratching) leading to deposition of insect's infected hemolymph containing numerous spirochetes on the abraded skin and mucous membranes
  - **Endemic RF:** It is transmitted by bite of an infected tick (*Ornithodoros* species).
- From the inoculated site, *Borrelia* spreads rapidly leading to bacteremia and fever. Host's immune system tries to eliminate the bacilli from the body
- However, the borrelial surface antigens frequently undergo **antigenic variation.** Each time, new antigens are produced which can evade host's immune system leading to repeated bacteremia and recurrent febrile episodes.

Table 37.6: Differences between epidemic and endemic relapsing fever

Characters	Epidemic relapsing fever	Endemic relapsing fever
Agent	*B. recurrentis*	*B. duttoni, B. hermsii*
Natural host	Humans	Rodents
Transmitted by	Louse–by crushing or rubbing	Tick bite
Distribution	East Africa (Sudan and Ethiopia)	North America, Central Asia, and Africa
Hemorrhage, CNS features	More common	Less common
Treatment	Doxycycline—single-dose	Doxycycline for 1 week

Clinical Manifestations

Both epidemic and endemic RF have similar manifestations although not identical (Table 37.6). Incubation period is about 7–8 days.

- **Recurrent febrile episodes** lasting for 3–5 days occur intervening with afebrile periods of 7–9 days. Subsequent episodes are shorter
- **Non-specific symptoms** may be present like alteration of sensorium, abdominal pain, vomiting and diarrhea
- **Hemorrhages:** Petechiae, epistaxis and blood-tinged sputum are more likely in epidemic RF
- **Neurologic features** such as meningitis, seizure, focal deficits, paraplegia and psychosis may occur in 10–30% of cases and are more common in epidemic RF.

Laboratory Diagnosis

- **Microscopy:** Microscopic features of *Borrelia* have been described earlier. Various methods are available to detect *Borrelia* from blood.
  - Peripheral thick or thin smear-stained by Wright- or Giemsa-stain (Fig. 37.6)
  - Direct fluorescent antibody test using monoclonal antibody is employed to identify the species
  - Dark ground microscope (low sensitivity)
  - Quantitative buffy coat (QBC) analysis is an alternative method with higher sensitivity
  - It is poorly gram-negative.
- **Culture:** During afebrile period, microscopy fails to detect *Borrelia*; hence, the confirmation is made by isolation of *Borrelia* from blood
- **Animal pathogenicity** testing can be done by intraperitoneal inoculation into white mice
- **Serology**
  - **ELISA** and **IFA** (indirect fluorescence assay) are available to detect serum antibodies. Fourfold rise of titer can be considered significant. However, false positive results may occur in other spirochete infections

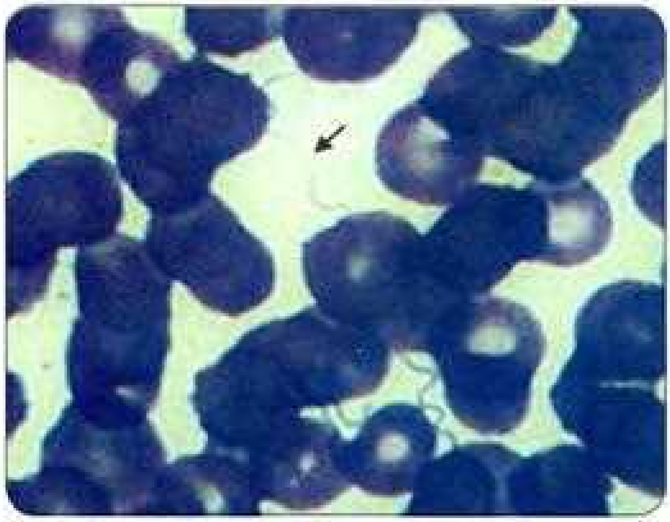


Fig. 37.6: Peripheral blood smear showing *Borrelia* species (Giemsa-staining) (arrow showing)

Source: Public Health Image Library, ID# 14495/Fort Collins/Centers for Diseases Control and Prevention (CDC), Atlanta (*with permission*).

- **GlpQ assay:** It is the most reliable serological method. It is an immunoblot assay detecting antibody against the recombinant GlpQ antigen (Glycerophosphodiester phosphodiesterase).

❖ **Molecular methods:** Multiplex Real-time PCR has been developed targeting *16S rRNA* and *GlpQ* genes to identify the various species of *Borrelia* causing RF.

TREATMENT — Relapsing fever

Antibiotics such as doxycycline or erythromycin are the drug of choice for relapsing fever. Recommended schedule is single dose for epidemic RF, and 7–10 days course for endemic RF.

LYME DISEASE

Agent

Lyme disease or Lyme borreliosis is caused by three genomospecies of *Borrelia*, collectively referred to as *Borrelia burgdorferi* sensu lato (i.e. *B. burgdorferi* in the general sense). They are:

❖ *Borrelia burgdorferi* sensu stricto (*B. burgdorferi* in the strict sense, hereafter referred to as *B. burgdorferi*)
❖ *Borrelia garinii*
❖ *Borrelia afzelii*.

Epidemiology

Rodents and deer are main reservoirs of Lyme disease. It is widespread in USA, but also reported from other parts of the world.

❖ All three genomospecies are found in Europe
❖ *B. burgdorferi* is the sole cause of Lyme disease in USA
❖ Latter two species; *B. garinii* and *B. afzelii* infections occur in Asia.

Fig. 37.7: Erythema migrans: Annular bull's eye pattern rash

Source: Public Health Image Library, Centers for Disease Control and Prevention (CDC), Atlanta, ID# 9874/J. Gathany (*with permission*).

Transmission

Lyme disease is transmitted by **tick bite** (*Ixodes ricinus* complex). All three stages of tick, i.e. larval, nymphal, and adult stages can transmit the infection.

❖ The spirochete expresses outer-surface protein A (OspA) in the midgut of the tick which is required for its survival in tick
❖ When the bacterium reaches the salivary gland of the tick, it expresses protein **OspC** that binds to a tick salivary-gland protein (**Salp15**). This attachment is crucial for transmission
❖ The tick must attach at least for 24 hours for transmission of *B. burgdorferi*.

Clinical Manifestations

Lyme disease occurs through four stages:

❖ **Stage 1: Early localized infection:** After an incubation period of 3–32 days, an annular maculopapular lesion develops at the site of the tick bite called **erythema migrans**, commonly involving thigh, groin, and axilla (Fig. 37.7). It may be absent in 20% of the cases
❖ **Stage 2: Early disseminated infection:** *B. burgdorferi* spreads hematogenously to many sites within days or weeks resulting in:
  - Secondary annular skin lesions
  - Musculoskeletal pain (arthralgia)
  - Profound malaise and fatigue
  - Neurological abnormalities, which occur in 15% of cases and include meningitis, encephalitis and a typical lymphocytic meningoradiculitis seen in cases from Europe and Asia; called ***Bannwarth's syndrome***

- Cardiac involvement occurs in 8% of cases, including atrioventricular block.

❖ **Stage 3: Late persistent infection (Lyme arthritis):** About 60% of untreated patients develop frank arthritis involving *large joints* (especially the knees), lasting for weeks or months in a given joint. Some cases of Lyme arthritis are refractory for treatment. **Acrodermatitis chronica atrophicans** is a late skin manifestation caused by *B.afzelii*, (affecting elderly women)

❖ **Post-Lyme syndrome** (Chronic): Few patients present with chronic fatigue symptoms and neurocognitive manifestations, develop after months to years of infection.

Laboratory Diagnosis

❖ **Isolation** of *B. burgdorferi* can be done by culturing specimens like skin lesions, blood or CSF in special medium called BSK medium (Barbour-Stoenner-Kelly). Cultures are incubated at 34°C and examined under dark field microscope weekly for two months

❖ **Genus atrophicans** is based on distinct properties of *Borrelia* such as morphology, size and motility as described earlier

❖ **Molecular methods:**
- PCR detecting specific DNA is much superior to culture for the detection of *B. burgdorferi* in joint fluid. But its sensitivity is poor for CSF, blood or urine samples. Common gene targets include 16S rRNA, *flaB*, *ospA* (outer surface lipoprotein)
- PCR-RFLP (restriction fragment length polymorphism) of the intergenic *rrf-rrl* region has been used for genomospecies detection.

❖ **Serology (antibody detection):** The most common method of diagnosis of Lyme disease is by characteristic clinical picture with a positive serological test
- **ELISA and western blot** formats are available detecting IgM and IgG separately
- In the first month of infection, both IgM and IgG are detected. As disease proceeds, IgM disappears and IgG response predominates
- **Fourfold rise of antibody** at 2-3 weeks' interval is more significant which obviates the false positive results
- **Two-test approach:** CDC recommends to perform an ELISA first → if found positive, it has to be confirmed by western blot
- **C6 peptide IgG ELISA** is recently introduced which has shown promising results. It is a second generation ELISA, uses *VlsE* lipoprotein antigen of *B. burgdorferi*.

❖ **↑WBC count:** Joint fluid examination reveals elevated polymorphonuclear cells whereas CSF shows lymphocytosis.

TREATMENT **Lyme disease**

- For all stages of Lyme disease except CNS and CVS infection: Oral doxycycline is the drug of choice, except for children where amoxicillin is given. Duration of treatment is as follows:
  - Localized skin infection (14 days)
  - Early disseminated infection (21 days)
  - Acrodermatitis (30 days)
  - Arthritis (30–60 days).
- For CNS or CVS infection: Ceftriaxone is given for 14–28 days.

VINCENT'S ANGINA

Vincent's angina (or trench mouth) is an acute ulcerative necrotising gingivostomatitis or oropharyngitis caused by symbiotic association of two organisms:

❖ *Borrelia vincentii* in association with *Leptotrichia buccalis* (formerly known as *Fusobacterium fusiforme*): An anerobic gram-negative bacillus, long, thin spindle-shaped with pointed ends (Fig. 37.8)

❖ Disease is characterized by inflamed pharyngeal mucosa covered by greyish membrane resembling diphtheria, but it peels off easily

❖ Both the agents are normal flora of mouth; however, they can be potential pathogens in the presence of underlying malnutrition or viral infections.

Laboratory Diagnosis

Demonstration of spirochetes and fusiform bacilli in stained smears of exudates from the lesion remains the mainstay of diagnosis. Cultivation is difficult but can be done in enriched media, incubated anaerobically.

TREATMENT **Vincent's angina**

Penicillin and metronidazole are effective.

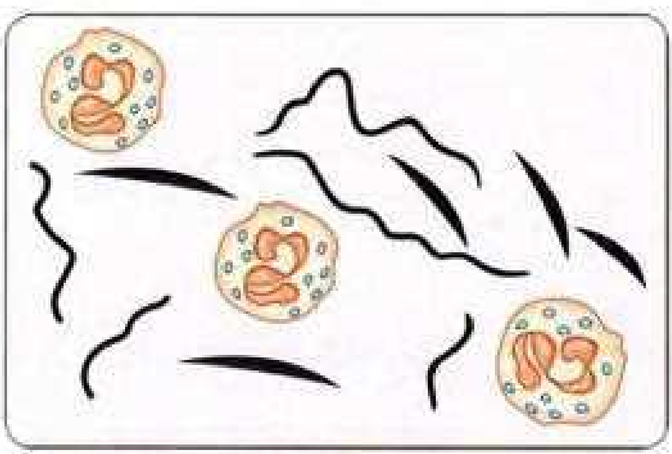


Fig. 37.8: Schematic picture showing *Borrelia vincentii* and *Leptotrichia buccalis*

LEPTOSPIRA

CLASSIFICATION

Phenotypic Classification

Species: *Leptospira*, comprises of two species:

1. L. *interrogans* (pathogenic for humans): It causes leptospirosis or Weil's disease involving liver and kidney.
2. *L. biflexa* (saprophyte).

Serovars and serogroups: *Leptospira* is antigenically complex and can be further classified on the basis of agglutination testing with specific antisera against the surface LPS (lipopolysaccharide) antigens.

- *L. interrogans* comprises of 26 serogroups (Table 37.7) which further consist of over 300 serovars
  - Serogroup Icterohaemorrhagiae has several serovars such as Icterohaemorrhagiae, Copenhageni, Lai, Naam and Mwogolo
  - New serogroups and serovars continue to be discovered
  - Although all serogroups and serovars are morphologically identical, produce similar clinical picture but they differ in their geographical distribution and in severity of infection.
- *L. biflexa* has 65 serovars arranged in 38 serogroups.

Genotypic Classification

DNA hybridization, *Leptospira* has been classified into 17 genomic species. Though it is considered to be the gold standard technique for species level identification of leptospires, it is seldom used because of its complexity.

LEPTOSPIRA INTERROGANS

L. interrogans have the size of 6-12 μm length × 0.1 μm width which allows them to pass through filters used to sterilize the culture medium.

- They are tightly and regularly coiled, with characteristic hooked ends (hence the species name *interrogans*—resembling interrogation or question mark)
- Spirals have a wave length (interval between spirals) of 0.5 μm and amplitude of 0.1 μm
- They possess a single endoflagellum attached at the pole and are highly motile exhibiting spinning and translational movements
- They cannot be seen under the light microscope due to their thinness (leptos, meaning fine or thin). They do not take up ordinary stains
- They may be observed by dark ground or phase contrast microscope or stained by sliver impregnation method and by immunofluorescence.

Table 37.7: *Leptospira serogroups*

Serogroups of *Leptospira interrogans*		
Australis	Grippotyphosa	Sarmin
Autumnalis	Hebdomadis	Sejroe
Ballum	Icterohaemorrhagiae	Semaranga
Bataviae	Javanica	Tarassovi
Canicola	Leptonema	Hurstbridge
Celledoni	Lyme	Ranarum
Cynopteri	Mini	Turneria
Djasiman	Pomona	Manhao
	Pyogenes	

Epidemiology

- **Mode of transmission:** Leptospirosis is zoonotic. Direct human-to-human transmission does not occur. It is transmitted by:
  - Indirect contact with water, moist soil and wet surfaces contaminated with animal urine or
  - Direct contact with urine and products of parturition, placenta of infected animals.
- **Source:** Although more than 100 animals can be infected; important sources of infection are rats, dogs, cattle and pigs. Even asymptomatic animals can transmit the infection via urine
- **Seasonality:** More common in rainy and post monsoon period
- **Risk factors** that promote transmission include:
  - Lower socioeconomic status
  - Urban and rural slum areas
  - Rainfall and floods
  - Occupational exposure: Agricultural workers (e.g. rice field planters), fishermen, sewer workers and all those persons who are liable to work in rodent infested environment are at increased risk
- **3R's:** The three important epidemiological determinants for leptospirosis include exposure to **rodents, rainfall** and **rice field**
- **Incidence:** The incidence rate ranges from 0.1-1/100,000 per year in temperate climates to 10–100/100,000 in tropical countries. During outbreak the incidence may reach over 100/100,000
- **Global distribution:** Leptospirosis is worldwide in distribution. Highest burden of the disease has been reported from area with high population density such as urban slums of Brazil, India and Thailand
- **In India:**
  - Leptospirosis has been reported more commonly from coastal districts of Andaman and Nicobar (hence leptospirosis is also called as **Andaman Hemorrhagic fever**), Gujarat, Kerala, Maharashtra and Tamil Nadu followed by **Andhra Pradesh,**

Table 37.8: Clinical stages of leptospirosis

	Mild anicteric febrile illness		Weil's disease	
	First stage 3–10 days (septicemic)	Second stage 10–30 days (immune)	First stage 3–10 days (septicemic)	Second stage 10–30 days (immune)
Clinical findings	• Fever • Myalgia • Headache • Conjunctival suffusion • Abdominal pain • Pharyngeal erythema without exudates • Vomiting	• Meningitis, • Uveitis, optic neuritis chorioretinitis • Rash • Fever • Peripheral neuropathy	• High-grade fever • Liver-Jaundice and raised liver enzymes • Hemorrhages: ➢ Pulmonary hemorrhage ➢ Petechiae and purpura ➢ Conjunctival hemorrhage ➢ Gastrointestinal hemorrhage • Kidney—Raised serum urea and creatinine and renal failure	
Isolation	From blood and CSF	From Urine	Blood and CSF	Urine
Serum IgM	Absent	Present	Absent	Present
Antibiotics	Susceptible to antibiotics	Refractory to treatment	Susceptible to antibiotics	Refractory to treatment

Karnataka, Dadar and Nagar Havelli, Daman and Diu, Puducherry, Goa and Odisha from time to time

- The serovars predominantly present in India are L. Andamana, L. Pomona, L. Grippotyphosa, L. Hebdomadis, L. Semoranga, L. Javanica, L. Autumnalis, L. Canicola.

Pathogenesis

There are two distinct phases of pathogenesis following leptospiral infection:

1. **First phase (septicemic phase):** After entering through the mucosa (conjunctival or oral) or abraded skin, *L. interrogans* spill over to the bloodstream and then disseminate hematogenously to various organs including brain, liver, lung, heart and kidney.
   - **Vascular damage:** Spirochetes can be found in the walls of capillaries, medium and large-sized vessels. The exact mechanism of vascular damage is not clear
   - Penetration and invasion of tissues is due to active motility and release of hyaluronidase.
2. **Second phase (immune phase):**
   - As antibodies develop, spirochetes disappear from the blood. Antigen antibody complexes are deposited in various organs
   - Renal colonization: Bacilli become adherent to the proximal renal tubular brush border and are excreted in urine.

Clinical Manifestations

The incubation period is around 10 (4–20) days. In general, the manifestations can be divided into two distinct clinical syndromes:

1. **Mild anicteric febrile illness:** It occurs in 90% of patients. It is biphasic; a septicemic phase occurs first, followed by immune phase.
2. **Weil's disease** (Hepato-renal-hemorrhagic syndrome): It is a severe form of icteric illness and occurs in 10% patients. Typical biphasic course may not be present. It is more severe and fulminant (Table 37.8).

LABORATORY DIAGNOSIS — Leptospirosis

Specimens: CSF, blood and urine

Microscopy

Dark ground or phase contrast microscope or silver impregnation staining: Reveals spirally coiled bacilli (tightly and regularly coiled), with characteristic hooked ends like umbrella handle.

Isolation

- ❑ Culture condition: 30°C for 4–6 weeks
- ❑ Medium: EMJH medium, Korthof's and Fletcher's media.

Animal inoculation: Samples are inoculated into hamsters and young guinea pigs.

Serology for antibody detection

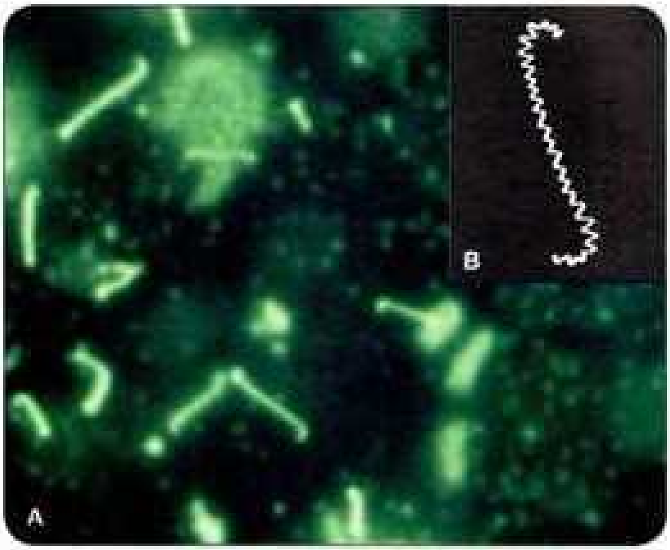
- ❑ Genus specific tests: Macroscopic slide agglutination test, latex agglutination test, ELISA, ICT
- ❑ Serovar specific test: Microscopic agglutination test.

Molecular methods:

- ❑ PCR detecting 16S or 23S rRNA or IS1533 genes
- ❑ PCR-RFLP and PFGE: to detect genomospecies
- ❑ Faine's diagnostic criteria
- ❑ Nonspecific findings: altered renal and liver function tests.

Laboratory Diagnosis

- ❖ **Specimens:** CSF and blood (in first 10 days of infection) and urine (between 10 and 30 days of infection) are useful specimens
- ❖ **Microscopy:** *Leptospira* are extremely thin; hence, cannot be seen under light microscope
  - **Wet films:** They may be observed under dark ground or phase contrast microscope (Fig. 37.9B)
  - **Staining:** They do not stain by ordinary stain, but can be stained by *silver impregnation stains* such as Fontana stain and modified Steiner technique
  - *L. interrogans* is 6–12 µm long and 0.1 µm wide
    - They are *tightly* and regularly coiled, with characteristic hooked ends like umbrella handle
    - Spirals have a wave length (width) of 0.5 µm and amplitude of 0.1 µm



Figs 37.9A and B: *Leptospira interrogans* (spirally coiled bacilli with hooked ends) **A.** dark ground microscopy of the mount following microscopic agglutination test, **B.** schematic diagram (viewed under dark-ground microscope).

Source: A. Public Health Image Library/ID#: 2888/ Mrs. M Gatton, Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

- They are highly motile; exhibit spinning and translational movements.
- **Disadvantage:** Microscopy is less sensitive and requires technical expertise. Serum proteins and fibrin strands in blood may resemble leptospires.

❖ **Isolation**

- **Culture condition:** *Leptospira* is obligate aerobe and slow growing. Cultures should be incubated at 30°C for 4–6 weeks at pH 7.2–7.5. Culture fluid should be examined under dark ground microscope for the presence of leptospires on the first, third and fifth day followed by weekly intervals up to 6 weeks
- **Culture media:** As *Leptospira* is highly fastidious, requires enriched media such as:
    - **EMJH medium** (Ellinghausen, McCullough, Johnson, Harris) is a semisynthetic liquid medium, most commonly used nowadays
    - It is composed of albumin fatty acid supplement added to the basal media containing 0.1% agar. Leptospiral growth produces a dense ring of organisms just at the under surface of the medium, called **Dinger's ring**
    - **Korthof's** medium with rabbit blood and **Fletcher's** semisolid medium can be used.
- **Advantages**
    - Isolation of *Leptospira* confirms the diagnosis
    - It is useful to maintain the stock culture of the *Leptospira* in the laboratories.
- **Disadvantages**
    - Culture technique is laborious, technically demanding and time-consuming

Fig. 37.10: ICT for Leptospira antibody

Source: Dept. of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (with permission).

- False-positive results: These may occur due to contamination of culture media with other organisms or saprophytic leptospires
- False-negative results: These may occur due to prior use of antibiotics, or incubating in improper temperature and pH.
- **Precautions taken** for obtaining pure culture:
    - Subculture in media containing antibiotics such as 5-fluorouracil which inhibits the contaminants
    - Filtration of urine sample through 0.22 µm filter and followed by inoculation on to selective culture media
    - Urine should be collected in phosphate buffer saline (PBS) with pH 7.2 to neutralize the acidic pH of urine.

❖ **Animal inoculation:** Samples may be inoculated into animals such as hamsters (4–6 weeks old) and young guinea pigs and peritoneal fluid is examined for presence of leptospires

❖ **Serology for antibody detection:**

- IgM antibodies appear early within one week of illness, reach peak levels in third or fourth week and then decline slowly and become undetectable within six months
- IgG antibodies appear later than IgM; reach peak level after few weeks of illness and may persist at low level for years.

Antibody detection tests can be broadly classified into:

- **Genus-specific tests** uses broadly reactive genus-specific antigen prepared from nonpathogenic *L. biflexa* Patoc 1 strain. They cannot detect the infecting serovar. Various tests available are:
    - Macroscopic slide agglutination test
    - Microcapsule agglutination test (MCAT)
    - Latex agglutination test
    - ELISA: It detects IgM and IgG separately
    - Lepto dipstick assay: It detects IgM antibodies
    - Immunochromatographic test (ICT): It detects IgM and IgG antibodies separately (Fig. 37.10).
- **Serovar-specific test:** Microscopic agglutination test (MAT) detects antibodies against specific serovars of *L. interrogans*
    - It is the gold standard method and the reference test for the diagnosis of leptospirosis

- Patient's serum is mixed in a microtitre plate with live antigen suspensions of various leptospiral serovars endemic in the locality, incubated for 2–4 hours at 30°C and then examined under dark ground microscopy for the presence of agglutination (Fig. 37.9A).

❖ **Cross agglutination and absorption test (CAAT):** It is done to detect the relatedness between the strains

❖ **Molecular methods:** PCR has been found particularly useful in severe disease, before seroconversion occurs
- Various genes such as 32-kDa lipoprotein (*lipL32*) gene, 16S or 23S rRNA or IS1533 insertion sequence are targeted
- However, PCR is not serovar-specific
- PCR-RFLP (Restriction fragment length polymorphism) or PFGE (Pulsed-field gel electrophoresis) are the methods followed to determine the genomo species of *Leptospira*.

❖ **Faine's criteria:** It is a WHO-approved guideline used for the diagnosis of leptospirosis. It is based on clinical, epidemiological and laboratory findings

❖ **Non-specific findings** such as:
- **Altered renal function:** Elevated levels of blood urea nitrogen and serum creatinine
- **Altered liver function:** Elevated bilirubin and liver enzymes in serum
- Urine sediment analysis may show increased leukocytes, erythrocytes, hyaline and granular casts.

TREATMENT — Leptospirosis

- **Mild leptospirosis** should be treated with oral doxycycline (100 mg twice a day for 7 days). Amoxicilin can be given alternatively
- **Severe leptospirosis:** Penicillin is the drug of choice (1.5 million units IV, four times a day for 7 days), alternatives being ceftriaxone or cefotaxime.

Prevention

Vaccine

Whole cell vaccines (mono- or polyvalent) containing specific serovars of *Leptospira* are available in many countries. SPIROLEPT manufactured by Sanofi-Pasteur is available for subcutaneous injection as two doses of 1 mL each at a 15-days interval, with the third dose 4–6 month after the first dose, followed by biannual revaccination. The efficacy rate is around 60–100%.

General Measures

❖ Chemoprophylaxis with doxycycline is recommended for anticipated short-term exposures, such as military training or travelling or fresh-water swimming
❖ General sanitation approaches including proper waste disposal
❖ Rodent control
❖ Avoidance of swimming in contaminated places
❖ Health education.

EXPECTED QUESTIONS

I. Essays:

1. Describe the clinical manifestations and laboratory diagnosis of syphilis.
2. Kishan, a young farmer was complaining of fever, headache, and myalgia. Gradually, he developed yellow discoloration of skin and sclera. On examination, he had conjunctival inflammation and hepatosplenomegaly. His blood count showed neutrophilia with a thrombocytopenia. Liver function tests showed an elevated conjugated bilirubin with mild elevation of transaminases. He was also found to be oliguric and uremic.
   a. What is the etiological agent and how is this disease transmitted?
   b. What is the typical clinical presentation and pathogenesis of this condition?
   c. How will you confirm the diagnosis?

II. Write short notes on:

1. Relapsing fever.
2. Lyme disease.
3. Yaws.

III. Multiple Choice Questions (MCQs):

1. **Which of the following tests confirms the diagnosis of leptospirosis?**
   a. Culture of urine on EMJH media.
   b. Testing serum by darkfield examination for the presence of leptospires.
   c. Testing acute and convalescent phase sera for anti-leptospiral antibodies by microscopic agglutination test.
   d. Culture of CSF on blood and chocolate agar.
2. **Which of the following statements about relapsing fever is correct?**
   a. Each relapse is associated with an antigenically distinct variant.
   b. Blood smears should be made when the patient is afebrile.
   c. Transmitted by flea bite.
   d. Caused by *Borrelia burgdorferi*.
3. **Weil's disease is caused by:**
   a. *Leptospira interrogans*
   b. *Borrelia recurrentis*
   c. *Treponema carateum*
   d. *Treponema pallidum*
4. **Bejel is caused by:**
   a. *Borrelia recurrentis*
   b. *Treponema endemicum*
   c. *Treponema pallidum*
   d. *Treponema carateum*

Answers

1. c **2.** a **3.** a **4.** b

Rickettsiae, Coxiella and Bartonella

38
CHAPTER

Chapter Preview

- General properties
- Family rickettsiaceae
  - *Rickettsia* species
  - *Orientia tsutsugamushi*
- Family anaplasmataceae- *Ehrlichia*, and others
- Former members
  - *Coxiella burnetii*
  - *Bartonella* species

GENERAL PROPERTIES

Rickettsiae comprise of group of small non-motile gram-negative coccobacilli that possess the following common characteristics:

- They are obligate intracellular organisms
- They are not cultivable in artificial media, although they can grow in cell lines, or by animal and egg inoculation
- They are transmitted by arthropod vectors, such as tick, mite, flea or louse.

Classification

The order Rickettsiales has two families:

1. Family Rickettsiaceae comprises of two pathogenic genera—*Rickettsia* and *Orientia*
2. Family Anaplasmataceae includes four genera—(1) *Ehrlichia*, (2) *Wolbachia*, (3) *Anaplasma* and (4) *Neorickettsia*.

Former members such as *Coxiella* and *Bartonella* are now excluded from the family because:

- *Coxiella* is not arthropod borne; infection is transmitted by inhalational mode
- *Bartonella* is not an obligate intracellular parasite; capable of growing in cell-free media. It also differs in genetic properties.

Rickettsiae Versus Viruses

Because of the small size and obligate intracellular properties, Rickettsiae were once thought to be viruses, however now they are confirmed to be bacteria due to having following characters:

- They possess gram-negative cell wall (however, they are poorly gram-stained, better stained with Giemsa or Gimenez stains)
- They contain both DNA and RNA
- They possess ribosomes for protein synthesis and enzymes for the Krebs cycle
- Rickettsiae multiply by binary fission
- They are susceptible to antibacterial agents
- Rickettsiae are large enough to be seen under the light microscope
- They are held back by bacterial filters.

History

- *Rickettsia* is named after Howard Taylor Ricketts (1911), who discovered that Rocky Mountain spotted fever is transmitted by tick
- *Rickettsia prowazekii* is named by Da Rocha Lima in honor of von Prowazek
- Both von Prowazek and H Ricketts died of typhus which they contracted during their study
- Charles Nicolle identified the role of body lice in the transmission of epidemic typhus.

FAMILY RICKETTSIACEAE

Family Rickettsiaceae comprises of two pathogenic genera *Rickettsia* and *Orientia* (Table 38.1).

GENUS RICKETTSIA

Species of *Rickettsia* can be categorized into two groups based on the clinical manifestations (Table 38.1):

1. Typhus group.
2. Spotted fever group.

Antigenic Structure

The cell wall of rickettsiae is similar to that of gram-negative bacteria, composed of peptidoglycan, lipopolysaccharide, and an outer membrane containing few outer membrane proteins.

Table 38.1: Features of Rickettsiaceae

Broad group	Species	Disease	Vector	Distribution	Rash	Eschar	LN	Weil-Felix test
Typhus group	*R. prowazekii*	Epidemic typhus	Louse (rubbing)	Worldwide (Africa and South America)	80% (All over the body except palm and sole)	–	–	OX19 ++++ OX2 +/-
		Brill-Zinsser disease						Negative or weakly positive
	R. typhi	Endemic typhus	Flea	Worldwide	80% (trunk)	–	–	OX19 ++++ OX2 +/-
Spotted fever group	*R. rickettsii*	Rocky mountain spotted fever (RMSF)	Tick	America	90% (extremities and trunk, more hemorrhagic)	<1%	+	OX19 ++ OX2 ++
	R. conorii	Indian tick typhus (ITT)	Tick	Europe, Asia	97%	50%	+	
	R. africae	African tick bite fever	Tick	Sub-Saharan Africa	50% (vesicular)	90%	++++	
	R. akari	Rickettsialpox	Mite (gamasid)	USA, Ukraine, Turkey, Mexico	100% (vesicular)	90%	+++	All negative
Scrub typhus	*Orientia tsutsugamushi*	Scrub typhus	Mite (trombiculid)	Asia, Australia	50%	35%	+++	OXK +++

- **Species specific outer membrane proteins (OMP):** They are highly immunogenic; surface cell antigens (*sca*) induce the synthesis of protective antibodies, thus can be used for vaccine as well as for diagnosis
  - OmpA is present only in spotted fever group of rickettsiae (coded by *sca5* gene)
  - OmpB is present in both spotted fever group and typhus fever group rickettsiae (coded by *sca4* gene).
- **Group specific alkali stable lipopolysaccharide (LPS) antigen:** It is found in some rickettsiae and is shared by certain strains of *Proteus* (OX19, OX2 and OXK strains). This antigenic cross reactivity serves as the basis of Weil-Felix reaction; the serological test used for the diagnosis of rickettsial infections.

Pathogenesis

- **Transmission:** All rickettsiae are transmitted to humans by arthropod vectors (Table 38.1)
  - **Tick and mite** borne rickettsiae are transmitted by biting, which leads to inoculation of organisms present in arthropod's saliva into the skin of the host during its blood meal
  - **Louse and flea** borne rickettsiae are transmitted by:
    - Autoinoculation following rubbing or scratching of abraded skin or mucosa contaminated by insect feces (seen in epidemic and endemic typhus), or
    - Aerosol (by inhaling dried louse or flea feces in the laboratory or as part of bioterrorism).
  - **Transovarial transmission:** Tick and mite can act as reservoir, maintain the organism, and pass to their offspring. Hence, they can serve as both vector as well as reservoir.
- **Spread:** Rickettsiae spread through the lymphatics from the portal of entry, multiply in the regional lymph nodes and then spread via bloodstream
- **Target sites:** For all rickettsiae, the final target site is the endothelial cells (in addition, *R. akari* and *O. tsutsugamushi*, attack the monocytes)
- **Phagocytosis:** Adhesion to the endothelial cells is mediated by outer membrane proteins; OmpA and OmpB present on rickettsial surface. Following adhesion, the organisms are phagocytosed
- **Intracellular locations:** Following phagocytosis, rickettsiae remain inside a vacuole. Later on, they vary in their intracellular locations:
  - *Rickettsia* and *Orientia* produce phospholipase A that lyses the vacuoles. They come out and are found free in the cytoplasm (in addition, spotted fever rickettsiae are also found free in the nucleus)
  - *Coxiella* and *Ehrlichia* continue to multiply in cytoplasmic vacuoles
    - *Coxiella* vacuole fuses with lysosome, but it is able to survive inside the acidic environment of the phagolysosome
    - In contrast, the ehrlichiae are maintained inside the vacuoles. They are killed if they fuse with the lysosomes.
- **Multiplication:** Inside the host cells, they multiply slowly by binary fission (generation time is about 9–12 hours)

- **Cell-to-cell spread:** Spotted fever rickettsiae can spread from cell-to-cell by actin polymerization. In contrast other rickettsiae accumulate in the cell until the lysis of the cell takes place
- **Reason for obligate intracellular survival:** It is not understood. However, it is observed that rickettsiae depend on the host cell for many reasons:
  - They lack many enzymes required for glycolysis, pentose phosphate pathways, purine and pyrimidine synthesis pathways and also lack genes coding for several amino acids
  - Although, they can produce their own adenosine triphosphate (ATP), but prefer to use the host cell ATP if available.
- **Endothelial cell injury:** This occurs via lipid peroxidation of host-cell membranes
  - In order to exploit the cell for its own growth, rickettsiae inhibit cell apoptosis by up regulating NF-kB pathway activation
  - The vascular endothelial cells enlarge, degenerate and cause thrombosis of the vessels leading to rupture and necrosis.
- **Release:** Once these bacteria are released from the host cells, they are unstable and die quickly. The exception is *Coxiella* which is highly resistant to desiccation and remains viable in the environment for months to years.

Epidemic Typhus (Louse-borne)

Epidemic typhus is caused by *R. prowazekii*.

- **Vector:** Human body louse, *Pediculus humanus corporis* acquires the organism while taking the blood meal from an infected patient. Rickettsiae multiply in the midgut epithelial cells of the louse and are shed in its feces
- **Mode of transmission:** (1) Autoinoculation of the organisms following rubbing or scratching of abraded skin or mucosa contaminated by louse feces, (2) rarely, by inhalation of louse feces, in the laboratory or during bioterrorism
- **Clinical manifestations:** Epidemic typhus is an acute febrile disease; accompanied by headache, myalgia, eye discharge and rashes occurring after an incubation period of 1-2 weeks
  - **Rash** begins on the upper trunk, usually on the fifth day, and then becomes generalized, involving the entire body except the face, palms and soles
  - **Myalgia** is usually severe, was referred to as sutama ("crouching") in Burundi outbreak, a designation reflecting the posture of the patients attempting to alleviate the pain
  - **Complications** include interstitial pneumonitis, CNS involvement like mental confusion and coma ('*typhus*' name comes from the Greek word *typhos* meaning smoky or hazy, describing the state of mind of the affected patients), myocarditis and acute renal failure.
- **Risk factors:** Outbreaks occur when louse population increases; especially in unhygienic conditions. Typical settings include refugee camps, prisons and overcrowded communities
- **Zoonotic cycle:** Eastern flying squirrels (*Glaucomys volans*) and their lice and fleas maintain *R. prowazekii* in the environment
- **Geographical distribution:** It is endemic in Africa (notably Burundi, Rwanda and Ethiopia) and South America (Peru, Bolivia and Ecuador). Burundi outbreak in 1997 had involved nearly 1 Lakh people in refugee camps. No cases have been reported from South East Asia since 1978 and Western Pacific since 1969
- **Brill-Zinsser disease:** It is a recrudescent illness occurring years after acute epidemic typhus. *R. prowazekii* remains latent for years; its reactivation occurs due to waning immunity, which leads to sporadic infection or outbreaks.

Endemic Typhus (Flea-borne)

Endemic (murine) typhus is caused by *R. typhi* infection.

- **Vectors:** The vector for endemic typhus is rat flea (*Xenopsylla cheopis*) or rarely by cat flea (*Ctenocephalides felis*)
- **Mode of transmission:** It is transmitted by inoculation on skin or inhalation of flea's dried feces; less frequently by the flea bite
- **Reservoir:** Rodents such as *Rattus rattus* and *R. norvegicus* species are the natural reservoirs; whereas the opossum/cat flea (*C. felis*) cycle is prominent in southern Texas and southern California
- **Clinical manifestations:** Incubation period is 1-2 weeks (average 11 days)
  - Symptoms are similar to epidemic typhus but milder and rarely fatal
  - Common symptoms include fever, headache, myalgia, anorexia and rash (involving the trunk more often than the extremities).
- **Geographical distribution:** It is endemic worldwide, especially in warm (often coastal) areas throughout the tropics having high rat infestations
  - Recent days, it is increasingly reported from South East Asia and Western Pacific
  - **India:** It has been reported from many places, such as Shimla, Kashmir, Mumbai, Jabalpur, Lucknow and Pune.

Rocky Mountain Spotted Fever

Rocky mountain spotted fever (RMSF) is caused by *Rickettsia rickettsii*.

- **Vector:** It is transmitted by various genera of ticks, such as:
  - *Dermacentor andersoni* in USA
  - *Amblyomma cajennense* in Central/South America
  - *Rhipicephalus sanguineus* in Mexico, Arizona and Brazil.
- **Transmission:** By the bite of an infected tick
- **Reservoir:** Ticks serve as vector as well as reservoir. Other mammals like dogs, small rodents can also act as reservoir
- **Clinical manifestations:** Incubation period ranges from 4 days to 14 days
  - RMS fever is an acute potentially fatal disease characterized by fever, headache and rash and frequently myalgia and anorexia
  - Rashes appear typically on extremities (wrist and ankles) and trunk. Initially they are maculopapular, later become hemorrhagic (Fig. 38.1)
  - **Complications:** They appear late, include: vascular damage, increased permeability, edema, hemorrhage, disseminated intravascular coagulation, interstitial pneumonitis, CNS involvement, myocarditis and renal failure
  - It is the most fatal rickettsial disease and is associated with higher mortality rate.
- **Geographical distribution:**
  - RMS fever is endemic in high tick population areas of USA, Central and South America
  - It is more common during tick season (summer in tropics) and among children and males.

Indian Tick Typhus

Indian tick typhus (ITT) is caused by *Rickettsia conorii*.

- **Vector:** Transmitted by tick bite (*Rhipicephalus sanguineus*)

Fig. 38.1: Characteristic rash seen in Rocky Mountain spotted fever
Source: Public Health Image Library, ID#:14489/Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*)

- **Clinical manifestations:** They are similar to that of RMS fever. In addition, an eschar is observed at the site of the tick bite in 50% of cases. Disease is more severe in patients with diabetes, alcoholism, or heart failure
- **Geographical distribution:** *R. conorii* is prevalent in Southern Europe, Africa and Southern Asia
  - Apart from ITT, disease caused by *R. conorii* has been given various other regional names such as:
    - Mediterranean spotted fever
    - Kenya tick typhus
    - Israeli spotted fever
    - Astrakhan spotted fever.
  - In India, ITT is widespread. Cases have been reported from Nagpur, Jabalpur, Sagar, Pune, Lucknow, Bengaluru and Secunderabad.

Other Tick-borne Fever

- **African tick-bite fever:** It is caused by *R. africae*, transmitted by tick bite (*Amblyomma hebraeum*) and is endemic in sub-Saharan Africa
- **Maculatum disease:** It is caused by *R. parkeri*, transmitted by tick bite (*Amblyomma maculatum*), seen in USA and South America
- **Japanese spotted fever:** It is caused by *R. japonica*, occurs in Japan and Korea
- **Queensland tick typhus:** It is due to *R. australis*, transmitted by Tick (*Ixodes holocyclus*)
- **Flinders Island spotted fever:** It is caused by *R. honei*.

Rickettsialpox

Rickettsialpox is caused by *Rickettsia akari*.

- **Vector:** Transmitted by bite of infected mites (*Liponyssoides sanguineus*) that are found on the surface of mice. Mites maintain the organisms by transovarian transmission
- **Reservoir:** Mice (*Mus musculus*) are the principal reservoir of *R. akari*
- **Clinical manifestations** of rickettsialpox are similar to any other rickettsial diseases, but differ from the later by the presence of:
  - Vesicular rashes (often confused for the rashes of chickenpox, hence so named)
  - Eschar (painless black crusted lesions surrounded by an erythematous halo), is present at the site of mite bite
  - Regional lymphadenopathy.
- **Geographical distribution:** *R. akari* is endemic in USA, Ukraine, Turkey and Mexico.

LABORATORY DIAGNOSIS — **Rickettsial infections**

Serology (antibody detection)

- Non-specific test (**Weil Felix test**)—Rickettsial antibodies detected against *Proteus* OX 19, OX 2 and OX K antigens
  - In epidemic and endemic typhus- ↑ OX 19 antibody

Contd...

Contd...

LABORATORY DIAGNOSIS	Rickettsial Infections
➢ In tick-borne spotted fever-↑OX 19 and ↑OX 2 antibodies ➢ In scrub typhus- ↑OX K antibody. ❑ Specific antibody detection – by Indirect IF, ELISA and Indirect immunoperoxidase assay **Histopathological examination** **Isolation**—by inoculating into cell lines (Vero, WI-38, HeLa), egg (yolk sac), or animal (guinea pig) **Neil Mooser reaction**—intraperitoneal inoculation into guinea pig, leads to testicular inflammation (positive tunica reaction)—Shown by *R.conori*, *R. akari* and *R. typhi* **PCR**—detecting gene encoding 56 kDa, 47 kDa ,16S rRNA or *Omp* genes.	

Laboratory Diagnosis of Rickettsiosis

Serology

Serology (antibody detection) is the mainstay of diagnosis of rickettsial diseases. They can be categorized into non-specific test (Weil-Felix test) and specific tests.

Weil-Felix Test

It is heterophile agglutination test works on the principle of antigenic cross reactivity. Though this test lacks high sensitivity and specificity but still serves as a useful and inexpensive diagnostic tool.

- Group specific alkali stable lipopolysaccharide (LPS) antigen found in some rickettsiae is also shared by certain strains of *Proteus* (OX19, OX2 and OXK strains). Hence, rickettsial antibodies are detected by using *Proteus* antigens
- **Procedure:** It is a tube agglutination test; serial dilutions of patient's serum are treated with non-motile strains of *P. vulgaris* OX19 and OX2 and *P. mirabilis* OXK
- **Results:**
  - In epidemic and endemic typhus—sera agglutinate mainly with OX19 and sometimes with OX2
  - In tick-borne spotted fever—antibodies to both OX19 and OX2 are elevated
  - In scrub typhus—antibodies to OXK are raised
  - The test is negative in rickettsialpox, Q fever, ehrlichiosis and bartonellosis
  - This test should be carried out only after 5-7 days of onset of fever. Titre of 1:80 is to be considered possible infection. However, baseline titers are needed to be standardized for each region.
- **False-positive titer** may be seen in presence of underlying *Proteus* infection. Hence, fourfold rise of antibody titer in paired sera is more meaningful than a single high titer
- **False-negative result** may occur due to excess antibodies in patient's sera (prozone phenomena). This can be obviated by testing with serial dilutions of patient's sera.
- Weil-Felix test being a nonspecific test should always be confirmed by specific tests.

Specific Antibody Detection Tests

- **Indirect immunofluorescence assay (IFA):** It is the gold standard and reference serologic test used for confirmation of the rickettsial diagnosis
  - Antibodies appear only after 7-10 days of infection
  - Titer of ≥1:64 is considered as significant
  - The sensitivity and specificity are 94–100% and 100% respectively.

  However, the cost and requirement of technical expertise limit the wide use of IFA
- **ELISA** (IgM capture ELISA): It is useful in early diagnosis (<1 week) with excellent sensitivity and specificity
- Indirect immunoperoxidase assay (IPA)
- Rapid tests such as ICT (immunochromatographic test) are available for diagnosis of scrub typhus; however not widely in use.

Other Methods of Diagnosis

- **Histological examination** of a cutaneous biopsy sample from a rash lesion can be done even during acute illness
- **Isolation:** Rickettsiae cannot be cultivated in cell free media
  - However, isolation can be done by cell lines (Vero, primary chick embryo, WI-38, HeLa), egg (yolk sac inoculation), or animal inoculation (guinea pig)
  - As rickettsiae are highly infectious, isolation should be attempted cautiously only in laboratories equipped with biosafety level III facilities.
- **Neil-Mooser reaction:** Specimen is inoculated intraperitoneally into male guinea pigs. The changes observed in the animal (over 3-4 weeks), varies among various rickettsial species
  - *R. rickettsii*—produces scrotal necrosis
  - *R. prowazekii*—produces only fever without any testicular inflammation
  - *R. typhi, R. conori* and *R. akari*—produce fever and positive tunica reaction (testicular inflammation).
- **Molecular tests:** Polymerase chain reaction (PCR) and real time PCR formats are available
  - They are rapid and specific; can detect specific rickettsial DNA (e.g. gene encoding the major surface antigens [56 kDa or 47 kDa], 16S rRNA or *Omp* genes) and 60-kDa heat shock protein (*groEL*) gene.
  - Useful specimens are: Whole blood, buffy coat fraction, skin rash biopsies, lymph node biopsies or tissue specimen
  - The results are best within the first week for blood samples as rickettsemia is present usually in first 7-10 days.

TREATMENT — Rickettsiosis

Doxycycline is the drug of choice for treatment of most rickettsial illnesses. It is given as 100 mg twice a day orally for 1–5 days. Chloramphenicol is used as alternative.

Prevention of Rickettsiosis

Preventive measures include:
- Vector control strategies such as use of insecticides
- Control of rodents and other animals
- Improvement of personal hygiene.

No vaccine is available at present against rickettsial infections.

GENUS ORIENTIA

Scrub Typhus

Scrub typhus is caused by *Orientia tsutsugamushi* (formerly classified under *Rickettsia*). It differs from *Rickettsia* by both genetically and in its cell wall composition (i.e. it lacks lipopolysaccharide layer).

- **Naming:** Scrub typhus is so named because as it can occur in areas where scrub vegetations consisting of low lying trees and bushes are encountered. However sandy, semi-arid and mountain desert areas can also be endemic harboring the vector
- **Affects military population:** Scrub typhus has been historically linked to war and military operation; thousands of cases among militarily population were reported in the Far East during the Second World War
- **Vector:** It is transmitted by the bite of infected trombiculid mites of genus *Leptotrombidium (L. akamushi* in Japan and *L. deliensis* in India)
  - Chiggerosis: Among all stages of mite, the larvae (called **chiggers**) are the only stage that feed on humans. Hence, scrub typhus is also called *chiggerosis*
  - Mites can maintain the organisms through transovarian transmission.
- **Clinical manifestations:** The classic presentation of scrub typhus consists of triad of an eschar (at the site of bite), regional lymphadenopathy and maculopapular rash. However, it is seen only in 40–50% of cases
  - Non-specific manifestations may appear early, such as fever, headache, myalgia, cough, and gastrointestinal symptoms
  - Complications, such as encephalitis and interstitial pneumonia due to vascular injury may occur rarely in the late stage.
- **Antigenic diversity:** Five major antigenic types have been identified—Boryon, Gilliam, Karp, Kato and Kawazaki. Because of this remarkable antigenic diversity exhibited by the organism, immunity wanes over 1-3 years
- **Epidemiology:** Among the rickettsial diseases, scrub typhus is most widespread
  - **Zoonotic tetrad:** Four elements are essential to maintain *O. tsutsugamushi* in nature:
    1. Trombiculid mites.
    2. Small mammals (e.g. field mice, rats, shrews).
    3. Secondary scrub vegetations or forests (hence named as scrub typhus).
    4. Wet season (when mites lay eggs).
  - **World scenario:** Scrub typhus is endemic to a part of the world known as the **"tsutsugamushi triangle"**, which extends from Northern Japan and far-eastern Russia in the north, to Northern Australia in the south, and to Pakistan in the west. Various countries included in this triangle are Japan, China, Philippines, and South-East Asia, including India, Pakistan, Afghanistan, tropical Australia, New Guinea, and Pacific Islands
  - **Indian scenario:** Scrub typhus is a re-emerging infectious disease in India. It is the most common rickettsial disease in India; prevalent in many parts of the country
    - There have been outbreaks in areas located in the sub-Himalayan belt, from Jammu to Nagaland
    - There were reports of scrub typhus outbreaks in Himanchal Pradesh, Sikkim and Darjeeling (West Bengal) during 2003-2004 & 2007, and occasional reports from Bihar, Rajasthan, Maharashtra in the past
    - Outbreaks of scrub typhus have also been reported in Southern India including Puducherry, Karnataka, Tamil Nadu and Kerala during the cooler months of the year
    - Though it is mainly reported from rural areas, however recently cases have been increasingly reported from urban areas also.
- **Treatment:** Doxycycline is the drug choice. Chloramphenicol or azithromycin is given alternatively
- **Vaccine:** An effective vaccine is not yet licensed for scrub typhus. However, several types of vaccine trials were attempted such as killed vaccine (using formalin-treated strains such as Karp and Gilliam strains), live attenuated (Gamma irradiated Karp strain) and subunit vaccines (targeting 22, 47, 56, 58, and 110-kDa antigens).

LABORATORY DIAGNOSIS — Scrub typhus

Serology (antibody detection):

Serology remains the mainstay of diagnosis. In primary infection IgM antibodies appear by the end of the 1st week, and IgG by end of the 2nd week, whereas in re-infection IgG antibodies are detectable by day 6, with IgM antibody titers being variable.

- ***Weil-Felix test:*** Nonspecific, detects high titers of heterophile antibodies to *Proteus* OXK antigens

Contd...

Contd...

LABORATORY DIAGNOSIS — Scrub typhus

- ***Indirect immunofluorescence antibody (IFA):*** It is specific, considered as the gold standard serological test
- ***ELISA*** using 56-kDa recombinant major surface protein antigens derived from Gilliam, Karp, and Kato strains. It is cost-effective and considered as alternative to IFA for acute diagnosis and seroprevalence
- ***Rapid tests*** such as ICT (immunochromatographic test) are available for diagnosis of scrub typhus. However these tests are not recommended at present as they need further evaluation
- ***Other formats*** available include: indirect immunoperoxidase test and western blot.

Culture

O. tsutsugamushi can be isolated from the clinical specimen such as egg (yolk sac), cell culture (Vero cells, MRC 5 cells, BHK21, L929 mouse fibroblast cells). Culture is time consuming (4 weeks) and technically demanding; hence not routinely performed.

Molecular test

Various formats have been developed such as PCR, nested PCR, LAMP (loop mediated isothermal amplification) targeting specific genes such as major 56-kDa gene, 47-kDa gene, 16S rRNA gene and 60-kDa heat shock protein (*groEL*) gene.

For more details, refer to 'laboratory diagnosis of rickettsial diseases', described later in this chapter.

FAMILY ANAPLASMATACEAE

EHRLICHIOSIS

Family Anaplasmataceae comprises of four obligatory intracellular organisms named *Ehrlichia*, *Wolbachia*, *Anaplasma* and *Neorickettsia* (Table 38.2).

- They reside in vertebrate reservoirs and target vacuoles of hematopoietic cells
- Few of them are pathogenic, such as:
  - *Ehrlichia chaffeensis:* It is the agent of human monocytic ehrlichiosis, infects predominantly mononuclear phagocytes
  - *Ehrlichia ewingii:* It infects neutrophils and causes human granulocytic ehrlichiosis
  - *Anaplasma phagocytophilum:* It infects neutrophils; causes human granulocytic anaplasmosis
  - *Neorickettsia sennetsu* infects the lymphocytes and cause mononucleosis like syndrome called human lymphocytic ehrlichiosis.
- **Clinical feature:** They produce an acute febrile disease that is generally characterized by headache, myalgia, arthralgia, cough, pharyngitis, lymphadenopathy, diarrhea, nausea, vomiting, abdominal pain and changes in mental status
- **Inclusions:** They reside inside the phagosome, multiply to produce the following three stages of growth—elementary body, initial body, and mulberry like inclusions called Morula
- **Morulae** in neutrophil can be detected in 20–75% of cases by Giemsa-stained peripheral blood film examination
- **Treatment:** Drug of choice for ehrlichiosis is doxycycline.

FORMER MEMBERS

Coxiella and *Bartonella* were previously under order Rickerttsiales, but now are separted.

COXIELLA BURNETII (Q FEVER)

Coxiella burnetii is an obligate intracellular organism that causes **'Q fever'**.

History

- For long time, the causative agent of the disease was unknown, hence was referred to as 'Query' or Q fever (by Edward Derrick). Later, the agent was identified as *Coxiella burnetii*
- *Coxiella burnetii* (formerly named as *Rickettsia diaporica*) was named after the two scientists Cox and Burnet who have contributed to its discovery.

Table 38.2: Features of family Anaplasmataceae

Characteristics	*Ehrlichia chaffeensis*	*Ehrlichia ewingii*	*Anaplasma phagocytophilum*	*Neorickettsia sennetsu*
Causes	Human monocytic ehrlichiosis (HME)	Human granulocytic ehrlichiosis (HGE)	Human granulocytic anaplasmosis (HGA)	Human lymphocytic ehrlichiosis (HLE)
Features	• Leukopenia • Thrombocytopenia • Elevated liver enzymes • Risk factor: Immunocompromised patients	• Features similar to HME but less severe • Risk factor: Immunocompromised patients	• Leukopenia • Thrombocytopenia	Mononucleosis like illness Atypical lymphocytosis Lymphadenopathy
Transmitted by	Tick (*Amblyomma americanum*)	Tick (*Amblyomma americanum*)	Tick (*Ixodes scapularis*)	Ingestion of fish carrying infected flukes
Reservoir	White-tailed deer (rarely dogs)	White-tailed deer and dogs	Mice, squirrels, and white-tailed deer	Not known
Distribution	USA	USA	USA	Japan and Malaysia

Source of Infection

Q fever is a zoonosis. The primary sources of human infection are infected cattle, sheep and goats. Wild animals and ticks are the reservoirs of infection.

Mode of Transmission

- The most common mode is by inhalation of infected dust from soil, previously contaminated by urine and feces of diseased animals
- By ingestion of contaminated milk
- Rarely, transplacental, blood transfusion or through skin abrasions/mucosa.

Geographical Distribution

Q fever is endemic in most parts of the world except New Zealand and Antarctica.

- In India, it is present in human and animal population in Rajasthan, Punjab, Haryana and Delhi
- In Rajasthan, the overall prevalence rate was found to be 18.6% in humans and 24.7% in animals.

Pathogenesis

C. burnetii escapes intracellular killing in macrophages by:

- Inhibiting the final phagosome maturation step (cathepsin fusion)
- Resistant to the acidic environment of phagolysosome by producing superoxide dismutase.

Infection with *C. burnetii* induces autoantibodies, particularly to cardiac and smooth muscles.

Clinical Manifestations

- **Acute Q fever:** After an incubation period of 3-30 days, patients present with hepatitis, interstitial pneumonia, fever, CNS involvement and pericarditis or myocarditis
- **Post Q fever fatigue syndrome:** It can occur following acute stage. Profound myalgia, headache, sweating, arthralgia, muscle fasciculation are the chief complaints
- **Latency:** *Coxiella* may remain latent in the tissues of patients for 2-3 years
- **Chronic Q fever:** It is characterized by endocarditis and usually occurs in patients underlying valvular heart disease, or immunosuppression. Fever is usually absent or of low grade.

Phase (Antigenic) Variation

Surface antigens, e.g. lipopolysaccharide (LPS) of *C. burnetii* shows phase variation.

- Fresh isolates possess Phase I antigen (LPS with a complex carbohydrate which blocks antibody interaction with surface proteins)
- On repeated passage in yolk sac, it switches over to Phase II (LPS is modified exposing the surface proteins that can react with antibodies).

Laboratory Diagnosis

C. burnetii is a small pleomorphic gram-negative coccobacillus. It is extremely fastidious, does not grow on routine media (it is one of the etiological agent for culture negative endocarditis). It is highly infectious, hence processing should be done in a biosafety level 3 laboratory.

- **Isolation:** Culture of *C. burnetii* must be done only in biosafety level 3 laboratories due to its extreme infectivity. It can be isolated by inoculation of specimens onto conventional cell cultures (monkey kidney cells, Vero cells) or by shell vial cell culture using human embryonic lung fibroblasts cell line, or yolk sacs (egg) or animals such as hamsters, mice or guinea pigs
- **Antibody detection:** Serology is the most commonly used diagnostic tool
  - **Indirect immunofluorescence assay** (IFA), also called as microimmunofluorescence (Micro-IF) is sensitive, specific and is the method of choice
    - IgM appears in 7-10 days of infection followed by IgG, which appears after 14-20 days of infection
    - In chronic infections, the IgG antibodies to phase I antigens are elevated (>1:6400 titer), whereas in acute Q fever, there is a rise of antibodies to phase II antigens (IgG ≥1:200 and IgM ≥1:50).
  - Complement fixation test can also be done detecting IgG antibodies to phase II antigens
  - Q fever sera do not cross-react with rickettsiae or *Proteus.*
- **Immunodetection of *C. burnetii* in tissues:** *C. burnetii* can be detected in tissues by immunoperoxidase staining targeting various antigens. This is especially informative in patients who are undergoing treatment for chronic Q fever
- **Molecular methods:** Strains of *C. burnetii* differ in their plasmids which they carry. QpH1 plasmids are found in acute Q fever isolates; whereas QpRS plasmids are found on the strains isolated from endocarditis patients. Other genes targeted are 16S rRNA, 23S rRNA, superoxide dismutase and *htpAB* genes.

TREATMENT — Q fever

- **Acute Q fever:** Doxycycline (100 mg twice daily for 14 days) is the drug of choice. Quinolones are also effective
- **Chronic Q fever:** Hydroxychloroquine is added to alkalinize the phagolysosome and to render doxycycline to act against the organism.

Prevention

Control measures include:

- **Vaccine:** Inactivated whole-cell vaccine (Q-Vax) is licensed in Australia. It is recommended for occupationally exposed workers

- Good animal husbandry practices should be followed such as proper disposal of animal excreta and aborted materials, isolation of aborting animals for 14 days
- Pasteurization of milk should be done by Flash method as *C. burnetii* survives Holder's method of pasteurization.

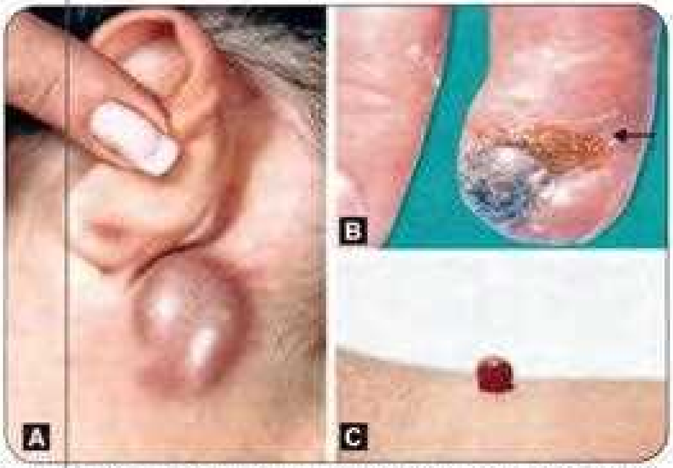
BARTONELLA

Bartonella species are fastidious, facultative intracellular, slow-growing, gram-negative bacteria that have ability to invade mammalian cells and RBCs. Among the 13 species infecting humans, three are most commonly identified pathogens—*B. bacilliformis*, *B. quintana*, and *B. henselae* (Table 38.3).

Bartonella henselae

Bartonella henselae is transmitted by cat scratch or bite. Cat fleas (*Ctenocephalides felis*) may be responsible for cat-to-cat (but not cat-to-man) transmission. It causes various diseases such as:

- **Cat-scratch disease (CSD):** Apart from *B. henselae* (most common cause), rare cases of CSD have also been associated with *Afipia felis* and *B. quintana*. It has two clinical forms:
  1. **Typical CSD:** This is more common form, characterized by subacute regional lymphadenopathy (most common being axillary/epitrochlear lymph nodes) and painless erythematous papule or pustule, that develops at the site of cat scratch (Fig. 38.2A).
  2. **Atypical CSD:** It is characterized by extranodal manifestations such as hepatitis, splenitis, and retinitis.
- **Bacillary angiomatosis (epithelioid angiomatosis):** It is an angioproliferative disorder, characterized by neovascular lesions involving skin and other organs (Fig. 38.2B)
  - It is associated with HIV and other severely immunocompromised conditions
  - It is caused by both *B. henselae* and *B. quintana*
    - Skin lesions are caused by both species



Figs 38.2A to C: A. Enlarged lymph node of cat-scratch disease; **B.** Bacillary angiomatosis; **C.** Verruga peruana

Source: Centers for Disease Control and Prevention/NEJM (with permission).

    - Hepatosplenic lesions are caused by *B. henselae*, whereas subcutaneous and lytic bone lesions are caused by *B. quintana*.
- **Bacillary peliosis:** It is another angioproliferative disorder caused by *B. henselae*, involving liver (peliosis hepatitis), spleen and lymph nodes
- **Bacteremia and endocarditis** may occur rarely.

Bartonella quintana

Bartonella quintana is transmitted to humans by body louse feces (autoinoculated into skin due to scratching). It causes trench fever, chronic bacteremia, endocarditis and bacillary angiomatosis.

Trench Fever (Quintan Fever)

- **Classical trench fever:** It was first seen causing epidemics in the trenches of World War I (1919) where it presented as periodic mild febrile illness lasting 4–5 days with 5-day intervals between the episodes (hence also called **5 days fever**). Thereafter, it was silent for decades

Table 38.3: Features of *Bartonella* species

Bartonella	Diseases	Reservoir	Transmission
B. henselae	• Cat-scratch disease • Bacillary angiomatosis • Bacillary peliosis • Bacteremia, endocarditis	Cats, Other felines	• Exposure to cat: by scratch or bite • Cat fleas associated with cat-to-cat transmission, but not cat-to-human transmission
B. quintana	• Trench fever • Chronic bacteremia, endocarditis • Bacillary angiomatosis	Humans	Louse (*Pediculus humanus corporis*)
B. bacilliformis	• Bartonellosis (Carrion's disease) • Oroya fever • Verruga peruana (Fig. 38.2C)	Humans	Sandfly (*Lutzomyia verrucarum*)

- **Re-emerged trench fever**: The disease has re-emerged in USA recently. It presents with chronic bacteremia and endocarditis, seen most often in homeless people.

Bartonella bacilliformis

Bartonella bacilliformis is transmitted by vector sandfly (*Lutzomyia*). Humans are the only known reservoir. It produces a biphasic disease:

- **Oroya fever or Carrion's disease:** It is the initial, bacteremic, systemic illness presenting with or without anemia
- **Verruga peruana:** It is a late-onset manifestation, characterized by cutaneous vascular lesions (Fig. 38.2C).

Laboratory Diagnosis of *Bartonella* Infections

- **Specimens** collected are blood, lymph node or skin biopsies
- **Microscopy:** Warthin-Starry silver nitrate staining and immunofluorescence staining can be used to detect *B. henselae* from lymph node smears
- **Culture:** *Bartonella* can be grown on blood agar at 37°C (except for *B. bacilliformis* at 30°C) in presence of 5% CO_2 and incubated for 12–15 days (maximum up to 45 days). Sensitivity can be increased after cell lysis or freezing the sample
- **Antibody detection:** Both indirect immunofluorescence assay (IFA) and enzyme immunoassay (EIA) based methods are available to detect antibodies against *B. henselae* and *B. quintana* separately
- **PCR:** It can be done to amplify the genes such as citrate synthase gene, 16S rRNA gene or heat-shock protein gene.
- **Treatment:** Refer Table 38.4.

Table 38.4: Treatment of bartonellosis

Bartonellosis	Antimicrobial of choice
Typical cat-scratch disease	Not routinely indicated; Azithromycin (for 5 days) for extensive LN†
Atypical cat-scratch disease	Doxycycline plus rifampin for 4–6 weeks
Trench fever, Endocarditis	Gentamicin 14 days plus doxycycline 6 weeks
Bacillary angiomatosis, Bacillary peliosis	Erythromycin or doxycycline for 3-4 months
Oroya fever	Chloramphenicol plus β-lactam for 14 days
Verruga peruana	Rifampin or streptomycin (10-14days)

EXPECTED QUESTIONS

I. Essay:

1. Mr Sarvanan, a 29-year-old military Jawan was brought to the hospital in a state of altered sensorium, which he had developed a few hours ago. There was history of high grade fever and headache associated with vomiting for the past 2 days. On examination he was febrile (102°F), his blood pressure was 90/60 mm Hg. There were petechial rashes noted throughout his body except palm and sole. On enquiry, he was found to have exposed to body lice. Similar symptoms were also reported from a few members of his battalion.
   a. What is the most probable diagnosis?
   b. List the other agents of the family to which the causative agent belongs to with their modes of transmission and the diseases they cause.
   c. How this disease is diagnosed in the laboratory?

II. Write short notes on:

1. Scrub typhus.
2. Cat-scratch disease.
3. Bacillary angiomatosis.
4. Ehrlichiosis.
5. Q fever.

III. Multiple Choice Questions (MCQs):

1. **Positive tunica reaction is produced by all, *except*:**
   a. *R. prowazekii* b. *R. typhi*
   c. *R. conorii* d. *R. akari*
2. **All of the following rickettsiae belong to spotted fever group, *except*:**
   a. *R. rickettsii* b. *R. conorii*
   c. *R. typhi* d. *R. akari*
3. **Tick is the vector for following rickettsial infections, *except*:**
   a. *R. rickettsii* b. *R. akari*
   c. *R. africae* d. *R. conorii*
4. **All are true about *Coxiella burnetii*, *except*:**
   a. It is obligate intracellular
   b. It causes 'Q fever'
   c. It is extremely fastidious
   d. It is killed by pasteurization

Answers

1. a **2.** c **3.** b **4.** d

Chlamydiae

39
CHAPTER

Chapter Preview

- General description
- *Chlamydia trachomatis*
- *Chlamydophila psittaci*
- *Chlamydophila pneumoniae*
- Laboratory diagnosis of chlamydial infections
- Treatment and prevention

GENERAL DESCRIPTION

Chlamydiae are obligate intracellular bacteria that cause a spectrum of diseases in man such as trachoma, lymphogranuloma venereum (LGV), conjunctivitis, pneumonia and psittacosis and can also cause widespread diseases in birds and mammals.

Classification

Based on genetic characteristics, family Chlamydiaceae has undergone recent taxonomic changes. Previously, *Chlamydia* was the only genus under the family. But now, it comprises of two genera:

1. *Chlamydia*: It has one pathogenic species, *C.trachomatis*.
2. *Chlamydophila*: It consists of:
   - Two pathogenic species—*C. psittaci* and *C. pneumoniae*
   - Several non-pathogenic animal species such as *C. pecorum*, *C. abortus*, *C. caviae* and *C. felis*.

Previous names: Based on the disease produced, chlamydiae were called previously as PLT agent (psittacosis-lymphogranuloma-trachoma) or TRIC organisms (trachoma-inclusion conjunctivitis).

Chlamydiae are Bacteria, Not Viruses

Chlamydiae were once thought to be viruses because of possessing a few viral properties, such as:

- They are obligate intracellular
- They cannot be grown in artificial media (however they can grow in cell lines, embryonated egg or animals)
- Filterable—small enough to pass through bacterial filters
- Produce intracytoplasmic inclusions.

However, chlamydiae are now confirmed to be bacteria, because they have many other properties similar to that of bacteria, as follows:

- Possess both DNA and RNA
- Their cell wall is similar to that of gram-negative bacteria (although they lack peptidoglycan layer)
- Multiply by binary fission
- Contain prokaryotic 70S ribosomes
- Capable of synthesizing their own nucleic acid, lipids and proteins
- Susceptible to a wide range of antibacterial agents.

Life Cycle

Chlamydiae exist in two distinct morphological forms—elementary body (EB) and reticulate body (RB) as shown in Table 39.1.

Chlamydiae have specific tropism for squamous epithelial cells and macrophages of the respiratory tract. The growth cycle is biphasic (i.e. alternates between EBs and RBs) and comprises of the following steps (Fig. 39.1).

Table 39.1: Features of elementary and reticulate body

Elementary body	Reticulate body
Extracellular form	Intracellular form
Infectious form	Replicating form
Metabolically inactive	Metabolically active
Rigid cell wall	Fragile cell wall
Small size (0.20–0.30 µm)	Large size (1–1.5 µm)
Nucleoid is electron dense	Nucleoid is diffuse
DNA and RNA contents are same	RNA content is more than DNA

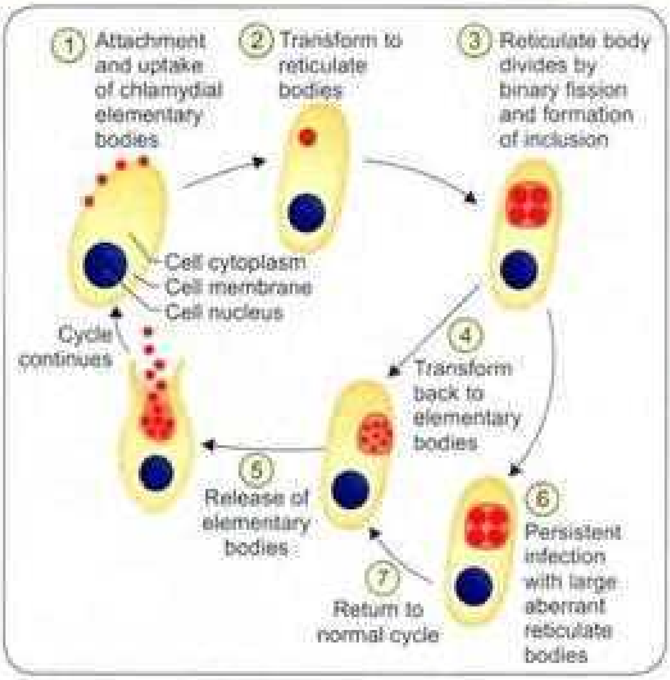


Fig. 39.1: Life cycle of *Chlamydia*

Abbreviations: EB, elementary body; RB, reticulate body; IFN, interferons.

- ❖ **Attachment:** Elementary bodies attach to the specific receptors on the surface of host cells following which they are endocytosed
- ❖ **Intracellular survival:** Elementary body resides inside the vacuole (phagosome), where the entire growth cycle is completed. They prevent phagosome-lysosome fusion by inserting chlamydial antigens on to the phagosomal membrane
- ❖ **Transform to reticulate bodies:** By 12 hours of infection the elementary bodies enlarge containing diffuse nucleoid and transform to reticulate bodies
- ❖ **Replication:** Reticulate bodies start to divide by binary fission within 18 hours. They are the metabolically active form, capable of synthesizing their own nucleic acid, lipids and proteins except ATP (lack enzymes of electron transport chain). Hence, chlamydiae are also called as **energy parasites** as they depend on host ATP for survival
- ❖ **Transform back to elementary bodies:** By 18–24 hours of infection, the reticulate bodies gradually reorganize to form elementary bodies
- ❖ **Inclusion body:** The vacuoles gradually increase in size to form inclusion body which can be readily detected by histologic stains
- ❖ **Release:** Mature inclusion body contains 100–500 elementary bodies which are ultimately released from the host cell by 48 hours
- ❖ **Persistent infection:** Sometimes, the development is arrested at the reticulate body stage, leading to persistent (or latent) infection
    - This plays an important role in ocular, chronic genital infections and possible role of *C. pneumoniae* in coronary atherosclerosis
    - Many factors, such as the interferon-γ or antibiotics (penicillin) or deprivation of tryptophan and cysteine may facilitate the arrest of life cycle.

Antigenic Structure

Chlamydiae possess the following antigens:

- ❖ **Genus/group specific antigen:** Chlamydial lipopolysaccharide (LPS) is genus specific; it is similar to the LPS present in the other gram-negative bacteria
    - In addition, family Chlamydiaceae carries a specific antigen—3-deoxy-manno-octulosonic acid
    - LPS is used in complement fixation test to detect genus specific antibodies
    - LPS is also important in the pathogenesis, by induction of TNF-α and other proinflammatory cytokines, leading to scarring and fibrosis.
- ❖ **Species specific protein antigens:** They are present at the envelope surface. They help in classifying chlamydiae into different species
- ❖ **Serovar-specific antigens:** They are the major outer membrane proteins (MOMP), encoded by *ompA* gene. They are used in micro-immunofluorescence test to detect serovar-specific antibodies
- ❖ **Other antigens:** Such as outer membrane complex proteins, type III secretory system and heat shock proteins play important role in pathogenesis.

CHLAMYDIA TRACHOMATIS

Chlamydia trachomatis is primarily a human pathogen, causing ocular, urogenital and neonatal infections.

Typing of Chlamydia

Biovars

Historically, based on the disease produced, *C. trachomatis* was subdivided into two strains or biovars (Table 39.2).

1. TRIC (Trachoma-inclusion conjunctivitis).
2. Lymphogranuloma venereum (LGV) biovar.

Serotyping

Based on antigenic structure of MOMP (and its gene *ompA*) of *C. trachomatis*, 19 serovars have been identified affecting humans.

- ❖ Serovars A, B, Ba and C are associated primarily with ocular disease called trachoma

Table 39.2: Features of *Chlamydia* infections

Species	Character	Biovar	Serotype(s)	Disease
C. trachomatis	• Forms compact inclusions mixed with glycogen matrix • Sensitive to sulfonamide • Natural human pathogen • Leaves the host cell with a scar	TRIC	A, B, Ba, C	Trachoma
			D-K (D, Da, E, F, G, H, I, Ia, J, Ja, K)	• Genital chlamydiasis • Inclusion conjunctivitis • Infant pneumonia
		LGV	L1, L2, L2a, L3	Lymphogranuloma venereum
C. psittaci	• Forms diffuse vacuolated inclusions without glycogen matrix • Resistant to sulfonamide • Natural pathogen of birds • Leaves the host cell by lysis	Nil	Many serotypes	• Psittacosis (Atypical interstitial pneumonia) • Transmission-inhalational route—pet birds (parrots) and poultry (turkeys and ducks) • No man-to-man transmission
C. pneumoniae TWAR agent	• Exclusive human pathogen • Forms inclusions without glycogen matrix • Resistant to sulfonamide	Nil	Only 1 serotype	• Community-acquired atypical (interstitial) pneumonia • Associated with: ➢ Atherosclerosis ➢ Asthma

Abbreviations: TRIC, trachoma inclusion conjunctivitis; LGV, lymphogranuloma venereum; TWAR agent, Taiwan acute respiratory agent.

- Serovars D-K are associated with oculogenital disease, which may be transmitted to neonates
- Serovars L1-L3 causes a sexually transmitted infection, lymphogranuloma venereum (LGV).

C. trachomatis Serovars A, B, Ba and C (Trachoma)

Trachoma is a chronic keratoconjunctivitis, caused by *C. trachomatis* serovars A, B, Ba and C.

- **Mode of transmission:** Trachoma is transmitted through direct contact (fingers and fomites) with discharges from the eyes of the infected patients or indirect contact through contaminated clothes or flies
- **Age:** Infection is acquired by 2-3 years of age and active disease is most common among young children
- **Acute infection** presents as:
  - Follicular conjunctivitis (inflammation of conjunctival lymphoid follicles) and papillary hyperplasia
  - Follicles rupture to leave shallow pits termed Herbert's pits
  - Cornea gets infected (keratitis).
- **Late stage (cicatrization):**
  - Recurrent infection leads to conjunctival scarring or cicatrization which may occur at sclera-conjunctiva junction (limbal scarring) or on palpebral conjunctiva
  - New vessel formation takes place (pannus)
  - Eyelashes become wet and turn inward (entropion), which may rub on the corneal surface (trichiasis)
  - Repeated rubbing of cornea may lead to opacity, which results in impaired vision or blindness.
- **WHO classification:** World Health Organization recommends a simplified grading system for trachoma, which is used clinically
- **Epidemiology:** Trachoma is a disease of developing nations
  - Worldwide, the hyperendemic areas of trachoma include sub-Saharan Africa, Middle East, and Southeast Asia including India
  - Trachoma still continues to be a leading cause of preventable infectious blindness worldwide.

C. trachomatis Serovars D–K

The infections produced by *C. trachomatis* serovars D-K are as follows.

Genital Infections

- **Nongonococcal urethritis (NGU):** *Chlamydia trachomatis* is the most common cause of nongonococcal urethritis (NGU), responsible for 30-50% of cases of NGU. It differs from gonococcal urethritis (GU) by:
  - Onset of symptoms (incubation period is 7-10 days, compared to 2-5 days for GU)
  - Symptoms: Mucopurulent discharge is followed by dysuria and urethral irritation (GU has purulent discharge).
- **Postgonococcal urethritis (PGU)**
  - *C. trachomatis* is the most common cause of PGU
  - Urethritis develops in men 2-3 weeks after recovery from GU
  - This occurs when patients with GU are treated with penicillin or cephalosporin alone without adding any antichlamydial drugs.
- **Epididymitis and proctitis:** *C. trachomatis* is the most common cause of epididymitis in males
- **Reactive arthritis (Reiter's syndrome):** It consists of conjunctivitis, urethritis (or, in females-cervicitis), arthritis, and characteristic mucocutaneous lesions
  - It occurs in 1-2% of cases of NGU, develops after1-4 weeks after genital infection
  - Men are more frequently affected than women (10:1)

- It is the most common cause of peripheral inflammatory arthritis in young men
- Large joints, particularly of the legs, or the sacroiliac joints are commonly affected
- Most of the patients possess HLA-B27 haplotype
- **Mechanism:** It is an immune-mediated inflammatory response to an infection at a distant site. *C. trachomatis* may act as a trigger organism that enhances immune response in susceptible individuals but is not responsible for all the cases
- Resolution usually occurs without specific treatment, but relapse is common.

❖ **In females:**
- Mucopurulent cervicitis is the most common manifestation
- It may progress to endometritis, salpingitis (fallopian tube), PID (pelvic inflammatory disease) and finally pelvic peritonitis
- Perihepatitis (Fitz-Hugh-Curtis syndrome).

Inclusion Conjunctivitis

C. trachomatis serovars D–K cause the following ocular infections:

❖ **Ophthalmia neonatorum** (or inclusion blennorrhea) occurs in the new borne
- *C. trachomatis* is more common cause of ophthalmia neonatorum than gonococcus
- Incubation period is longer for chlamydial infection (6–21 days) and discharge is mucopurulent compared to gonococcus (48 hours incubation period, purulent and crusted discharge).

❖ **Adult inclusion conjunctivitis:** It is an acute follicular conjunctivitis, that may occur in adults following swimming (swimming pool conjunctivitis).

Infant Pneumonia

❖ It is an interstitial pneumonia that develops within 3 weeks to 3 months of birth

❖ Infection spreads from conjunctiva to pharynx via the nasolacrimal duct

❖ Infection via the eustachian tube may cause otitis media.

C. trachomatis Serovar (L1, L2, L2a, and L3)

C. trachomatis serovar L1, L2, and L3 are the agents of lymphogranuloma venereum.

Lymphogranuloma Venereum

Lymphogranuloma venereum is an invasive systemic sexually transmitted infection, characterized by:

❖ **First stage:** Painless papule, ulcer or vesicle develops on the penis or vulva after an incubation period of 3 days to 6 weeks

❖ **Second stage:**
- Inguinal lymph nodes in the groin become enlarged, tender and soft (called **bubo**)
- Fistulae-buboes may breakdown and discharge may spread externally as chronic fistulae
- Systemic symptoms may develop such as fever, headache and myalgia.

❖ **Third stage:** Occurs in untreated cases, especially in women and homosexual men
- Rectal stricture or rectovaginal and rectal fistulae may occur
- Esthiomene—the vulva, scrotum or penis may undergo edematous granulomatous hypertrophy ('esthiomene' in Greek meaning 'eating away')
- Elephantiasis of the vulva or scrotum may occur due to impaired lymphatic drainage and is endemic in tropics, notably Africa and India.

❖ **Epidemiology:** Though the incidence of LGV is decreasing; it is still endemic in Southeast Asia (including India), South America and Caribbean.

❖ **Frei test:** Previously, the Frei test was used to diagnose LGV by demonstrating type IV hypersensitivity reaction (inflammatory nodule of <6 mm), when the antigen injected intradermally into forearm.

CHLAMYDOPHILA PSITTACI

C. psittaci is a pathogen of parrots and other psittacine birds causing psittacosis. A similar disease of non-psittacine birds was previously called ornithosis, but now it is merged with psittacosis.

❖ **Reservoirs:** Pet birds (parrots, parakeets, macaws, and cockatiels) and poultry (turkeys and ducks) act as natural reservoir of infection and are involved in transmission of infection to humans (Table 39.2)

❖ **Mode of transmission:** *C. psittaci* can be transmitted to humans by:
- Inhalation of aerosols from avian nasal discharges and from infectious avian fecal or feather dust
- Direct contact with infected birds
- There is no person to person transmission.

❖ **Clinical manifestations:** Incubation period is usually 5–19 days. It can present as:
- Respiratory manifestation is the most common form, varies from a mild influenza-like syndrome to a fatal pneumonia
- Septicemia occurs which may lead to meningoencephalitis, endocarditis, pericarditis, arthritis and gastrointestinal symptoms
- Typhoid-like syndrome characterized by fever, hepatosplenomegaly and Horder's spots (rashes resembling the rose spots of typhoid fever).

- **Epidemiology:** Due to control of birds and improved veterinary-hygienic measures, cases of psittacosis are now rare.

CHLAMYDOPHILA PNEUMONIAE

C. pneumoniae is an exclusively human pathogen. It is transmitted from person to person by inhalational route. It causes various manifestations (refer Table 39. 2).

- **Atypical pneumonia:** *C. pneumoniae* is a common cause of atypical (interstitial) pneumonia accounting for 10% of cases of community-acquired pneumonia
  - Symptoms are similar to that caused by *Mycoplasma pneumoniae* such as fever, non-productive cough and absence of leukocytosis
  - Upper respiratory tract involvement is frequent such as pharyngitis and sinusitis.
- **Atherosclerosis:** There is strong evidence of association between *C. pneumoniae* and atherosclerosis of coronary and other arteries
  - Antibodies are often elevated and *C. pneumoniae* has been recovered from atheromatous plaques
  - One hypothesis says that antibodies to outer membrane protein of *C. pneumoniae* may cross-react with human proteins resulting in an autoimmune reaction.
- **Asthma and COPD:** *C. pneumoniae* may cause exacerbations of bronchial asthma and COPD (chronic obstructive pulmonary disease).

LABORATORY DIAGNOSIS — Chlamydial infections

- **Specimen:** Depends on the type of lesions
- **Microscopy:** Detects chlamydial inclusion bodies
  - Gram staining, Lugol's iodine and other stains such as Castaneda, Machiavello or Gimenez stains
  - Direct IF: Used for direct detection of inclusion bodies.
- **Antigen detection (LPS antigens):** By enzyme immunoassays
- **Culture:** It was the gold standard method in the past
  - Egg (yolk sac), mice inoculation and cell line culture
  - Cell lines of choice-
    - McCoy, HeLa (for *C. trachomatis*)
    - HEp2 (for *C. pneumoniae*).
- **Nucleic acid amplification tests (NAAT)**, e.g. PCR
  - The most sensitive and specific method
  - Currently the diagnostic assays of choice.
- **Serology (antibody detection):**
  - CFT using group specific LPS antigen
  - Micro-IF test detects antibody against species and serovar specific MOMP antigen.

LABORATORY DIAGNOSIS OF CHLAMYDIAL INFECTIONS (TABLE 39.3)

Specimen Collection

- Scrapings or swabs from infected sites: As chlamydiae are intracellular, the sample must contain cells. Hence, firm scraping or swabbing of the site is required. Recommended specimens are:
  - Urethral swab for NGU
  - Endocervical swab for cervicitis

Table 39.3: Laboratory diagnosis of chlamydial infections

Clinical presentation	Specimen	Recommended laboratory test
Genital infections		
Urethritis (NGU) and cervicitis (*C. trachomatis* D–K)	Urethral swab or endocervical swab	NAAT, direct detection (EIA and DIF)
	First-catch urine in the morning	NAAT, direct detection (EIA and DIF)
Pelvic inflammatory disease/ Fitz-Hugh–Curtis syndrome (*C. trachomatis* D–K)	Endocervical swab, fallopian/ peritoneal swab	NAAT, antigen detection (EIA and DIF), culture
Lymphogranuloma venereum (*C. trachomatis* L1–L3)	Serum	Antibody detection (ELISA, MIF, CFT)
	Scraping from ulcer base	Direct detection (EIA and DIF), culture
	Lymph node aspirate	Culture
Ocular infections		
Trachoma (*C. trachomatis* A–C)	Conjunctival swab (upper)	NAAT, direct detection (EIA and DIF), culture
Ophthalmia neonatorum (*C. trachomatis* D–K)	Conjunctival swab (lower)	
Pulmonary infections		
Infant pneumonia (*C. trachomatis* D–K)	Serum	IgM antibody detection (EIA, MIF)
	Nasopharyngeal aspirate	NAAT, direct detection (EIA and DIF), culture
Psittacosis (*C. psittaci*)	Serum	Antibody detection (MIF, CFT)
Community-acquired pneumonia (*C. pneumoniae*)	Serum	Antibody detection (MIF)
	Respiratory secretions	Direct detection (EIA and DIF)

Abbreviations: CFT, complement fixation test; DIF, direct immunofluorescence test; EIA, enzyme immunoassay; MIF, microimmunofluorescence; NAAT, nucleic acid amplification tests.

- Conjunctival swabs for ocular infections-upper conjunctiva for trachoma and lower conjunctiva for ophthalmia neonatorum.

❖ First catch urine samples in the morning contain greatest amount of urethral secretions, hence it is the preferred specimen for urethritis or cervicitis
❖ Nasopharyngeal aspirate and respiratory secretions for pneumonia
❖ Bubo aspirate for LGV.

Microscopy

❖ **Gram staining:** Though, chlamydiae are gram-negative they are poorly stained
❖ **Presumptive diagnosis:** Routine Gram-staining often reveals sterile pyuria (i.e. elevated neutrophils without any organisms, including gonococci). In such a case any other diagnostic test should be performed for confirmation. Presumptive diagnosis is usually made based on neutrophil count:
- NGU, post gonococcal urethritis, epididymitis, reactive arthritis more than 4 neutrophils per oil immersion field (OIF)
- Cervicitis more than 20 neutrophils per OIF
- Proctitis more than 1 neutrophils per OIF.

❖ **Other stains:** Such as Castaneda, Machiavello or Gimenez stains are better methods to detect chlamydiae from samples. The inclusion bodies can be detected in the cytoplasm
❖ **Lugol's iodine:** The inclusion bodies of *C.trachomatis* can be stained with Lugol's iodine because of the presence of glycogen matrix. Whereas the inclusion bodies of *C. psittaci* are diffuse vacuolated, without glycogen matrix, hence do not take up the iodine stain
❖ **Inclusion bodies:** They are given various names such as:
- Halberstaedter-Prowazek (H-P) body in trachoma
- Miyagawa corpuscle in LGV
- LCL body (Levinthal-Cole-Lillie) body in psittacosis.

Direct Immunofluorescence Test (DIF)

DIF is used as for direct detection of inclusion bodies in clinical material, particularly from the genital tract and eye or can also be used for culture confirmation.

❖ Swabs are rolled on to a teflon-coated slide, and then fixed in methanol. Fluorescent tagged monoclonal antibodies directed against group-specific LPS antigen or species-specific MOMP antigens are added
❖ Though, DIF is sensitive, the specificity is low because of the non-specific fluorescence.

Enzyme Immunoassays (Antigen Detection)

EIA detects chlamydial group specific antigens (LPS) from the samples by using specific monoclonal antibodies.

❖ It is sensitive (60–80%), rapid, easy to perform
❖ However, the specificity is low and has to be confirmed by NAAT or DIF.

Culture

Chlamydiae cannot be cultivated in artificial media. They can grow only in embryonated egg (yolk sac), animal (mice) and cell line.

❖ Both egg and mice inoculation methods are no longer in use
❖ Mice inoculation was used in the past for isolation of *C. psittaci* and LGV serovars of *C. trachomatis*. Others are not infective to mice
❖ **Cell line culture** is the traditional method of diagnosis in the past, was considered as the gold standard method
- Though highly specific, it is less sensitive (90% compared with NAATs), time consuming, technically demanding and labor intensive
- **Choice of the cell line** depends on the species:
  - *C. trachomatis* recommended cell lines are McCoy, HeLa 229, buffalo, green monkey and baby hamster kidney (BHK-21) cell lines (Figs 39.2 and 39.3)
  - *C. pneumoniae* can be isolated from HEp2 or human fibroblast cell line
  - *C. psittaci* although grow well in cell culture, isolation should not be attempted in the routine laboratory because of the risk of laboratory infection.
- **Procedure:**
  - Cell lines should be in their stationary phase of growth before inoculation of specimens. This may be achieved by treatment with γ-radiation or idoxyuridine or cycloheximide

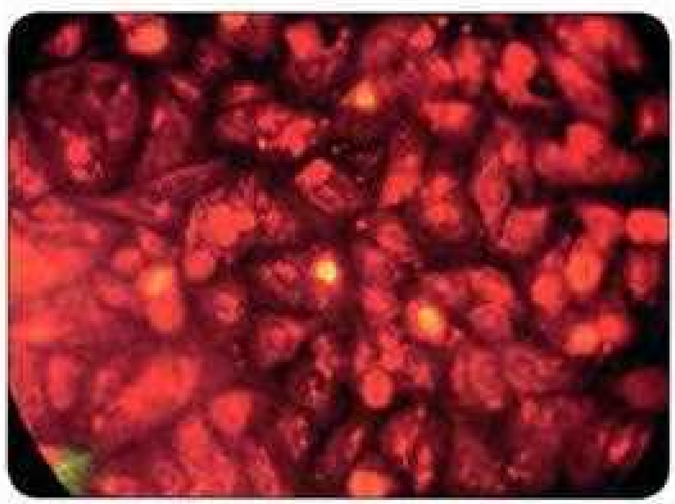


Fig. 39.2: HeLa cells infected with Type-A *Chlamydia trachomatis*

Source: Public Health Image Library, ID#/3847/Joe Miller/Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

Fig. 39.3: *Chlamydia trachomatis* inclusion bodies (brown) in a McCoy cell culture

Source: Public Health Image Library/ ID#: 3802, Dr E Arum; Dr N Jacobs, Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

- **Promote contact:** Pre-treatment of cell lines with diethylaminoethanol (DEAE) dextran or centrifugation after inoculation of specimen should be done to promote contact between chlamydiae and the cells, thus increasing the chance of isolation
- **Incubation:** Cultures are incubated in 10% CO_2 for 48–72 hours (shorter for *C. trachomatis* and longer for others)
- **Detection:** Cell lines are then stained to demonstrate the presence of inclusions as described under microscopy (Fig. 39.3).

Nucleic Acid Amplification Tests (NAAT)

NAAT have revolutionized the diagnosis of chlamydial infections.

- **Advantages:** NAAT is highly sensitive and specific, takes less time, and detects even few copies of DNA from the sample. It can also differentiate the species and serovars
- NAATs are currently the diagnostic assays of choice for chlamydial infection as recommended by the CDC, replacing the so called gold standard culture methods
- **Genes targeted** are *C. trachomatis* specific genes such as opacity protein gene or 16S or 23S rRNA
- Various methods available are:
  - Polymerase chain reaction (PCR)
  - Real time PCR
  - FilmArray respiratory panel.

Serology (Antibody Detection)

Serological tests are useful for LGV, infant pneumonia and psittacosis (systemic infections).

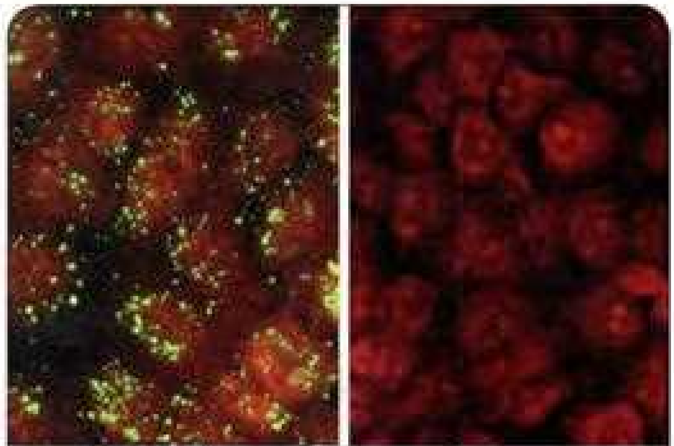


Fig 39.4: Anti-*Chlamydia* microimmunofluorescence test (MIF)

Source: EUROIMMUN AG Pvt Ltd.

- **Complement fixation test (CFT)** using LPS antigen was used in the past; which is group specific and cannot distinguish between species. Titer of ≥1:64 is considered significant
- **ELISA** based formats are also available using recombinant LPS antigen
- **Microimmunofluorescence (MIF)** test uses the species and serovar specific MOMP (major outer membrane protein) antigen (Fig. 39.4)
  - Serovar and species-specific antigens are spotted onto slides and incubated with serial dilutions of patient's serum. After incubation and washing, antigen antibody complex is detected by fluorescein tagged antihuman globulin
  - MIF is the best antibody detection test available at present. It can detect IgM and IgG separately
  - Still MIF is not widely used, because the procedure is highly technically demanding and labor intensive
  - Single high titer of ≥1:512 is diagnostic, however fourfold rise of titer at 2–3 weeks interval is more significant.

TREATMENT — ***Chlamydia* infections**

Chlamydia Trachomatis

- **For uncomplicated genital infection or trachoma or adult conjunctivitis:**
  - Azithromycin is the drug of choice given as single dose of 1 gram tablet, per oral
  - Alternatively doxycycline, tetracycline, erythromycin or ofloxacin can be given for at least a duration of 7 days
  - Both the sex partners should be treated
  - Ceftriaxone should be added to the regimen as co-infection with gonococcus may be present in most of the cases.
- **For complicated genital infection:** Doxycycline (100 mg twice daily), or erythromycin (500 mg four times daily) are the drugs of choice, given for:

Contd...

Contd...

- 2 weeks for PID, epididymitis
- 3 weeks for LGV.

- **For neonatal infections (ophthalmia neonatorum and infant pneumonia):**
  - Erythromycin is given orally at a dosage of 50 mg/kg per day in four divided doses, for 2 weeks
  - Topical ointments (erythromycin) can be used in addition to oral therapy for eye infections but relapse is common if used alone.

Chlamydophila Psittaci

Tetracycline is the drug of choice, given 250 mg four times a day for at least 3 weeks to avoid relapse. Erythromycin (500 mg four times a day, per oral) is given as alternate.

Contd...

Chlamydophila Pneumoniae

Tetracycline or erythromycin (500 mg four times a day) is recommended for 10–14 days.

Prevention

Control measures for prevention of genital infections include:

- Periodic screening of high risk groups; such as young women having multiple sex partners
- Treatment of both the sex partners
- Use of barrier methods of contraception such as condoms.
- Abstain from sex till 7 days after starting the treatment.

EXPECTED QUESTIONS

I. Essay:

1. A 27-year-woman had developed mucopurulent discharge, followed by development of dysuria and urethral irritation. She had a history of multiple sexual partners. Microscopy of the urethral swab revealed sterile pyuria and presence of compact inclusion bodies which are later stained by Lugol's iodine.
   a. What is the most probable etiological diagnosis?
   b. What are the other manifestations produced by the causative agent?
   c. How is this infection diagnosed in the laboratory?

II. Write short notes on:

1. Psittacosis.
2. Lymphogranuloma venereum.
3. Nongonococcal urethritis.
4. Reiter's syndrome.

III. Multiple Choice Questions (MCQs):

1. **Lugol's iodine is used to stain the inclusion body of:**
   a. *Chlamydia trachomatis*
   b. *Chlamydophila psittaci*
   c. *Chlamydophila pneumoniae*
   d. All of the above
2. **All of the following are true, *except*:**
   a. Elementary body is metablically active
   b. Reticulate body is the replicating form
   c. Reticulate body is intracellular form
   d. Elementary body is infectious form
3. **The most commonly used method for isolation of *Chlamydia*:**
   a. Culture on artificial media
   b. Culture on Vero cell line
   c. Inoculation into guinea pig
   d. Culture on McCoy cell line
4. **The most sensitive and specific test for *Chlamydia* diagnosis:**
   a. Direct immunofluorescence test (DIF)
   b. Culture on McCoy cell line
   c. Nucleic acid amplification tests (NAAT)
   d. Microimmunofluorescence (MIF) test
5. **Elementary body—all are true, *except*:**
   a. Extracellular form
   b. Infectious form
   c. Replicative form
   d. Rigid cell wall
6. **Chlamydiae—all are true, *except*:**
   a. Obligate intracellular
   b. Produce intracytoplasmic inclusions
   c. They can be grown in artificial media
   d. Multiply by binary fission
7. **Reactive arthritis syndrome consists of all, *except*:**
   a. Conjunctivitis b. Urethritis
   c. Polyarthritis d. Myocarditis
8. **Serotype specific antigen of *Chlamydia* is:**
   a. Lipopolysaccharide
   b. Envelope surface protein
   c. Major outer membrane proteins (MOMP)
   d. Heat shock protein
9. **Serotype specific antibody detection test for *Chlamydia* is:**
   a. Micro-IF b. CFT
   c. Chemiluminescence d. IHA
10. **Frei test is done for:**
    a. Trachoma
    b. Lymphogranuloma venereum
    c. Non-gonococcal urethritis
    d. Chlamydia conjunctivitis
11. **Which of the following is associated with pathogenesis of atherosclerosis?**
    a. *C. trachomatis* b. *C. psittaci*
    c. *C. pneuminiae* d. *C. picorum*

Answers

1. a 2. a 3. d 4. c 5. c 6. c 7. d 8. c 9. a 10. b 11. c

Mycoplasma and Ureaplasma

40
CHAPTER

Chapter Preview

- Introduction and Classification
- *Mycoplasma pneumoniae*
- Urogenital mycoplasmas

INTRODUCTION

Mycoplasmas are the smallest microbes capable of free-living in the environment and self-replicating on artificial culture media. They have the following characteristics as mentioned below:

- They resemble to viruses in certain properties such as:
  - Size: They are very small, 150–350 nm in size
  - They are filterable by bacterial filters
- They differ from viruses as:
  - They are **free living** in the environment
  - They can grow on artificial cell-free culture media.
- They **lack a rigid cell wall**, which is replaced by a triple-layered cell membrane containing sterol
- They are completely resistant to antibiotics acting on cell wall such as β-lactams
- **Pleomorphic**: They are highly pleomorphic, exist in coccoid, bacillary or filamentous or even in helical forms (*Spiroplasmas*)
- They are poorly gram-negative, better stained by Giemsa stain
- They reproduce by binary fission and budding
- They are non-sporing and non-flagellated, usually non-motile. However, **gliding motility** is described in some species which is due to their specialized tip structures
- **Contaminants of cell cultures:** Mycoplasmas are common contaminants of continuous cell cultures, thus interfere with the growth of viruses in cell cultures.

Mycoplasmas and L-form

- L-forms (after Lister Institute, London) as described by Kleineberger (1935) are cell wall deficient forms. They lose their cell wall either spontaneously or on exposure to cell wall acting antibiotics (like β-lactams), and usually revert back once the antibiotic exposure is withdrawn
- Mycoplasmas lack cell wall permanently and differ from L-forms in many ways (Table 40.1). It has been suggested that mycoplasmas may represent stable L-forms of bacteria but genetic, antigenic and biochemical properties do not support this hypothesis.

History

Pleuropneumonia-like organisms (PPLO): Nocard and Roux (1898) were the first to isolate mycoplasma as a filterable and highly pleomorphic microorganism from bovine pleuropneumonia.

- Later on, it was termed as *Mycoplasma* (derived from *Myco* meaning fungus-like, forming branching filaments; *plasma*, denoting their plasticity of shape)
- **Eaton's agent:** It refers to the most pathogenic species, i.e. *Mycoplasma pneumoniae*; which was first isolated by Monroe Eaton (1944).

Classification

Mycoplasmas belong to class Mollicutes (mollis—soft; cutis—skin, in Latin), order Mycoplasmatales, which in turn comprises of the five families. The term mycoplasmas and class Mollicutes, are often used interchangeably.

Table 40.1: Comparison of *Mycoplasma* and L-forms

Mycoplasma	L-forms
Stable, cell wall is absent permanently	**Two types:** **Unstable L- forms:** They usually revert back to normal state once the antibiotic exposure is withdrawn **Stable L-forms:** They are permanently cell wall deficient but resemble parent bacteria both biochemically and antigenically
Absence of precursors of peptidoglycan	Though cell wall deficient, remnants of cell wall components can be demonstrated
Cell membrane contains sterols	Sterols absent
Filterable like viruses	Not filterable
Mycoplasma can cause disease in cell wall deficient form	L-forms do not cause disease but they play an important role in persistence of infection during antibiotic therapy

- Members of most of the families infect plants, insects and animals, except family Mycoplasmataceae which is of human importance
- Family Mycoplasmataceae comprises of two genera—(1) *Mycoplasma* and (2) *Ureaplasma;* both infect humans and animals
- Out of 16 species of *Mycoplasma* and *Ureaplasma* infecting humans, five are established pathogens, rest are normal flora of oral and urogenital tract. The human pathogenic species are:
  - *Mycoplasma pneumoniae* causing pneumonia
  - Others cause genital tract infections, such as:
    - *Mycoplasma hominis*
    - *Mycoplasma genitalium*
    - *Ureaplasma urealyticum*
    - *Ureaplasma parvum.*

MYCOPLASMA PNEUMONIAE

M. pneumoniae is the causative agent of **primary atypical pneumonia**.

Antigens

Mycoplasma possesses several cell membrane antigens which probably play an essential role in the host response to infection.

- **Glycolipid antigen**: It is nonspecific, found in diverse tissues, forms the basis of detection of heterophile antibodies (cold agglutination test)
- **Membrane bound proteins** (e.g. cytadhesin P1 protein): They help in attachment to host cell surface.

Pathogenesis

Pathogenesis of *M. pneumoniae* involves the following events:

- **Adhesion**: Attachment to respiratory mucosa is the most important step in pathogenesis, which is mediated by membrane bound adhesion proteins that are clustered to form complex terminal organelle at the tip of the organism
- **Induces injury to host respiratory tissue:** This is mediated by producing:
  - Hydrogen peroxide
  - **Cytotoxin**: It is a recently identified toxin, has ADP ribosylating and vacuolating properties similar to pertussis toxin
  - **Lipoproteins:** They are present in the cell membrane and appear to induce inflammation.

Host Immunity

Host immunity plays a crucial role in controlling *Mycoplasma* infection.

- **Cellular immunity:** This has an immunopathogenic role, exacerbating pneumonia caused by *Mycoplasma*
- **Humoral immunity**: It does not protect from early disease; however, it provides protection against disseminated *Mycoplasma* infections such as arthritis, meningitis, and osteomyelitis.

Epidemiology

Mycoplasma pneumoniae infection occurs worldwide.

- **Transmission:** *M. pneumoniae* is transmitted by respiratory droplets expectorated during coughing
- **Facilitating factors**: The transmission is favored by close contacts as in families, military bases, boarding schools, and summer camps
- Infections tend to be endemic, with periodic epidemics every 4-7 years
- Incubation period is about 2-4 weeks.

Clinical Manifestations

M. pneumoniae produces various infections; which are as follows:

Upper Respiratory Tract Infections (URTI)

URTI manifests as pharyngitis, tracheobronchitis or rarely otitis media. It is acute in onset and is 20 times more common than pneumonia.

Pneumonia

M. pneumoniae causes **"atypical"** community acquired interstitial pneumonia similar to pneumonia caused by other agents, such as *Chlamydophila pneumoniae, Legionella pneumophila* and viral pneumonia.

- This is also referred to as Eaton agent pneumonia, primary atypical pneumonia and walking pneumonia
- Pneumonia develops in 3-13% of infected individuals; its onset is usually gradual
- It is characterized by wheeze or rales, dry cough and peribronchial pneumonia with thickened bronchial markings and streaks of interstitial infiltration on chest X-ray.

Atypical pneumonia

It is also called as interstitial pneumonia as here the interstitial space is infected in contrast to typical pneumonia which involves the alveoli.

Clinically atypical pneumonia manifests as non-productive dry cough whereas, productive cough with purulent sputum is characteristic of alveolar pneumonia.

Atypical pneumonia can be caused by the following agents:

- *M. pneumoniae*
- *Chlamydophila pneumoniae*
- *Legionella pneumophila*
- Agents of viral pneumonia.

Extrapulmonary Manifestations

They are rare, occur either as a result of active *Mycoplasma* infection (e.g. septic arthritis) or due to postinfectious autoimmune phenomena (e.g. Guillain-Barré syndrome). Various manifestations include:

- **Neurologic:** Meningoencephalitis, encephalitis, Guillain-Barré syndrome and aseptic meningitis

- **Dermatologic:** Skin rashes including erythema multiforme major (Stevens-Johnson syndrome)
- **Cardiac:** Myocarditis, pericarditis
- **Rheumatologic:** Reactive arthritis
- **Hematologic:** Anemia and hypercoagulopathy.

LABORATORY DIAGNOSIS ***Mycoplasma pneumoniae***

- **Specimen:** Throat swabs and nasopharyngeal aspirates bronchial brushing, BAL and lung biopsies.
- **Culture:**
  - Solid medium containing PPLO agar: Produces fried egg appearance colonies
  - Liquid medium containing PPLO broth: Produces turbidity and a color change.
- **Antigenic detection:** By Direct IF, antigen capture ELISA
- **Antibody detection in serum:**
  - Specific CFT, indirect-IF, LAT and ELISA using protein P1 antigens
  - Nonspecific: Cold agglutination test and *Streptococcus* MG test.
- **Molecular methods:** Detects 16S rRNA and P1adhesin gene.

Laboratory Diagnosis

Specimen Collection and Transport

Ideal specimens are throat swabs and nasopharyngeal aspirates, bronchial brushing, bronchoalveolar lavages (BAL) and lung biopsies. Sputum is not very useful as it contains too many contaminants.

- Specimens must be placed immediately into the following transport media to avoid drying:
  - **Standard *Mycoplasma* fluid medium** containing fetal bovine serum, gelatine and penicillin
  - **Viral transport medium,** added with ampicillin and cefotaxime.
- Transportation should be immediate. If delay is expected, then specimens should be stored at 4°C for 48 hours and beyond that at -70°C.

Culture

Primary isolation of *Mycoplasma* requires complex media, such as:

- **Standard solid medium:** Containing PPLO agar, horse serum and penicillin
- **Standard liquid medium:** Containing PPLO broth, glucose and penicillin and phenol red (indicator)
- **Diphasic medium**: Contains both standard solid phase and liquid phase media as described above
- **SP-4 medium:** It is more complex and contains fetal bovine serum
- **Hayflick modified medium:** Containing heart infusion broth.

Specimens are inoculated in culture media and incubated at 37°C for 5-7 days or sometimes even up to 1-3 weeks. Growth is detected as follows:

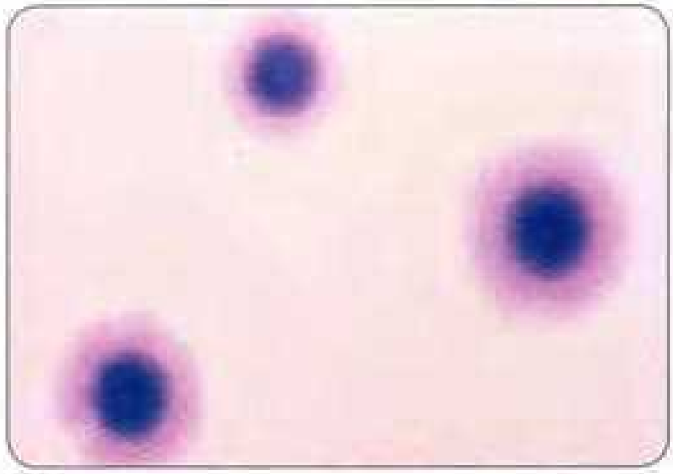


Fig. 40.1: *Mycoplasma* colonies (typical fried egg appearance)

Source: Public Health Image Library, ID# 11024/Dr E Arum; Dr N Jacobs/Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

- **In liquid medium:** *M. pneumoniae* growth is detected by turbidity and a color change (red to yellow) of phenol red indicator due to fermentation of glucose. *Ureaplasma* and some other mycoplasmas do not ferment glucose and show only turbidity
- **In solid medium:** The colonies appear as described below
  - **Shape:** Colonies are embedded on agar surface and consist of a central opaque granular area of growth, surrounded by a flat, translucent peripheral growth-**described as fried egg appearance.** (Fig. 40.1)
  - **Size:** Colony size varies from 200-500 µm for mycoplasmas and 15-60 µm for ureaplasmas
  - **Colonies can be examined by:**
    - Hand lens
    - Dienes' staining: Plate is flooded with alcoholic solution of methylene blue and azure, and examined under low power microscope. Mycoplasmas retain color for at least 2 days and appear intense royal blue, where as ureaplasmas appear reddish to greenish-blue.
  - Culture of respiratory specimens has a sensitivity of ≤60% and specificity of 100%.

Identification

Colonies of *M. pneumoniae* may be further identified by:

- Most strains of *M. pneumoniae* produce hemolytic colonies
- **Hemadsorption test:** *M. pneumoniae* agglutinates guinea pig red blood cells (RBCs) and the colonies on agar adsorb RBCs to their surface
- **Tetrazolium reduction test:** *M. pneumoniae* colonies reduce the colorless tetrazolium compound to red colored formazan
- **Growth inhibition test:** The growth of *M. pneumoniae* is inhibited by adding specific antisera.

Antigenic Detection

- Direct immunofluorescence test: Detects the *Mycoplasma* antigens directly in the clinical specimens

- Capture ELISA assay is available using monoclonal antibodies against P1 adhesion antigen.

Antibody Detection in Serum

Specific Antibody Detection Tests

Antibodies specific to *M. pneumoniae* protein and glycolipid antigens can be detected after about 1 week of illness, and peak at 3–6 weeks and then decline gradually. Serological assays have a sensitivity and specificity of 55–100%.

IgM antibodies are frequently elevated in children with acute infection where as in adults, IgA-antibody detection remains the method of choice for the diagnosis of acute infection.

Various specific antibody detection tests are as follows:

- **Complement fixation test (CFT)**: It detects antibodies to glycolipid antigen. It was the reference test in the past; now not in use
- **Alternative techniques** with greater sensitivity are:
  - Immunofluorescence assays
  - Latex agglutination assays
  - ELISA using protein P1 antigens.

Nonspecific Antibody Detection Tests

Mycoplasma possesses certain heterophile antigens such as surface glycolipid haptens that cross react with 'I' antigens of the RBCs or carbohydrate antigens of group F *Streptococcus* cell wall. This property can be used to detect heterophile antibodies in patient's sera by using non-specific antigens.

- **Cold agglutination test:** It uses human O blood group RBC ('I' antigen) and test is carried out at 4°C
- ***Streptococcus* MG tests:** It uses killed suspension of *Streptococcus* MG (group F *Streptococcus*).

However, these tests are less commonly used nowadays as they are neither specific nor sensitive (positive only in 30–50% of cases).

Molecular Methods

- PCR targeting *M. pneumoniae* specific 16S rRNA gene and P1 adhesion gene is available. PCR has a sensitivity of 65–90% and specificity of 90–100%
- Multiplex PCR has been developed detecting the common agents of atypical pneumonia—*M. pneumoniae, Chlamydophila pneumoniae* and *Legionella pneumophila*
- Real-time PCR: It is useful for quantitative detection of *M. pneumoniae*.

TREATMENT ***Mycoplasma pneumoniae***

- Macrolides are drug of choice (oral azithromycin, 500 mg on day 1, then 250 mg on days 2 to 5)
- Alternative drugs are as follows:
  - Doxycycline
  - Respiratory fluoroquinolones such as levofloxacin, moxifloxacin and gemifloxacin (not ciprofloxacin).

UROGENITAL MYCOPLASMAS

M. hominis, M. genitalium, Ureaplasma (U. urealyticum, and U. parvum) are associated with urogenital tract disease.

- They frequently colonize female lower urogenital tract such as vagina, periurethral area and cervix
- Transmission: They are transmitted mostly by sexual contact or mother to fetus during birth.

Clinical Manifestations

The manifestations of urogenital mycoplasmas are as follows:

- Non-gonococcal urethritis and epididymitis (mainly due to *Ureaplasma* and *M. genitalium*)
- Pyelonephritis (*M. hominis*), and urinary calculi (*Ureaplasma*)
- Pelvic inflammatory disease (mainly due to *M. hominis*)
- Postpartum and postabortal infection
- Non-urogenital infections (rare, due to *M. hominis*) such as: Brain abscess, wound infections or neonatal meningitis.

Laboratory Diagnosis

Culture and PCR are the appropriate methods for diagnosis of urogenital mycoplasmas. *Ureaplasma* forms very tiny colonies of 15–50 µm size, hence it was previously named as T-form *Mycoplasma*.

TREATMENT **Urogenital *Mycoplasma***

- Macrolides (azithromycin) are the drug of choice for *Ureaplasma* and *M. genitalium* infections
- Doxycycline is the drug of choice for *M. hominis*
- However, resistance has been reported to both the drugs.

EXPECTED QUESTIONS

I. Write short notes on:

1. Primary atypical pneumonia.
2. Comparison of *Mycoplasma* and L-forms.

II. Multiple Choice Questions (MCQs):

1. Which is not a property of *Mycoplasma*?

a. Susceptibility to beta lactams
b. Have both DNA and RNA
c. Can grow in cell-free media
d. Extracellular survival

2. The agent of primary atypical pneumonia is:

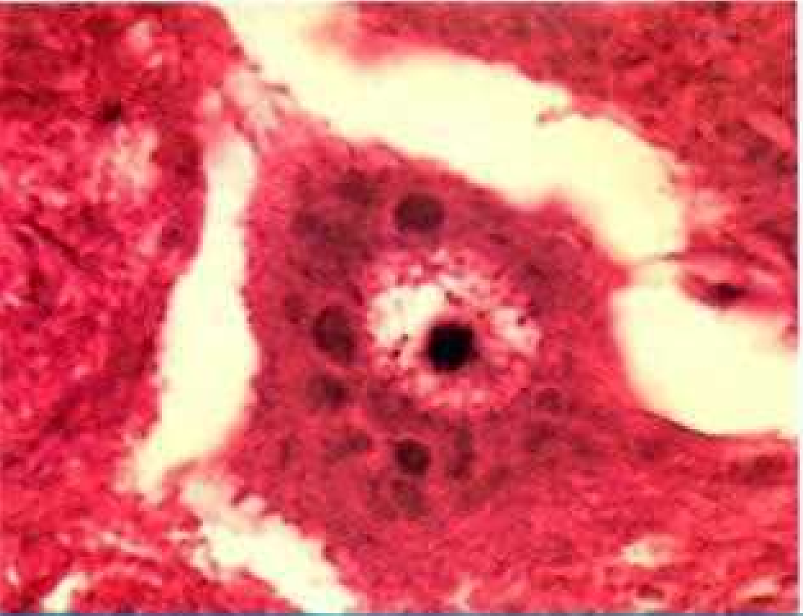
a. *Legionella pneumophila*
b. *Klebsiella pneumoniae*
c. *Mycoplasma pneumoniae*
d. *Streptococcus pneumoniae*

3. Fried egg colonies are produced by:

a. *Bacillus cereus*
b. *Haemophilus influenzae*
c. *Neisseria subflava*
d. *Mycoplasma pneumoniae*

Answers

1. a **2.** c **3.** d



Virology

SECTION 4

Section Outline

General Properties of Viruses

41
CHAPTER

Chapter Preview

- Morphology of virus
- Nomenclature and classification
- Viral replication
- Viral genetic modifications
- Virus host interactions
- Laboratory diagnosis of viral diseases
- Treatment of viral diseases
- Immunoprophylaxis for viral diseases

Viruses are the smallest unicellular organisms that are obligate intracellular. Viruses are the most primitive microorganisms infecting man. They differ from bacteria and other prokaryotes in many ways.

Viruses differ from bacteria by:

- They are obligate intracellular
- They possess either DNA (deoxyribonucleic acid) or RNA (ribonucleic acid), but never both
- Filterable: They are smaller than bacteria, can be passed through the bacterial filters
- They cannot be grown on artificial cell free media (However, they can grow in experimental animals, embryonated eggs or tissue culture)
- They multiply by a complex method, but not by binary fission as seen in bacteria
- Viruses do not have a proper cellular organization
- They do not have cell wall or cell membrane or cellular organelles including ribosomes
- They lack the enzymes necessary for protein and nucleic acid synthesis
- They are not susceptible to antibacterial antibiotics.

MORPHOLOGY OF VIRUS

The entire virus particle called as virion, comprises of a **nucleic acid** (DNA or RNA) surrounded by a protein coat called as **capsid**, together known as the nucleocapsid. Some viruses also have an outer **envelope** (Fig. 41.1).

Nucleic Acid

Viruses have only one type of nucleic acid, either DNA or RNA but never both. Accordingly, they are classified as DNA viruses and RNA viruses. The nucleic acid may be single or double stranded, circular or linear, segmented or unsegmented.

Capsid

Capsid is composed of a number of repeated protein subunits (polypeptides) called capsomeres. Functions of capsid include:

- It protects the nucleic acid core from the external environment, e.g. nucleases
- In non-enveloped viruses, it initiates the first step of viral replication by attaching to specific receptors on the host cells, thus facilitating the entry of the virus
- It is antigenic and specific for each virus.

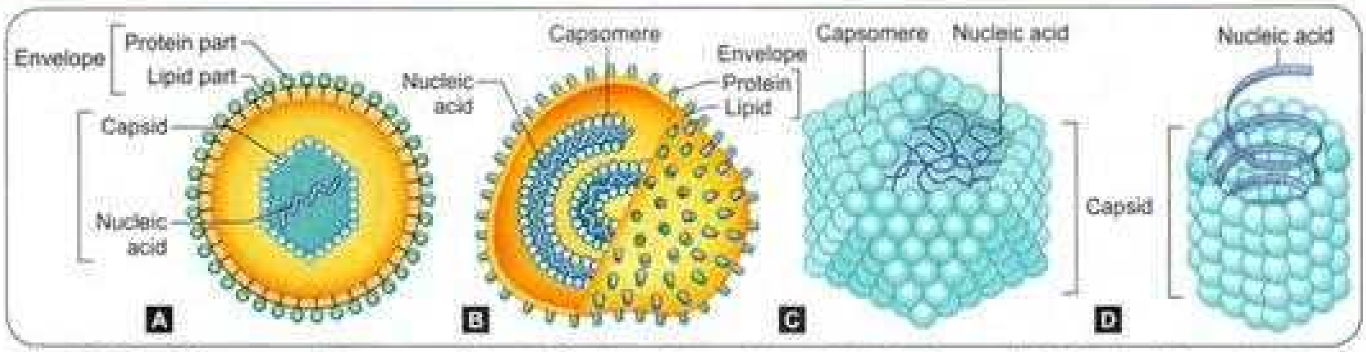
Symmetry

Depending upon the arrangement of capsomeres surrounding the nucleic acid, three types of symmetries are described.

1. **Icosahedral (cubical) symmetry:** The capsomeres are arranged as if they lay on the faces of an icosahedron, which has 20 triangular facets and 12 corners or vertices. Such viruses have a rigid structure. Viruses that are smaller than 50 nm may appear spherical under electron microscope. All DNA viruses (except poxviruses) and most of the RNA viruses have icosahedral symmetry (Figs 41.1A and C).
2. **Helical symmetry:** The capsomeres are coiled surrounding the nucleic acid in the form of a helix or spiral. Such viruses are often flexible. Example include few RNA viruses such as—myxoviruses, rhabdoviruses, filoviruses, bunyaviruses, etc. (Figs 41.1B and D).
3. **Complex symmetry:** Poxviruses do not have either of the above symmetry, but they possess a complex symmetry.

Envelope

Certain viruses possess an envelope surrounding the nucleocapsid. Envelope is lipoprotein in nature.



Figs 41.1A to D: Structure and symmetry of virus. **A.** Enveloped virus with icosahedral nucleocapsid; **B.** Enveloped virus with helical nucleocapsid; **C.** Non-enveloped virus with icosahedral nucleocapsid; **D.** Non-enveloped virus with helical nucleocapsid

- The lipid part is derived from the host cell membrane and the protein part is virus coded, made up of subunits called **peplomers,** which project as spikes on the surface of the envelope
- Some viruses may have more than one kind of peplomers, e.g. influenza viruses possess hemagglutinin and neuraminidase peplomers
- Enveloped viruses are more susceptible to heat and lipid solvents like ether
- Peplomers are antigenic. They can also bind to specific receptors on the host cells, thus facilitating the entry of the virus.

Most Viruses are Enveloped Except

- Non-enveloped DNA viruses—parvovirus, adenovirus and papovavirus
- Non-enveloped RNA viruses—picornavirus, reovirus, calicivirus, hepatitis A virus and hepatitis E virus.

Size of the Viruses

Viruses are extremely small, vary from 20–400 nm in size. Smallest virus is parvovirus (20 nm) and largest being poxvirus (400 nm).

- Because of the small size, viruses can pass through bacterial filters and they cannot be visualized under light microscope
- **Size of the viruses** (Fig. 41.2) can be determined by:
  - Electron microscope (best method)
  - Ultracentrifugation
  - Passage through membrane filters of different pore sizes.

Genomic size: Retroviruses have the largest genome (7-115 kb) whereas smallest genome is found in Hepatitis D virus (1.6 kb), Hepatitis B virus (3.2 kb) and Parvovirus (5.6 kb).

Shapes of the Viruses

Most of the animal viruses are **roughly spherical** with some exceptions.

Shapes of the Viruses

- **Rabies virus:** Bullet shaped
- **Ebola virus:** Filamentous shaped
- **Poxvirus:** Brick shaped
- **Adenovirus:** Space vehicle shaped
- **Rotavirus:** wheel shaped
- **Tobacco mosaic virus:** Rod shaped

Viruses Differ from Viroids, Prions and Virusoids

The entire viral particle is called as virion; comprising of protein capsid coat and nucleic acid. There are some incomplete viral particles such as viroid, prion and virusoid.

- **Viroids** comprise of naked, circular, small ssRNA without a capsid. They are mostly restricted to plants. They depend on host enzymes for replication. Hepatitis D virus in humans is similar to viroids
- **Prions** consist of abnormal infectious protein molecules without nucleic acid (described in Chapter 49)

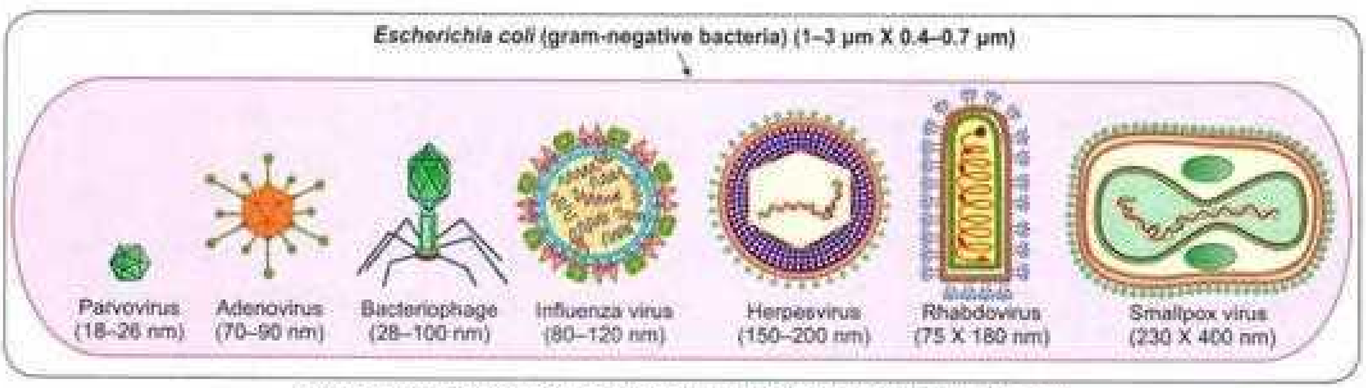


Fig. 41.2: Comparison of sizes of various viruses with that of *Escherichia coli*

- They are highly resistant to physical and chemical agents
- Prions produce slow infections in humans having long incubation period (in years) called prion disease (a neurodegenerative condition of brain).

- **Virusoids** are plant pathogens similar to viroids, made up of circular ssRNA without protein, but are dependent on other plant viruses for replication and encapsidation.

NOMENCLATURE AND CLASSIFICATION

International Committee on Taxonomy of Viruses (2000) had proposed a classification for viruses (Table 41.1).

- Viruses are grouped into families (ending with suffix '*viridae*') on the basis of morphology, genome structure, and strategies of replication
- Viruses infecting humans belong to 24 families, out of which important ones are listed below (Table 41.1)
- Most of the families are further classified into genera (ending with suffix -'*virus*') based on physicochemical or serologic differences
- Some families (Poxviridae, Herpesviridae, Parvoviridae and Paramyxoviridae) have subfamilies, which in turn consist of genera.

Table 41.1: Classification of viruses

Family	Nucleic acid	Envelope	Symmetry	Size (nm)	Representative viruses
DNA viruses	**DNA**				
herpesviridae	ds, linear	Yes	Icosahedron	150–200	• Herpes simplex virus (HSV)-1 • Herpes simplex virus (HSV)-2 • Varicella-zoster virus • Epstein-Barr virus (EBV) • Cytomegalovirus (CMV) • Human herpesvirus 6, 7 and 8
Hepadnaviridae	ds, circular, incomplete	Yes	Icosahedron	40–48	• Hepatitis B virus
Parvoviridae	ss, linear	Absent	Icosahedron	18–26	• Parvovirus B19
Papovaviridae	ds, circular	Absent	Icosahedron	45–55	• Human papillomavirus • JC virus and BK virus
Poxviridae	ds, linear	Yes	Complex	230 x 400	• Variola (smallpox) • Molluscum contagiosum virus
Adenoviridae	ds, linear	Absent	Icosahedron	70–90	• Human adenovirus
RNA viruses	**RNA**				
Picornaviridae	ss, +ve sense	Absent	Icosahedron	28–30	• Poliovirus • Coxsackievirus • Echovirus • Enterovirus • Rhinovirus • Hepatitis A virus
Caliciviridae	ss, +ve sense	Absent	Icosahedron	27–40	• Norwalk agent • Hepatitis E virus
Togaviridae	ss, +ve sense	Yes	Icosahedron	50–70	• Rubella virus • Eastern equine encephalitis virus • Western equine encephalitis virus
Flaviviridae	ss, +ve sense	Yes	Icosahedron	40–60	• Yellow fever virus • Dengue virus • St Louis encephalitis virus • West Nile virus • Hepatitis C virus
Coronaviridae	ss, +ve sense	Yes	Helical	120–160	• Coronaviruses
Rhabdoviridae	ss, -ve sense	Yes	Helical	75 x 180	• Rabies virus • Vesicular stomatitis virus
Filoviridae	ss, -ve sense	Yes	Helical	80 x 1000	• Marburg virus • Ebola virus

Contd...

Contd...

Family	Nucleic acid	Envelope	Symmetry	Size (nm)	Representative viruses
RNA viruses	**RNA**				
Paramyxoviridae	ss, -ve sense	Yes	Helical	150–300	• Parainfluenza virus • Mumps virus • Measles virus • Respiratory syncytial virus (RSV) • Newcastle disease virus • Metapneumovirus
Orthomyxoviridae	ss, -ve sense, 7 or 8 segments	Yes	Helical	80–120	• Influenza viruses- A, B, and C
Bunyaviridae	ss, -ve sense, 3 circular segments	Yes	Helical	80–120	• Hantavirus • California encephalitis virus • Sandfly fever virus
Arenaviridae	ss, -ve sense 2 circular segments	Yes	Helical*	50–300	• Lymphocytic choriomeningitis virus • Lassa fever virus • South American hemorrhagic fever virus
Reoviridae	ds, 10–12 segments	Absent	Icosahedron	60–80	• Rotavirus • Reovirus • Colorado tick fever virus
Retroviridae	Two identical copies of +ve sense ss linear RNA	Yes	Icosahedron (spherical)	80–110	• HTLV (Human T Lymphotropic virus) • HIV (Human immunodeficiency virus)

*Doubtful

Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; ds, double stranded; SS, single stranded; +ve, positive; –ve negative.

VIRAL REPLICATION

Viruses do not undergo binary fission (seen in bacteria), but undergo a complex way of cell division. Replication of viruses passes through seven sequential steps:

Attachment → Penetration → Uncoating → Biosynthesis → Assembly → Maturation → Release.

1. Adsorption/Attachment

It is the first and the most specific step of viral replication. It involves receptor interactions. The viruses have attachment sites on their envelopes or capsid proteins that bind to the complementary receptor sites present on the host cell surface.

- **HIV:** Viral surface glycoprotein gp 120 binds to CD4 molecules on the host cells
- **Influenza:** Viral hemagglutinin (an envelope protein) binds specifically to glycoprotein receptors present on the surface of respiratory epithelium.

2. Penetration

After attachment, the virus particles penetrate into the host cells either by:

- **Phagocytosis (or viropexis):** It occurs through receptor mediated endocytosis resulting in the uptake of virus particles within the endosomes of the host cytoplasm
- **Membrane fusion:** Some enveloped viruses (e.g. human immunodeficiency virus or HIV) enter by fusion of their envelope proteins with the plasma membrane of the host cell so that only the nucleocapsid enters into the cytoplasm, whereas the viral envelope remains attached to the host cell membrane
- **Injection of nucleic acid:** Bacteriophages (viruses that infect bacteria) cannot penetrate the rigid bacterial cell wall, hence only the nucleic acid is injected; while the capsid remains attached to the cell wall.

3. Uncoating

By the action of lysosomal enzymes of the host cells, the viral capsid gets separated and the nucleic acid is released into the cytoplasm. This step is absent for bacteriophages.

4. Biosynthesis

In this step, the following viral components are synthesized:

- Nucleic acid
- Capsid protein
- Enzymes required for various stages of viral replication.
- Regulatory proteins to shut down the host cell metabolism.

Site of Nucleic Acid Replication

- In DNA viruses, the DNA replication occurs in the nucleus except in poxviruses, which synthesize DNA in the cytoplasm
- In RNA viruses, the RNA replication occurs in cytoplasm except in retroviruses and orthomyxoviruses, which synthesize RNA in the nucleus.

DNA Viruses

Biosynthesis of DNA viruses involves the following basic steps (Fig. 41.3):

- Transcription of parental DNA to form early messenger RNA (mRNA)
- Early mRNA undergoes translation to produce early non-structural proteins
- **Viral DNA replication:** Early non-structural proteins shutdown the host metabolism and help in the replication of parental DNA to form copies of progeny DNA
- Progeny DNA undergoes transcription to form late mRNA, which are further translated to form late structural proteins (i.e. capsids and envelope proteins).

Minor Differences in Replication Among DNA Viruses

- **Site:** DNA replication occurs in nucleus (except in poxviruses), whereas the mRNA transcription and protein translation take place in the cytoplasm
- Most DNA viruses use host enzymes for transcription, except poxviruses which use their own viral enzymes
- The linear dsDNA of herpesviruses becomes circular inside the host cell and then replicates by **rolling circle mechanism**. However, DNA of other viruses remain either linear or circular as they are inside the viruses (i.e. adenoviruses, poxviruses, parvoviruses have linear DNA and papovaviruses and hepadnaviruses have circular DNA)

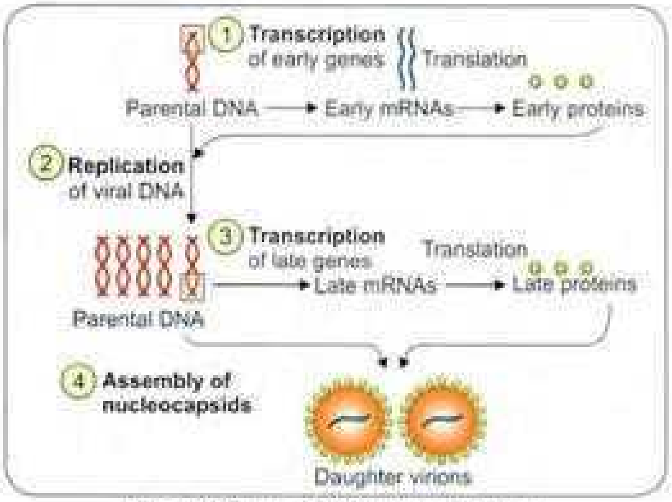


Fig. 41.3: Steps of DNA virus biosynthesis

- **Hepadnaviruses** (e.g. Hepatitis B virus) contain partially dsDNA that is first converted to fully dsDNA by viral polymerases. Then the dsDNA undergoes transcription to produce mRNA and a pregenomic RNA. DNA does not directly undergo replication but the progeny DNA are formed by reverse transcription of pregenomic RNA (Discussed in detail in Chapter 50)
- **Parvoviruses** have a negative sense ssDNA, which first gets duplexed to form dsDNA using host enzymes. Then the remaining steps are the same.

RNA Viruses

The process of biosynthesis varies among RNA viruses depending whether the genomic RNA is positive/negative sense and single/double stranded.

Type I (Positive Sense Single Stranded RNA Viruses)

They have an RNA with same polarity as mRNA, hence, they can directly translate to form early proteins (Fig. 41.4A).

- **Synthesis of progeny RNA:** Early proteins have RNA polymerase activity that direct replication (transcription) of (+)ssRNA → (-) ssRNA → (+) ssRNA
- Late proteins are formed by translation of (+)ssRNA.

Type II (Negative Sense Single Stranded RNA Viruses)

Their RNA polarity is opposite to that of mRNA, hence, they cannot directly translate into proteins (Fig. 41.4B).

- The (-) ssRNA transcribes into (+) ssRNA first, using viral RNA polymerases
- Then the (+)ssRNA translates to form proteins and also it acts as template and undergoes replication to form copies of (-)ssRNA.

Type III (Double Stranded RNA Viruses)

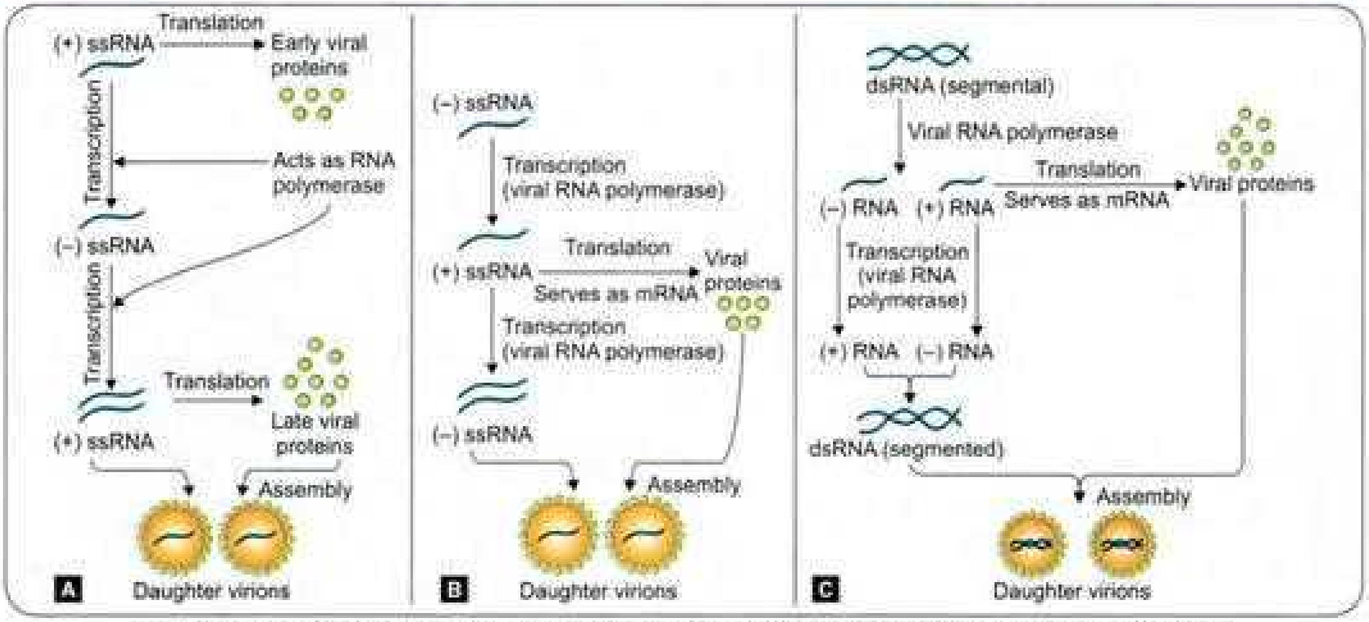
Reoviruses have dsRNA which is usually segmented with each segment coding for one polypeptide (Fig 41.4C).

- The (+) strand RNA can act as mRNA and undergoes translation to form proteins by using viral enzymes
- Both the (+) and (-) strands serve as templates for the synthesis of complementary strands to form the duplex.

Type IV (Retroviruses)

HIV and other retroviruses possess two copies of linear non-segmented (+)ssRNA and enzymes such as reverse transcriptase (RT) and integrase (Fig 41.5).

- After entry into the host cell, the ssRNA gets reverse transcribed to form ssDNA by viral RT which acts as RNA dependent DNA polymerase. DNA : RNA hybrid is formed
- Reverse transcriptase has also ribonuclease activity by which it digests the RNA from DNA:RNA hybrid
- The resulting ssDNA is converted to dsDNA by the DNA polymerase activity of the same RT enzyme
- dsDNA is transported to the nucleus where it gets integrated into the host chromosome by viral integrase



Figs 41.4A to C: Replication of—**A.** Positive sense ss RNA virus; **B.** Negative sense ss RNA virus; **C.** ds RNA virus

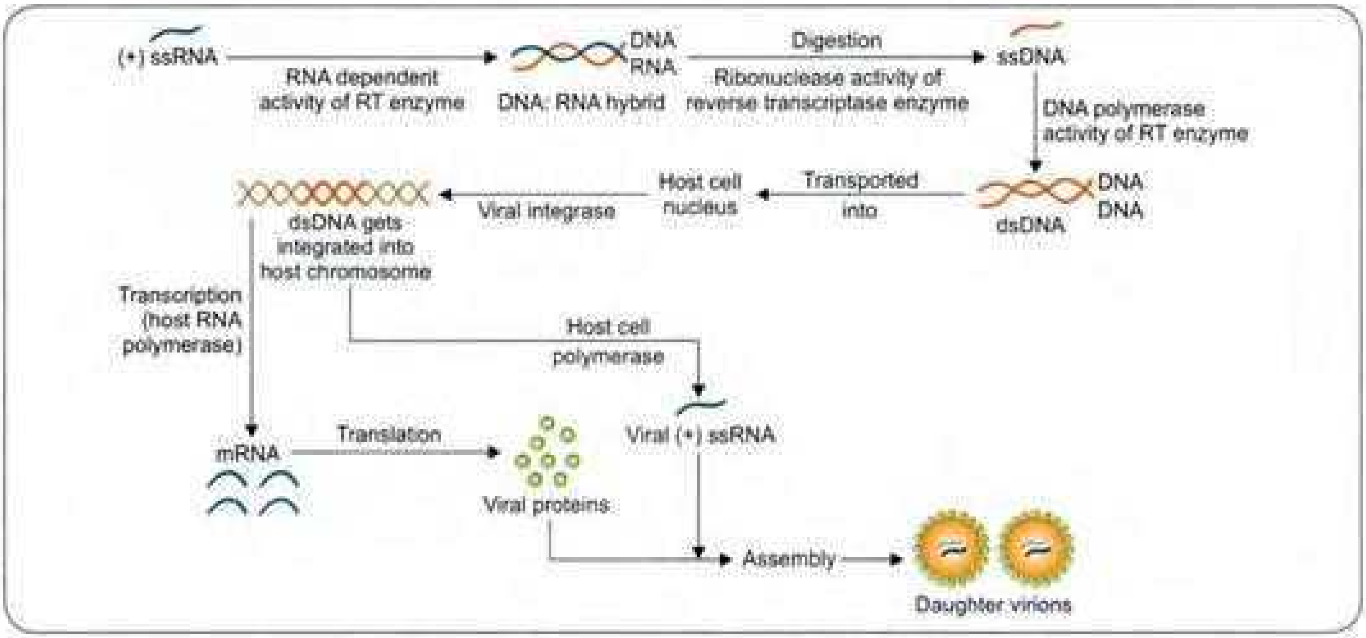


Fig. 41.5: Replication retroviruses (e.g. HIV)

- The integrated DNA serves as template for production of mRNA (which are translated into proteins) and genomic progeny RNA.

5. Assembly

Viral nucleic acid and proteins are packaged together to form progeny viruses (nucleocapsids). Assembly may take place in the host cell nucleus or cytoplasm.

- DNA viruses are assembled in the nucleus except hepadnaviruses and poxviruses (in cytoplasm)
- RNA viruses are assembled in the cytoplasm.

6. Maturation

Following assembly, maturation of daughter virions take place either in the host cell nucleus or cytoplasm or membranes (Golgi or endoplasmic reticulum or plasma membrane).

7. Release

Release of daughter virions occur either by:

- **Lysis of the host cells:** As shown by non-enveloped viruses and bacteriophages
- **Budding through host cell membrane:** As shown by enveloped viruses—during budding, they acquire a part of the host cell membrane to form the lipid part of their envelopes
- Envelope is acquired either from plasma membrane (influenza virus) or from nuclear membrane (e.g. herpesviruses)
- Viral glycoproteins are then inserted into the envelopes
- Excess viral glycoproteins are synthesized to saturate cell receptors so that the viruses will not stick to the host cell following release.

Eclipse phase: It is defined as 'interval between the penetration of the virus into the host cell till the appearance of first infectious virus progeny particle'.

- During this period, the virus cannot be demonstrated inside the host cell
- The duration of eclipse phase is about 15 to 30 minutes for bacteriophages and 15–30 hours for most of the animal viruses.

Abnormal Replicative Cycles

Incomplete Viruses

These are formed as a result of defective assembly where the proteins are assembled without nucleic acid. These viruses are not infective. Such a defective assembly in influenza virus leads to production of progeny viral particles having high hemagglutinin titer but low infectivity; which is known as **Von Magnus phenomenon**.

Pseudovirions

The capsid occasionally encloses the host cell nucleic acid instead of viral nucleic acid. Such pseudovirions are noninfective and cannot replicate.

Abortive Infection

It occurs when viruses enter into wrong host cells (non-permissive cells). Here, the parent virus is normal, but still does not perform some of the steps of viral replication.

Defective Viruses (Dependoviruses)

Such viruses are genetically defective. They cannot perform all the steps of viral replication by themselves, but they need a second helper virus, which can supplement the genetic deficiency. Examples of defective viruses are:

- Hepatitis D virus (requires the help of hepatitis B virus)
- Adeno-associated satellite viruses (require the help of adenoviruses).

VIRAL GENETIC MODIFICATIONS

Similar to other living objects, viruses also follow laws of genetics. Several properties of viruses (e.g. virulence, antigenicity, capsid production) are under genetic control. The viruses show genetic modifications by two principal methods—(1) mutations and (2) interactions between viral genes or their gene products (proteins).

Mutation

Mutations occur during every viral infection, at a frequency of 10^{-4} to 10^{-8} mutations per base pair per generation. However, mutation becomes evident only if it induces some readily observable property or leads to survival or death of the virus.

- Mutants occur spontaneously or may be induced chemically (e.g. 5-fluorouracil) or by physical agents such as UV light or irradiation
- **Types of mutations:** The mutants may be of various types as described in bacteria (Chapter 6). A special class of mutant is seen among viruses called **conditional lethal mutant** which has great applications in virology.

> **Conditional lethal mutant** can grow only in specific conditions called permissive conditions, but cannot grow in other conditions.
> **Temperature sensitive mutant** (*ts* mutant) is a type of conditional lethal mutant that can grow at a low (permissive) temperature (28–31°C), but not at higher (restrictive) temperature (37°C). *ts* mutants have been used for preparation of live viral vaccines (e.g. *ts* influenza vaccine).

Interactions Between Viral Genes

When two or more virus particles infect the same host cell, there occurs a variety of both genetic and non-genetic interactions.

Genetic Recombination

It occurs between two different but related viruses of the same family infecting a host cell simultaneously.

- The two viruses exchange segments of nucleic acids between them so that a hybrid (recombinant virus) results
- Such hybrids possess new genes not found in both the parent viruses, are genetically stable and able to replicate.

Reassortment

It is a type of recombination seen in segmented RNA viruses such as influenza, rota, bunya, and arena viruses.

- When two strains of influenza virus infect a host cell, gene exchanges take place between the RNA segments resulting in production of reassortants.

- Reassortment is probably the most important method by which the pandemic strains of the influenza virus originate in nature (e.g. H1N1 strain in 2009).

Genetic Reactivation

Genetic reactivation can take place in two ways.

1. Marker Rescue

It occurs when a host cell is infected with an active virus and a different but related inactive virus simultaneously. A portion of the genome of the inactivated virus combines with that of the active virus, so that certain markers of the inactivated virus are rescued and the progeny viruses appear viable.

Example: The epidemic strains of influenza (which usually do not grow in eggs) when mixed with inactivated standard laboratory stains, the resultant progeny carry the virulence property of epidemic strain but growth characteristics of laboratory strain, hence can be grown in eggs.

2. Multiplicity Reactivation

It occurs when many inactive viruses interact in the same host cell to produce a stable viable virus.

Example: This usually occurs when viruses are inactivated by UV rays (e.g. UV irradiated vaccines). Such inactivated viruses have damages in different genes. Thus, from the total genetic pool, the healthy genes may get combined to form a complete undamaged genome. Therefore, UV irradiation is no longer recommended for producing inactivated viral vaccines.

Viral Interference

Viral Interference is another important example of interaction between viruses (see below in the box).

Viral Interference

When two viruses infect a host cell or a cell line, sometimes it leads to inhibition of one of the virus. Interference does not occur with all viral combinations; many viruses may infect and multiply together in a host cell.

Several mechanisms may be responsible for viral interference:

- First virus may inhibit the entry of second virus either by:
  - Blocking the host cell receptors, e.g. retroviruses, enteroviruses or
  - Destroying the host cell receptors, e.g. influenza virus by producing neuraminidase.
- First virus may compete with the second for components needed for replication apparatus (e.g. polymerase)
- The first virus may induce the host cell to produce interferon which prevents replication of the second virus.

Interference and Oral Polio Vaccine (OPV)

Viral interference is classically observed with OPV which contains three live attenuated serotypes of poliovirus.

Contd...

Contd...

- OPV serotypes interfere with the spread of wild poliovirus thus played a crucial role in control of the polio outbreaks
- On the other hand, interference in between the three OPV serotypes or interference by pre-existing enteric viruses with OPV serotypes may result in vaccine failure.

VIRUS HOST INTERACTIONS

PATHOGENESIS OF VIRAL INFECTIONS

Most of the viral infections progress through the following steps inside the human body:

- Transmission (entry into the body)
- Primary site replication
- Spread to secondary site
- Manifestations of the disease.

Transmission

Viruses enter into the human body through various routes (Table 41.2).

Primary Site of Replication

- Some viruses are restricted to the portal of entry where they multiply and produce local diseases. They spread locally over the epithelial surfaces, but there is no viremia or spread to distant sites. They have a shorter incubation period and shorter duration of immunity
- On the other hand, most viruses multiply locally to initiate a silent local infection, which is followed by the spread via lymphatics to regional lymph nodes (most viruses) or via blood (e.g. poliovirus) or via neuronal spread to reach CNS (e.g. rabies virus).

Spread of Virus (Fig. 41.6)

- **Primary viremia:** Viruses spread to the blood stream either from the primary sites or from the lymph nodes. In blood, viruses may remain as free in plasma or may be cell-associated in lymphocytes or macrophages
- **Secondary site replication:** Viruses are then transported to the reticuloendothelial system (bone marrow, endothelial cells, spleen and liver) where further multiplication takes place. Secondary sites are called as the central foci for viral multiplication
- **Secondary viremia:** From the spleen and liver, viruses spillover into the blood stream leading to *secondary viremia* which result in the onset of non-specific symptoms
- **Target organs:** Via the blood stream, they reach the target organs (lung, brain, skin, etc.). Certain viruses (e.g. rabies) affecting brain, there is no viremia. Instead, virus reaches the target organ via neuronal spread
- **Tropism** of the viruses for specific organs (Table 41.3) determines the pattern of systemic illness (e.g. hepatitis

Table 41.2: Transmission and spread of viruses

Mode of transmission	Produce local infection at the portal of entry	Spread to distant sites from the portal of entry
Respiratory route (probably the most common route)	**Produce respiratory infection** • Influenza virus • Parainfluenza virus • Respiratory syncytial virus (RSV) • Rhinovirus • Adenovirus • Coronavirus • Herpes simplex virus (HSV) (rare)	• Measles virus • Mumps virus • Rubella virus • Varicella-zoster virus • Cytomegalovirus (CMV) • Parvovirus • Smallpox virus
Oral route	**Produce gastroenteritis** • Rotavirus • Adenovirus-40,41 • Calicivirus • Astrovirus	• Poliovirus • Coxsackie virus • Hepatitis virus—A and E • Cytomegalovirus • Epstein-Barr virus (EBV)
Cutaneous route	**Produce skin lesions** • Herpes simplex virus-1 (HSV-1) • Human papillomavirus (HPV) • Molluscum contagiosum virus	• Herpes simplex virus
Vector bite	—	Arboviruses such as: • Dengue virus (*Aedes*) • Chikungunya virus (*Aedes*) • Japanese encephalitis virus (*Culex*) • Yellow fever virus (*Aedes*) • Kyasanur Forest disease virus (Tick)
Animal bite	—	Rabies virus
Sexual route	**Produce genital lesions** • Herpes simplex virus-2 (HSV-2) • Human papillomavirus (HPV)	• Hepatitis B, C and rarely D viruses • Human immunodeficiency virus (HIV)
Blood transfusion	—	• Hepatitis B, C and rarely D viruses • HIV • Parvovirus
Injection	—	• Hepatitis B, C and rarely D viruses • HIV
Transplacental route	**Produce congenital manifestations in fetus** • Rubella virus • Cytomegalovirus • Herpes simplex virus • Varicella-zoster virus • Parvovirus	**Transmitted through placenta to fetus, without congenital manifestations** • Measles virus • Mumps virus • Hepatitis B, C and rarely D viruses • HIV
Conjunctival route	• Adenovirus • Enterovirus 70 • Coxsackie virus A-24 • Herpes simplex virus	—

viruses have tropism for hepatocytes, and produce hepatitis as the primary disease). Tropism in turn depends on the presence of host cell receptors specific for the viruses.

Virus Shedding

Shedding of infectious virus is a necessary step to maintain viruses in the environment or hosts. Shedding may occur at various stages of disease.

- **Portal of entry** is the site for shedding for those viruses that produce local infection, e.g. influenza virus is shed in respiratory secretions
- **Blood:** The viruses that spread through vector bite (arboviruses) or by blood transfusion or needle pricks (hepatitis B) are shed in blood
- **Near the target tissue/organ:** Skin (varicella-zoster), salivary gland (mumps), kidney (CMV)

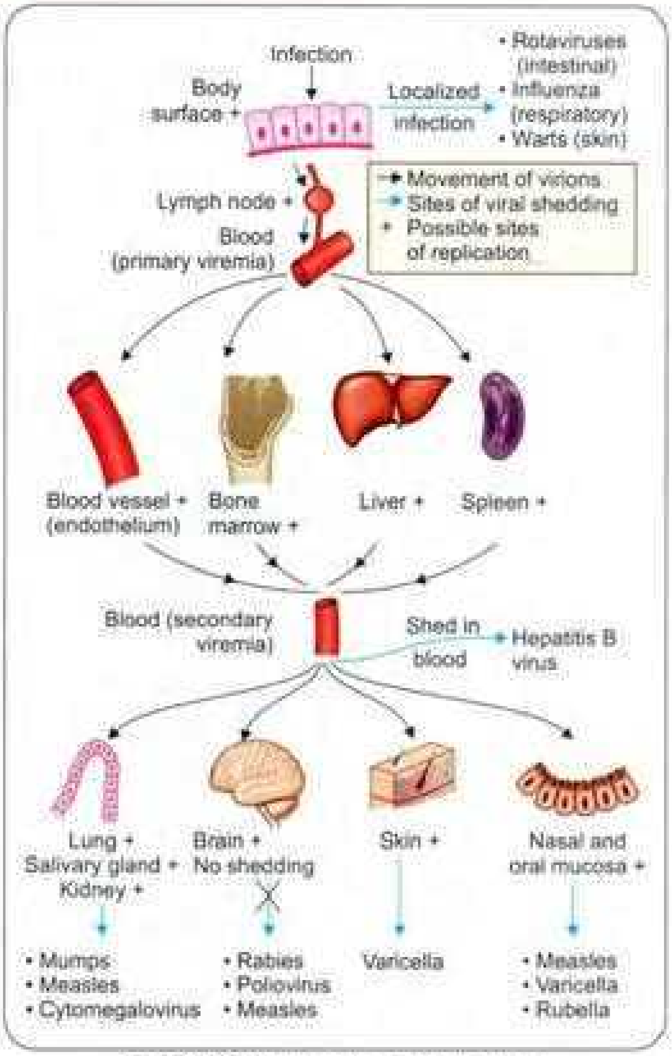


Fig. 41.6: Pathogenesis of viral infections

Concept adapted and modified from Jawetz, Melnick, & Adelberg's Medical Microbiology; McGraw-Hill Education (with permission).

❖ **No viral shedding:** Humans are the dead end for certain viruses infecting central nervous system, such as rabies.

Manifestations of Viral Infections

Incubation Period

The incubation period is the time interval between entry of the virus into the body and appearance of first clinical manifesation. Therefore, incubation period depends on the distance between site of entry and the target organ.

❖ It is shorter if the virus produces lesions near to the site of entry, e.g. influenza virus
❖ It is longer if the target organ is much far from the site of entry, e.g. poliovirus and rabies virus
❖ **Exception:** There are many exceptions to this rule. For example, both dengue and hepatitis B virus are blood borne, but they greatly vary in incubation periods (4-5 days and 30-180 days respectively). This is because there are many other factors that affect the incubation period such as host immune response, nature of the virus, etc.

Table 41.3: Cell tropism of human viruses

Cell type associated	DNA viruses	RNA viruses
Lymphocytes	Epstein-Barr virus Cytomegalovirus Hepatitis B virus JC virus BK virus	Mumps Measles Rubella HIV
Monocytes-macrophages	Cytomegalovirus	Poliovirus HIV Measles
Neutrophils	—	Influenza virus
Red blood cells	Parvovirus B19	Colorado tick fever virus
None (free in plasma)	—	Togavirus Picornavirus

Depending on the clinical outcome, viral infections can be categorized into:

❖ Inapparent (subclinical) or
❖ Apparent (clinical or overt) infections which may be acute, subacute or chronic infection
❖ Latent or persistent infections (described later in this chapter).

VIRAL PATHOGENESIS AT CELLULAR LEVEL

At the cellular level, the virus can produce three types of infections in a host cell which in turn depends on the nature of the virus and the cell infected.

Failed Infection (Abortive Infection)

It occurs if the virus infects the host cells which are non-permissive (i.e. absence of surface receptors or machineries to support viral replication).

Cell Death (Cytocidal or Lytic Infection)

Viruses adopt different mechanisms to induce host cell death such as:

❖ Inhibition of host cell DNA by herpesvirus
❖ Inhibition of host cell protein synthesis by poliovirus
❖ **Fusion (syncytia formation):** Glycoproteins of some enveloped viruses (paramyxoviruses, herpesviruses, and retroviruses) are expressed on host cell surface which triggers the fusion of neighboring cells to form multi-nucleated giant cells called syncytia, that allows the virus to spread from cell to cell and escapes antibody neutralization

- Disruption of cytoskeleton by non-enveloped viruses and herpesvirus
- Immune mediated lysis: The expression of viral antigens on the host cell surface can lead to binding of antibodies followed by complement or natural killer (NK) cell mediated lysis
- Inducing apoptosis of the host cells
- Release of progeny viruses by lysis of the host cells (e.g. poliovirus).

Infection without Cell Death

Infection without cell death may occur in two ways.

1. Steady State Infection

The virus and host cell enter into a peaceful coexistence, both replicating independently without any cellular injury.

2. Persistent Viral Infection

The virus undergoes a period of latency which may be of various types:

- **Latent infection with periodic exacerbations:** Seen with members of Herpesviridae family
- **Cell transformation:** Oncogenic viruses such as hepatitis B virus or Epstein-Barr virus or human papillomavirus induce host cell transformation and the transformed cells divide indefinitely leading to tumor production
- **Latency in HIV infection:** Viral genome gets integrated with host cell chromosome and undergoes long period of clinical latency
- **Latency in slow virus infection:** Slow viruses have an unsual long incubation period (years)
- **Persistent tolerant infection:** The classical example is lymphocytic choriomeningitis virus infecting mice. Here, the host is immunologically tolerant to the virus, does not show any immune response, but the virus is readily demonstrable in the tissues. Disease sets in when the tolerance is interrupted.

Morphological Changes in the Host Cells

Certain viruses induce characteristic changes in the appearance and staining properties of the target cells which can be detected by histopathological staining.

- **Damage to the host cell chromosomes:** For example, formation of chromatin rings surrounding the nuclear membrane in host cells infected with HSV or adeno-virus
- **Formation of inclusion body:** See box below and Table 41.4.

Inclusion Body

They are the aggregates of virions or viral proteins and other products of viral replication that confer altered staining property to the host cell.

Role in Laboratory Diagnosis

Inclusion bodies are characteristic of specific viral infections. They have distinct size, shape, location and staining properties by which they can be demonstrated in virus infected cells under the light microscope.

Location

They may be present either in the host cell cytoplasm or nucleus or both (Table 41.4).

- **Intracytoplasmic inclusion bodies:** They are generally acidophilic and can be seen as pink structures when stained with Giemsa or eosin methylene blue stains (e.g. most poxviruses and rabies)
- **Intranuclear inclusion bodies:** They are basophilic in nature. Cowdry (1934) had classfied them into:
  - Cowdry type A inclusions: They are variable in size and have granular appearance
  - Cowdry type B inclusions: They are more circumscribed, amorphous or hyaline spheres; multiple in number.
- **Bosth intracytoplasmic and intranuclear inclusions.**

HOST IMMUNE RESPONSES

The outcome of viral infection is determined by several viral and host factors. Viral factors include inoculum size, virulence of the virus, serotype, and cytopathic effect of the virus. Host factors can be classified into non-specific or specific immunological responses.

Non-Specific Immune Responses

There are various non-specific immunological responses, as described below.

- **Host immune status:** Low immunity predisposes to many viral infections, such as CMV. On the other hand, certain viral infections require an adequate host immune response (e.g. dengue).

Table 41.4: Inclusion bodies

Intracytoplasmic inclusion bodies
• Negri bodies—seen in rabies virus • Paschen body—seen in variola virus • Guarnieri bodies—seen in vaccinia virus • Bollinger bodies—seen in fowlpox virus • Molluscum bodies—seen in molluscum contagiosum virus • Perinuclear cytoplasmic body—seen in reovirus
Intranuclear inclusion bodies
Cowdry type A inclusions
• Torres body—seen in yellow fever virus • Lipschultz body—seen in herpes simplex virus
Cowdry type B inclusions
• Poliovirus • Adenovirus
Intracytoplasmic and intranuclear inclusion bodies
• Owl's eye appearance seen in cytomegalovirus • Measles virus

- **Prior immunity to the virus:** Prior immunity to the virus due to vaccination or past infection can protect the individual from further infection. Some viral infections such as smallpox, chickenpox, measles, mumps, and rubella provide lifelong immunity
- **Permissiveness of host cells:** The host cell should bear the surface receptors or machineries required for viral replication
- **Nutritional status:** Malnutrition lowers the host immunity and thus predisposes to many viral infections, e.g. measles. However, in certain viral infections such as dengue, malnutrition has a paradoxical effect
- **Other comorbid diseases:** Presence of other comorbid diseases can influence the immune status of the individual and may predispose to viral infections
- **Genetic makeup:** Certain individuals are more prone to develop some viral infections. This depends on the genetic makeup of the individual
- **Age:** Most viral infections are common at extremes of age, i.e. childhood and old age. However, sexually transmitted viral infections are common among young adults
- **Hormones:** Corticosteroids administration supresses the immunity, hence predisposes to most viral infections
- **Body temperature:** Fever may act as a natural defense mechanism as most viruses are inhibited by temperature above 39°C. Exception is, febrile blisters seen in herpes infection
- **Phagocytosis:** Macrophages play a crucial role in clearing of the viruses from the bloodstream. In contrast, neutrophils do not phagocytose viruses effectively
- **Interferons:** See below.

Interferons

Interferons (IFNs) are the cytokines, produced by host cells on induction by viral or nonviral inducers.

Classification

Interferons are classified into three groups, designated as IFN-α, β and γ (Table 41.5).

- IFN α and β are produced by many cell types and have antiviral action, hence called as type-I or viral interferons
- IFN γ is produced mainly by lymphocytes, especially T cells and NK cells. It is not antiviral, but acts on macrophages and other immune cells, hence called as type-II or immune IFN.

Mechanism of Action

IFN has **no direct action** on viruses and it does not protect the virus-infected cell that produces it. However, it induces the other host cells to produce certain proteins called **translation inhibition proteins** (TIPs), that inhibit viral protein synthesis by selectively inhibiting the translation of viral mRNA, without affecting cellular mRNA. TIPs include:

- **Protein kinase:** Inactivates elongation factor-2 and thus prevents formation of the initiation complex needed for viral protein synthesis
- **Oligonucleotide synthetase:** Activates endonuclease (RNase) which in turn degrades viral mRNA
- **Phosphodiesterase:** Inhibits peptide chain elongation
- **Nitric oxide synthetase:** It is specifically induced by IFN-γ. It has no antiviral action, but acts on macrophages and other immune cells.

Table 41.5: Properties of interferons (IFNs)

Property	IFN-α	IFN-β	IFN-γ
Formerly called as	Leukocyte IFN	Fibroblast IFN	Immune IFN
Type of designation	Type I	Type I	Type II
Produced by host cell	Most cell types (mainly macrophages)	Most cell types (mainly by fibroblasts)	Lymphocytes (mainly T_H1 cells, rarely CD8 T cells, NK cells)
Inducing agent	Viruses; dsRNA	Viruses; dsRNA	Mitogens
Actions	Antiviral action ↑MHC-I expression Activates NK cells Anti-proliferative function	Antiviral action ↑MHC-I expression Activates NK cells Anti-proliferative function	Immunoregulatory function Stimulates macrophages ↑MHC-I and II expression Anti-proliferative function
Stability at pH 2.0	Stable	Stable	Labile
Chromosomal location of genes	9	9	12
IFN receptor	IFN -α/β receptor	IFN -α/β receptor	IFN-γ receptor
IFN receptor genes located on chromosome number	21	21	6

Abbreviations: T_H, T helper; NK, natural killer cells, MHC, major histocompatibility complex.

Interferons are Host Specific but not Virus Specific

- IFNs produced by one species can protect the cells of the same or related species only
- IFNs induced by one virus (or even non-viral inducers) can be protective against the same or unrelated viruses. However, viruses vary in their susceptibility to interferon.

Inducers

Both viral and nonviral agents can induce IFN synthesis. Viruses vary in their capacity to induce IFNs.

- In general, RNA viruses and avirulent viruses are strong inducers of IFNs than DNA viruses or virulent (cytocidal) viruses
- Examples of potent inducers are:
  - Viruses: Togaviruses, vesicular stomatitis virus, Sendai virus and NDV (Newcastle disease virus)
  - Nucleic acids (double-stranded RNA)
  - Synthetic polymers (e.g. Poly I:C)
  - Bacterial endotoxin
- IFN-γ production is not induced by viruses; it is induced by mitogen or antigen contact.

Quicker Induction

Interferon induction is much quicker than the antibody response. IFN synthesis begins within an hour of induction and reaches high levels in 6–12 hours.

Resistance

IFNs are proteins, hence, they are inactivated by proteases, but not by nucleases or lipases. They are heat stable and also stable to wide ranges of pH (except IFN-γ which is labile at pH2).

Interferon Assay

Estimation of IFN levels is based on their biological activity, such as ability to inhibit plaque formation by a sensitive virus. They cannot be detected serologically as they are poorly antigenic.

Preparations of Interferons

- **Human IFNs:** As IFNs are host specific, human IFNs prepared commercially by DNA recombinant technology are the best for human use
- **Pegylated IFNs** are the IFN-α linked to polyethylene glycol. This linkage results in slower absorption, decreased clearance, and more sustained serum concentrations; hence, they can be administered once a week.

Application

Interferons are used in the following clinical conditions:

- IFN-α is used:
  - Topically—in rhinovirus infection, genital warts and herpetic keratitis
  - Systemically—in chronic hepatitis B, C and D infections, hairy cell leukemia and Kaposi's sarcoma.
- IFN-β is used in multiple sclerosis
- IFN-γ is used in chronic granulomatous disease and osteopetrosis.

Specific Immunological Responses

In general, viral antigens are potent immunogens and can induce both cell-mediated immunity (CMI) and antibody-mediated immunity (AMI).

Cell-mediated Immune Response (CMI)

As viruses are intracellular, CMI plays a vital role to provide immunity against most viral infections. Viruses are processed by the host cells and the viral peptide antigens are presented to the helper T cells. Following activation, helper T cells differentiate into either T_H1 or T_H2 subtypes which in turn secrete specific cytokines that modulate the immune response to viral infections in the following ways:

- Activation of macrophages with enhanced phagocytic ability (mediated by IFN-γ secreted by T_H1 cells)
- Activation of cytotoxic (T_C) T cells (by IL-2 secreted by T_H1 cells)-T_C cells can cause lysis of virus infected cells by producing perforins and granzymes
- Activation of NK cells by IL-2 secreted by T_H1 cells leads to cytotoxic killing of virus infected host cells
- Stimulation of B cells to produce antibodies (mainly by cytokines of T_H2 cells).

At times, CMI may contribute to the viral pathogenesis. In order to kill the viruses, T cells and NK cells also kill the virus-infected host cells thus may lead to tissue injury (e.g. liver injury seen during hepatitis virus infections).

Antibody-Mediated Immune Response

Antibodies are important in providing immunity against viral infection. However, antibodies cannot act when viruses are inside the host cells.

- IgG and IgM play a major role in blood and tissue spaces respectively
- IgA acts at the mucosal surfaces and provides mucosal immunity by preventing the virus entry at local sites such as respiratory, intestinal and urogenital tracts.

Antibodies may act in the following ways:

- Neutralize viral surface antigens, thus preventing viral attachment to the host cells. (However, it is important to note that antibodies cannot neutralize the internal antigens.)
- Antibody may attach to viral antigens on the surface of infected cells, rendering these cells prone to:
  - Complement mediated lysis

- Destruction by phagocytes: Opsonization of virus which in turn enhances phagocytosis by macrophages
- Natural killer cell mediated lysis (by antibody dependent cell mediated cytotoxicity or ADCC).

Antibody-mediated immune response can work even in other way round:

- The non-neutralizing antibodies against dengue virus can facilitate subsequent dengue infection with a different serotype by a peculiar phenomenon called as ADE (antibody-dependent enhancement)
- Antibodies can combine with viral antigens and cause immune complex mediated tissue injuries.

LABORATORY DIAGNOSIS OF VIRAL DISEASES

Laboratory diagnosis of viral infections is useful for the following purposes:

- **To start antiviral drugs** for those viral infections for which specific drugs are available such as herpes, CMV, HIV, influenza and respiratory syncytial virus (RSV)
- **Screening of blood donors** for HIV, hepatitis B and hepatitis C-helps in prevention of transfusion-transmitted infections
- **Surveillance purpose:** To assess the disease burden in the community by estimating the prevalence and incidence of viral infections
- **For outbreak or epidemic investigation**, e.g. influenza epidemics, dengue outbreaks—to initiate appropriate control measures
- **To start post-exposure prophylaxis** of antiretroviral drugs to the health care workers following needle stick injury (Chapter 56)
- **To initiate certain measures:** For example,
    - If rubella is diagnosed in the first trimester of pregnancy, termination of pregnancy is recommended
    - If newborn is diagnosed to have hepatitis B infection, then immunoglobulins (HBIG) should be started within 12 hours of birth.

LABORATORY DIAGNOSIS — Viral Diseases

Direct Demonstration of Virus
- Electron microscopy
- Immunoelectron microscopy
- Fluorescent microscopy
- Light microscopy:
    - Histopathological staining: To demonstrate inclusion bodies
    - Immunoperoxidase staining.

Contd...

Contd...

LABORATORY DIAGNOSIS — Viral Diseases

Detection of Viral Antigens

By various formats such as ELISA, direct IF, ICT, flow through assays.

Detection of the Specific Antibodies
- Conventional techniques such as HAI, neutralization test and CFT
- Newer diagnostic formats such as ELISA, ICT, flow through assays.

Molecular Methods to Detect Viral Genes
- Nucleic acid probe—for detection of DNA or RNA by hybridization
- PCR—for DNA detection by amplification
- RT-PCR—for RNA detection
- Real time PCR—for DNA quantification
- Real time RT-PCR—for RNA quantification.

Isolation of Virus by
- Animal inoculation
- Embryonated egg inoculation
- Tissue cultures: Organ culture, explant culture, cell line culture (primary, secondary and continuous cell lines).

DIRECT DEMONSTRATION OF VIRUS

Electron Microscopy

Detection of viruses by electron microscopy (EM) is increasingly used nowadays. Specimens are negatively stained by potassium phosphotungstate and scanned under EM.

- **Shape:** Viruses can be identified based on their distinct appearances; for example:
    - Rabies virus—bullet-shaped
    - Rotavirus—wheel-shaped
    - Coronavirus—petal-shaped peplomers
    - Adenovirus—space vehicle-shaped
    - Astrovirus—star-shaped peplomers.
- **Direct detection from specimens:** Direct detection of viruses by EM is preferred as primary tool for diagnosis in the following situations:
    - For viruses that are difficult to cultivate; for e.g.
        - Agents of viral gastroenteritis such as rotavirus, coronavirus, adenovirus, calicivirus from diarrheal stool
        - Hepatitis A and E viruses from feces
        - Cytomegalovirus from urine (infants).
    - As an alternative to tissue culture as tissue culture is time consuming and technically demanding; for e.g:
        - Vesicular rashes: Herpes simplex virus and varicella-zoster virus detection from vesicular fluid

- Meningitis: Detection of enteroviruses and mumps virus from CSF.

❖ **Virus detection from tissue culture:** EM can also be used for detection of viral growth in tissue cultures

❖ **Drawbacks:** EM is highly expensive, has low sensitivity with a detection threshold of 10^7 virions/mL. The specificity is also low.

Immuno-electron Microscopy

The sensitivity and specificity of EM can be improved by adding specific antiviral antibody to the specimen to aggregate the virus particles which can be centrifuged. The sediment is negatively stained and viewed under EM.

Fluorescent Microscopy

Direct immunofluorescence (Direct-IF) technique is employed to detect viral particles in the clinical samples.

❖ **Procedure:** Specimen is mounted on slide, stained with specific antiviral antibody tagged with fluorescent dye and viewed under fluorescent microscope

❖ **Clinical applications:**
- Diagnosis of rabies virus antigen in skin biopsies, corneal smear of infected patients
- Syndromic approach: Rapid diagnosis of respiratory infections caused by influenza virus, rhinoviruses, respiratory syncytial virus, adenoviruses and herpesviruses can be carried out by adding specific antibodies to each of these viruses
- Detection of adenovirus from conjunctival smears.

Light Microscopy

Light microscopy is useful in the following situations.

❖ **Inclusion bodies:** Histopathological staining of tissue sections may be useful for detection of inclusion bodies which helps in the diagnosis of certain viral infections (see Table 41.4), e.g. Negri bodies detection in brain biopsies of patients or animals died of rabies

❖ **Immunoperoxidase staining:** Tissue sections or cells coated with viral antigens are stained using antibodies tagged with horse radish peroxidise following which hydrogen peroxide and a coloring agent (benzidine derivative) are added. The color complex formed can be viewed under light microscope.

DETECTION OF VIRAL ANTIGENS

Various formats are available for detection of viral antigens in serum and other samples such as enzyme-linked immunosorbent assay (ELISA), immunochromatographic test (ICT), flow through assays, etc. Some important antigen detection tests include:

❖ HBsAg and HBeAg antigen detection for hepatitis B virus infection from serum

❖ NS1 antigen detection for dengue virus infection from serum

❖ p24 antigen detection for HIV infected patients from serum

❖ Rotavirus antigen detection from diarrheic stool

❖ CMV specific pp65 antigen detection in peripheral blood leukocyte.

DETECTION OF VIRAL ANTIBODIES

Antibody detection from serum is one of the most commonly used method in diagnostic virology. Appearance of IgM antibody or a four-fold rise of titer of IgG antibody indicates recent infection; whereas presence of IgG antibody (without a recent rise) indicates chronic or past infection. Various techniques available are described below:

Conventional Diagnostic Techniques

These are less commonly used nowaday. Examples include:

❖ Heterophile agglutination test (e.g. Paul-Bunnell test for Epstein-Barr virus)

❖ Hemagglutination inhibition (HAI) test for influenza virus and arbovirus infection

❖ Neutralization test (for poliovirus and arbovirus infections)

❖ Complement fixation test or CFT (for poliovirus, arbovirus and rabies virus infections).

Newer Diagnostic Formats

Newer techniques such as ELISA, ICT, flow through assays are widely used for antibody detection against most of the viral infections, for example:

❖ Anti-HBc, Anti-HBs and Anti-HBe antibodies in serum for hepatitis B infection

❖ Anti-Hepatitis C antibodies in serum

❖ Antibodies against HIV-1 and HIV-2 antigens from serum

❖ Anti-Dengue IgM/IgG antibodies from serum.

MOLECULAR METHODS

Advent of molecular techniques has eased the diagnosis of viral infections. They are more sensitive, specific and yield quicker results than culture.

Nucleic Acid Probe

It is an enzyme or radio-labeled nucleic acid sequence complementary to a part of nucleic acid sequence of the target virus.

❖ When added to the clinical specimen, it hybridizes to the corresponding part of viral nucleic acid

❖ Depending on the type of label attached to the probe, the hybridized-labeled probe can be subsequently detected by colorimetric methods (dot blot hybridization) or gamma counting

- Both DNA and RNA probes are commercially available
- Nucleic acid probes have a low sensitivity compared to polymerase chain reaction (PCR) as it directly detects the viral genes in the specimen, without amplification.

Polymerase Chain Reaction

PCR has revolutionized the diagnostic virology. It involves three basic steps—(1) viral DNA extraction from the specimen, (2) amplification of specific region of viral DNA to 10^7 folds, (3) detection of amplified products by gel electrophoresis.

Reverse Transcriptase PCR (RT-PCR)

RT-PCR is used for the detection of RNA viruses. After RNA extraction, the viral RNA is reverse transcribed to DNA, which is then subjected to amplification similar to that followed in PCR.

Both PCR and RT-PCR cannot quantify the viral nucleic acid load in the specimen.

Real Time PCR

It has the advantage of quantifying viral nucleic acid in the samples, hence used to monitor the treatment response, e.g., monitoring the response to antiretroviral therapy. More so, it takes much less time than PCR as the amplification is visualized on real time basis.

ISOLATION OF VIRUS

Viruses cannot be grown on artificial culture media. They are cultivated by animal inoculation, embryonated egg inoculation or tissue cultures.

- Being labor intensive, technically demanding and time consuming, virus isolation is not routinely used in diagnostic virology
- The specimen should be collected properly and immediately transported to the laboratory. Refrigeration is essential during transportation as most viruses are heat labile. Type of specimen collected depends on the virus suspected.

Animal Inoculation

Because of the ethical issues related to use of animals, animal inoculation is largely restricted only for research purpose.

- **Research use:** To study viral pathogenesis or viral oncogenesis or for viral vaccine trials
- **Diagnostic use:** Primary isolation of certain viruses which are difficult to cultivate otherwise; such as arboviruses and coxsackieviruses
- **Procedure:** Infant (suckling) mice are used for the isolation of viruses. Specimens are inoculated by intracerebral or intraperitoneal routes. Mice are observed for signs of disease or death. Later on, they are sacrificed and the tissue sections are subjected to histological examination
- Following intracerebral inoculation into suckling mice:
  - Coxsackie-A virus produces flaccid paralysis
  - Coxsackie-B virus produces spastic paralysis.

Egg Inoculation

Embryonated hen's eggs are used for cultivation of viruses. Eggs were first used for viral cultivation by Good pasture in 1931 and the method was further developed later by Burnet.

Specimens can be inoculated by four different routes (Fig. 41.7) into embryonated 7 to 12 days old hen's eggs and then incubated for 2–9 days.

Yolk Sac Inoculation

It is preferred for arboviruses (e.g. Japanese B encephalitis virus, Saint Louis encephalitis virus, and West Nile virus) and some bacteria such as *Rickettsia*, *Chlamydia* and *Haemophilus ducreyi*. Growth of the encephalitis viruses may result in death of the embryo.

Amniotic Sac

It is mainly used for the primary isolation of the influenza virus and viral growth is measured by detection of hemagglutinin antigens in amniotic fluid.

Allantoic Sac

It is a larger cavity, hence is used for better yield of viral vaccines.

- **Example of egg derived vaccines** are influenza vaccine, yellow fever (17D) vaccine and Rabies (Flury strain) vaccine
- **Duck eggs** are bigger than hen's eggs, therefore produce better yield of rabies virus for preparation of inactivated non-neural vaccine.

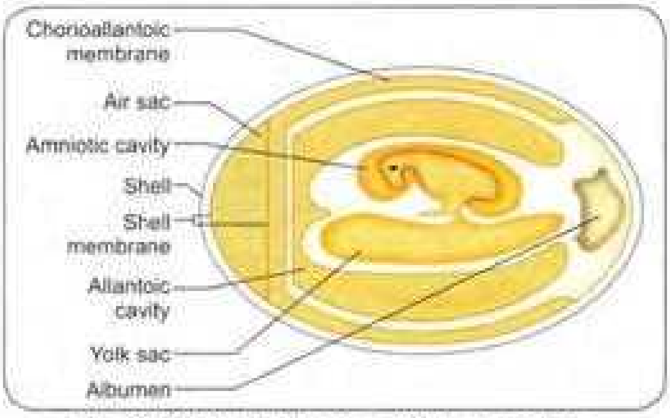


Fig. 41.7: Schematic diagram of embryonated egg

Chorioallantoic Membrane

It is preferred for poxviruses and other viruses such as HSV. Viruses produce visible lesions called as **pocks** on chorioallantoic membrane (CAM).

- **Pock counting:** Each pock is derived from a single virion. So, the number pocks would represent the number of viral particles present in the inoculum
- Pocks produced by different viruses have different morphology. For example,
  - Vaccinia pocks are more hemorrhagic and necrotic than pocks of variola virus
  - Pocks of HSV-2 are larger than HSV-1.
- **Ceiling temperature:** It is the maximum temperature above which the pock formation is inhibited. Viruses vary in their ceiling temperature, e.g. variola (37°C) and vaccinia (41°C).

Tissue Culture

Steinhardt was the first to use tissue culture in virology (1913) who maintained the vaccinia virus in fragments of rabbit cornea. Enders, Weller, and Robbins (1949) were able to culture poliovirus in tissue cultures of non-neural origin and that was the turning point following which tissue culture was widely used in diagnostic virology.

Tissue culture can be of three types:

1. **Organ culture:** It was previously used for certain fastidious viruses that have affinity to specific organs; for example, tracheal ring culture for isolation of corona virus.
2. **Explant culture:** Fragments of minced tissue can be grown as 'explants', e.g. adenoid explants used for adenoviruses. This method is obsolete now.
3. **Cell line culture:** This is the only isolation method which is in use now. The preparation of cell lines and the types of cell lines have been described below.

Preparation of the Cell Lines

Tissues are completely digested by treatment with proteolytic enzymes (trypsin or collagenase), followed by mechanical shaking so that the components are completely dissociated into individual cells.

- **Viral growth medium:** The cells are then washed, counted, and suspended in viral growth medium which contains balanced salt solution added with essential amino acids and vitamins, salts and glucose supplemented by 5-10% of fetal calf serum and antibiotics. Medium is buffered with bicarbonate to maintain a pH of 7.2-7.4 and phenol red is added as pH indicator
- **Tissue culture flasks:** The viral growth medium containing cells is dispensed in tissue culture flasks. (Fig. 41.8)
- **Monolayer sheet formation:** On incubation, the cells adhere to the glass surfaces of the flask and then they divide to form a confluent monolayer sheet of cells within a week covering the floor of tissue culture flask
- **Incubation:** Tissue culture flasks are incubated horizontally in presence of CO_2, either as a stationary culture or as a roller drum culture. Rolling of the culture bottle in *roller drums* provides better aeration which is useful for isolation of fastidious viruses (e.g. rotavirus).

Types of Cell Lines

The cell line cultures can be classified into three types based on their origin, chromosomal characters, and maximum number of cell divisions that they can undergo.

1. **Primary cell lines:** They are derived from normal cells freshly taken from the organs and cultured.
   - They are capable of very limited growth in culture, maximum up to 5-10 divisions
   - They maintain a diploid karyosome
   - Useful for both primary isolation as well as growth of the viruses for vaccine production
   - Common examples include:
     - Monkey kidney cell line—useful for isolation of myxoviruses, enteroviruses and adenoviruses
     - Human amnion cell line
     - Chick embryo cell line.
2. **Secondary or diploid cell lines:** They can divide maximum up to 10-50 divisions before they undergo senescence (death). They are also derived from the normal host cells and they maintain the diploid karyosome.
   Common examples: Diploid cell lines are derived from human fibroblasts and are useful for isolation of some fastidious viruses as well as for viral vaccine preparation.
   - Human fibroblast cell line: It is excellent for the recovery of CMV (Fig. 41.9)
   - MRC-5 and WI-38 (human embryonic lung cell strain):
     - Used for preparation of various viral vaccines, e.g. vaccines for rabies, chickenpox, hepatitis-A and MMR vaccines

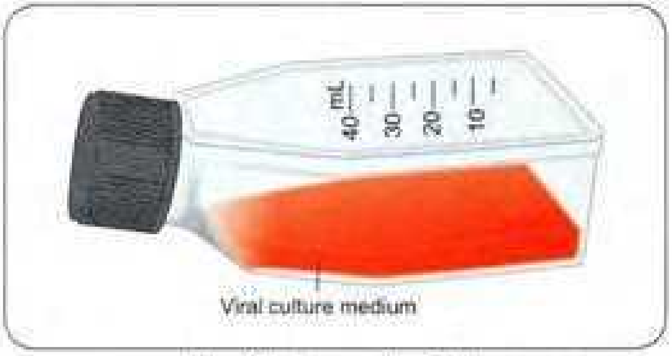


Fig. 41.8: Tissue culture flask

- They also support the growth of spectrum of viruses (e.g. HSV, VZV, CMV, adenoviruses, and picornaviruses).

3. **Continuous cell lines** (see the box below).

Continuous Cell Lines

They are derived from cancerous cell lines, hence are immortal (capable of indefinite growth). They also possess altered haploid chromosome.

They are easy to maintain in the laboratories by serial subculturing for indefinite divisions. This is the reason why continuous cell lines are the most widely used cell lines.

***Common examples include* (Fig. 41.10)**

- HeLa cell line (Human carcinoma of cervix cell line)
- HEp-2 cell line (Human epithelioma of larynx cell line)—widely used for RSV, adenoviruses and HSV
- KB cell line (Human carcinoma of nasopharynx cell line)
- McCoy cell line (Human synovial carcinoma cell line)—useful for isolation of viruses, as well as *Chlamydia*
- Vero cell line (Vervet monkey kidney cell line)—used for rabies vaccine production
- BHK cell line (Baby hamster kidney cell line).

Detection of Viral Growth in Cell Cultures

Following methods are used to detect the growth of the virus in cell cultures.

Cytopathic Effect (CPE)

It is defined as the morphological change produced by the virus in the cell line detected by light microscope.

Cytopathic viruses: Not all, but few viruses can produce CPE and those are called as cytopathic viruses. The type of CPE is unique for each virus and that helps for their presumptive identification (Table 41.6).

Viral Interference

The growth of a non-CPE virus in cell culture can be detected by the subsequent challenge of the cell line with a known CPE virus.

- The growth of the first virus would inhibit infection by the second virus by a mechanism known as viral interference

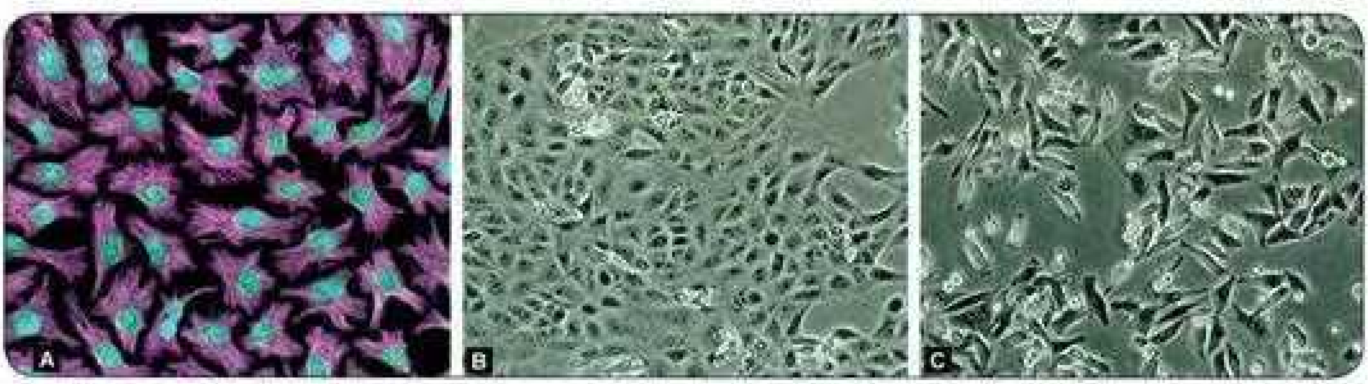
Fig. 41.9: Human lung fibroblast cell line (Normal)

Source: American Type Culture Collection (ATCC), USA (*with permission*).

Table 41.6: Viral cytopathic effects (CPE)

Types of cytopathic effect (CPE)	Virus
Rapid crenation and degeneration of the entire cell sheet	Enteroviruses
Syncytium or multinucleated giant cell formation	Measles, RSV, HSV
Diffuse roundening and ballooning of the cell line	HSV
Cytoplasmic vacuolations	SV 40 (Simian vacuolating virus-40)
Large granular clumps resembling bunches of grapes	Adenovirus

Abbreviations: RSV, respiratory syncytial virus; HSV, herpes simplex virus.



Figs 41.10A to C: Continuous cell lines (normal, uninfected). **A.** HeLa cell line; **B.** Vero cell line; **C.** HEp-2 cell line

Source: American Type Culture Collection (ATCC), USA (with permission).

- For example, rubella is a non-CPE virus but prevents the replication of enteroviruses which are known to produce CPE.

Hemadsorption

Hemagglutinating viruses (e.g. influenza virus) when grown in cell lines, they produce hemagglutinin antigens which are coated on the surface of the cell lines and can be detected by adding guinea pig erythrocytes to the cultures. The process of adsorption of erythrocytes to the surfaces of infected cell lines is known as hemadsorption.

Direct Immunofluorescence Assay

Virus infected cells are mounted on a slide and stained with specific antibodies tagged with fluorescent dye and viewed under fluorescent microscope for the presence of viral antigens on the surface of infected cells.

Immunoperoxidase Staining

Cells coated with viral antigens are stained by immunoperoxidase tagged specific antibodies and viewed under light microscope.

Electron Microscopy

The viruses can also be demonstrated in infected cell lines by EM.

Viral Genes Detection

The presence of specific viral genes in culture fluid can be detected by using PCR or nucleic acid probes.

VIRAL ASSAYS

Viral assays are used for quantification of viral particles, which can be grouped into physical and biological methods.

Physical Methods

All these methods, estimate the total virus count (or viral antigen or gene count) and cannot distinguish between infectious and non-infectious virus particles.

- **Real time PCR:** It can determine the number of viral genome copies in a sample
- **Antigen detection assay** such as radioimmunoassays (RIA) and ELISA can be standardized to quantitate the amount of virus in a sample. However, these tests may detect free viral proteins that are not assembled into particles
- **Hemagglutination assay:** It is an easy and rapid method of quantitating hemagglutinating viruses (e.g. influenza virus). The viral hemagglutinin antigens can agglutinate RBCs by binding to the specific receptors
- **Electron microscopy:** Virus particles can be counted directly by visualizing under the electron microscope by comparing with a standard suspension of latex particles of similar size.

Biological Methods

Biological methods detect the infectious virions only. Both qualitative (end point biological assays) or quantitative (plaque assay and pock assay) methods are available.

End Point Biological Assays

These assays depend on the measurement of animal death/lesion, or CPE produced in tissue culture when serial dilutions of the viral suspension are inoculated into animals or cells. The titer is expressed as the **50 percent infectious dose (ID_{50})**, which is the highest dilution of virus that produces the effect in 50% of the cells or animals inoculated.

Plaque Assay

It is the most widely used assay for quantifying infectious viruses.

- Monolayer of cell line is inoculated with suitable dilutions of the virus
- After allowing time for adsorption of virus, the cell line is covered with an agar layer so the viruses would spread only to the immediate surrounding cells, but the spreading of the virus throughout the culture will be prevented
- Multiple cycles of replication and cell killing produce a small area of infection called *plaque*
- **Plaque counting:** As single plaque arises from a single infectious virus particle, hence the number of plaques counted would represent the quantitative infectivity titer of the virus suspension.

Pock Assay

Certain viruses such as variola, vaccinia and herpes form pocks on chorioallantoic membrane (CAM) of embryonated eggs. Number of pocks on CAM represents the approximate number of infectious viral particles present in the dilution inoculated.

TREATMENT OF VIRAL DISEASES

Unlike most bacteria, viruses are obligate intracellular and they use host machinery and enzymes for replication. Viral chemotherapy therefore was considered impracticable, as it was believed that it would inhibit cellular metabolism. Nevertheless, intense research made it possible to develop various antiviral drugs that can inhibit various steps of viral replication by targeting viral machineries without affecting host enzymes and without being toxic to host cells. However, the antiviral drugs are limited, not available against most of the viral diseases. Drugs currently approved for various viral diseases are listed in Table 41.7.

Table 41.7: Commonly used antiviral drugs, their mechanism of action and spectrum of action

Antiviral drugs	Mechanism of action	Active against
Anti-herpesvirus drugs		
Acyclovir, valacyclovir, penciclovir	Inhibit Viral DNA polymerase	HSV1>HSV2>VZV and EBV
Famciclovir	Inhibit Viral DNA polymerase	HSV, VZV and HBV
Ganciclovir, valganciclovir	Inhibit Viral DNA polymerase	CMV and EBV
Cidofovir	Inhibit Viral DNA polymerase	HSV and CMV
Foscarnet	Inhibit Viral DNA polymerase	HSV and CMV (including resistant strains)
Fomivirsen	Inhibit mRNA of CMV	CMV (including resistant strains)
Docosanol (topical)	Inhibit the fusion of the human host cell with envelope of herpes virus	HSV (recurrent herpes labialis)
Trifluridine (topical)	Inhibits viral DNA polymerase	Herpes keratitis (eye drops)
Anti-influenza virus drugs		
Oseltamivir, zanamivir	Neuraminidase inhibitor	H1N1 flu, Avian flu, Seasonal flu
Amantadine, rimantadine	Matrix protein inhibitor	Seasonal flu
Anti-hepatitis drugs		
Telbivudine, tenofovir, lamivudine, adefovir, entecavir	Nucleoside analogues	For hepatitis B infection
Interferon alfa (2a and 2b**)	Indirectly inhibits viral protein synthesis	For hepatitis B and C infection
Grazoprevir, paritaprevir, simeprevir	NS3/4A (proteases) inhibitors	For hepatitis C infection
Dasabuvir, sofosbuvir	NS5B (polymerases) inhibitors	For hepatitis C infection
Daclatasvir, ledipasvir, velpatasvir	NS5A inhibitors	For hepatitis C infection
Ribavirin*	Nucleoside inhibitor	For hepatitis C infection
Anti-retroviral therapy (ART)- Refer Chapter **48** Table 48.6		

Abbreviations: HSV, herpes simplex virus; VZV, varicella-zoster virus; CMV, cytomegalovirus; EBV, Epstein-Barr virus; NtRTI, nucleotide reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitors.

*Ribavirin has action against influenza, parainfluenza, respiratory syncytial virus, HCV, and HIV-1.

**Intralesional injection of interferon alfa-2b may be used for treatment of condylomata acuminata.

IMMUNOPROPHYLAXIS FOR VIRAL DISEASES

Viral Vaccines (Active Immunization)

Since viral antigens are potent immunogens, viral vaccines confer prolonged and effective immunity. Vaccines for viral infections may be available either in live, killed or in subunit forms. For certain viruses, both live and killed vaccines are available (Table 41.8).

Killed Viral Vaccines

Killed vaccines are available for various viruses (Table 41.8).

- **Preparation:** They are prepared by inactivating viruses with heat, phenol, formalin or beta propiolactone. Ultraviolet irradiation is not recommended because of the risk of multiplicity reactivation. For example, Rabies vaccine
- **Advantages:** They are more stable and are considered safe when given in immunodeficiency or in pregnancy
- **Disadvantages:** Killed vaccines are associated with more adverse side effects due to reactogenicity, which can be reduced to some extent by purification of viruses.

Subunit Vaccines

In subunit vaccines, only a particular antigen of the virus is incorporated (Table 41.8).

- **Preparation:** Subunit vaccines are prepared by DNA recombinant technology. The gene coding for the desired antigen is integrated into bacteria or yeast chromosome. Replication of the bacteria or yeast yields a large quantity of desired antigens, e.g. hepatitis B vaccine
- Unlike killed vaccines, there is no local side effects associated with subunit vaccines.

Live Vaccines

Live vaccines are available for various viruses (Table 41.8).

Table 41.8: Vaccines for viral infections

	Examples	Derived from
Inactivated vaccine		
Rabies neural vaccine	Semple vaccine	Sheep brain derived, inactivated with phenol
	BPL vaccine	Sheep brain derived, beta propiolactone inactivated
	Infant mouse brain vaccine	Neural tissue of newborn mice
Rabies Non-neural vaccine	PCEC (purified chick-embryo cell) vaccine	Chicken fibroblast cell line
	HDC (human diploid cell) vaccine	Human fetal lung fibroblast cell line (WI-38 and MRC-5)
	Purified Vero cell (PVC) vaccine	Vero cell line
Kyasanur Forest Disease (KFD)	Killed KFD vaccine	Formalin-inactivated chick embryo vaccine
Subunit vaccine		
Hepatitis B	HBsAg (Hepatitis B surface antigen)	Yeast (recombinant DNA technology)
Papilloma	L1 protein	Yeast (recombinant DNA technology)
Both live and inactivated vaccines		
Poliovirus	Live Oral Polio Vaccine (OPV)	Monkey kidney cell line (Vero)
	Killed Injectable Polio Vaccine (IPV)	Monkey kidney cell line
Japanese B encephalitis	Nakayama strain (killed)	Formalin inactivated mouse brain derived
	Beijing strain (killed)	Formalin inactivated mouse brain derived
	SA 14-14-2 strain (live)	Primary hamster kidney cell line
Influenza	Killed vaccine	Embryonated chicken egg
	Live attenuated (intranasal)	Embryonated chicken egg
Yellow fever	17D live attenuated	Embryonated chicken egg
	Dakar strain (killed)	Mouse brain derived
Hepatitis A	Inactivated	Human fetal lung fibroblast cell line (WI-38 and MRC-5)
	Live attenuated	Human diploid cell line (H2 and L-A-1)
Live attenuated vaccine		
Mumps	Jeryl-Lynn strain	Embryonated chicken eggs and chicken embryo fibroblast cell line
Measles	Edmonston-Zagreb Strain	Chicken embryo fibroblast cell line
Rubella	RA 27/3 Strain	Human fetal lung fibroblast cell line (WI-38 and MRC-5)
Chickenpox	Oka strain of varicella-zoster	Human fetal lung fibroblast cell line (WI-38 and MRC-5)
Smallpox	Live vaccinia virus	Calf lymph
Rotavirus	Live attenuated	Vero cell line
Adenovirus	Live	Human fetal lung fibroblast cell line (WI-38 and MRC-5)

- **Preparation:** Most of the live vaccines are prepared by attenuation by serial passages. (Exception is smallpox vaccine where the naturally occurring vaccinia viruses were used for vaccination)
- **Advantages:** Live vaccines provide a stronger and long-lasting immunity, mimicking immunity produced after natural infection. They are administered as a single dose (except OPV)
- **Disadvantages:** Live vaccines are risky in immunodeficiency or in pregnancy. They are less stable than killed vaccines.

Passive Immunization (Immunoglobulin)

Passive immunization is indicated when individual is immunodeficient or when an early protection is needed (i.e. for post-exposure prophylaxis). However, as there is no memory cells involved, passive immunization have no role in prevention of subsequent infections.

- Previously used horse derived immunoglobulins were less effective with more side effects due to local hypersensitivity reactions; hence, they are now replaced by human immunoglobulins
- Currently, human immunoglobulins are available for the following viral infections:
  - Mumps
  - Measles
  - Hepatitis B
  - Rabies
  - Varicella-zoster.

Combined Immunization

Simultaneous administration of vaccine and immunoglobulin in post-exposure prophylaxis is extremely useful. It is recommended for:

- Hepatitis B (neonates born to HBsAg positive mothers or for unvaccinated people following exposure)
- Rabies (for exposures to severe class III bites).

EXPECTED QUESTIONS

I. Essay:

1. Discuss in detail laboratory diagnosis of viral infections.

II. Write short notes on:

1. Replication of viruses.
2. Methods of detecting viral growth in cell cultures.
3. Interferons.
4. Inclusion bodies.
5. Viral vaccines.

III. Multiple Choice Questions (MCQs):

1. Which of the following virus is enveloped?
a. Poliovirus b. Adenovirus
c. Herpesvirus d. Parvovirus B19

2. All of the following are RNA viruses, *except*:
a. Human adenoviruses
b. Enterovirus
c. Coxsackievirus
d. Hepatitis A virus

3. All of the following viruses are transmitted by respiratory route, *except*:
a. Influenza virus
b. Rotavirus
c. Respiratory syncytial virus
d. Rhinovirus

4. All of the following are continuous cell lines, *except*:
a. HeLa cell line
b. Chick embryo cell line
c. HEp-2 cell line
d. KB cell line

5. Suckling mice are used for isolation of:
a. Coxsackievirus b. HIV
c. Hepatitis B virus d. Poliovirus

6. All of the following are intracytoplasmic inclusion bodies, *except*:
a. Negri bodies
b. Molluscum bodies
c. Cowdry type A inclusions
d. Guarnieri bodies

7. Which of the following vaccine is a killed vaccine?
a. Mumps vaccine b. Measles vaccine
c. Rubella vaccine d. Semple vaccine

8. The largest virus in size is?
a. Herpes simplex virus
b. Hepatitis B virus
c. Poxvirus
d. Adenovirus

9. The smallest virus in size is?
a. Picornaviruses b. Parvovirus
c. Hepatitis D virus d. Adenovirus

10. Which of the following DNA virus is single-stranded?
a. Parvovirus b. Hepatitis B virus
c. Adenovirus d. Poxvirus

11. Which of the following RNA virus has double stranded RNA?
a. Retroviruses b. Hepatitis C virus
c. Rotavirus d. Influenza virus

12. Which of the following virus-shape combination is wrong?
a. Ebolavirus-filamentous
b. Adenovirus- space vehicle shaped
c. Poxvirus- brick shaped
d. Rotavirus- bullet shaped

13. Which one of the following vaccine is contra-indicated in children with egg allergy?
a. MMR b. BCG
c. DPT d. Yellow Fever

14. Human fibroblast cell line is used for cultivation of:
a. Adenovirus b. Poliovirus
c. CMV d. Measles

15. Both intranuclear and cytoplasmic inclusion is seen in:
a. Poxvirus b. Herpesvirus
c. Measles virus d. Mumps virus

16. Which is NOT a continuous cell line for viruses:
a. Vero b. HEp-2
c. WI-38 d. HeLa

17. Viral inclusion bodies are all, *except*:
a. Psamomma bodies b. Molluscum bodies
c. Negri bodies d. Bollinger bodies

Answers

1. c **2.** a **3.** b **4.** b **5.** a **6.** c **7.** d **8.** c **9.** b **10.** a **11.** c **12.** d **13.** d **14.** c **15.** c **16.** c **17.** a

Herpesviruses

42
CHAPTER

Chapter Preview

- General properties
- Herpes simplex virus
- Varicella-zoster virus
- Cytomegalovirus
- Epstein-Barr virus
- Less common herpesviruses
  - Human herpesvirus 6
  - Human herpesvirus 7
- Human herpesvirus 8
- Herpes simian B virus

GENERAL PROPERTIES

Herpesviridae comprises of a group of viruses that possess a unique property of establishing latent or persistent infections in their hosts and later on undergoing periodic reactivation.

Morphology

Herpesviruses are large (150-200 nm size), spherical in shape with icosahedral symmetry.

- **Nucleocapsid:** They possess a linear double-stranded DNA comprising of 125-240 kbp nucleotides surrounded by a capsid composed of 162 capsomeres (Fig. 42.1)

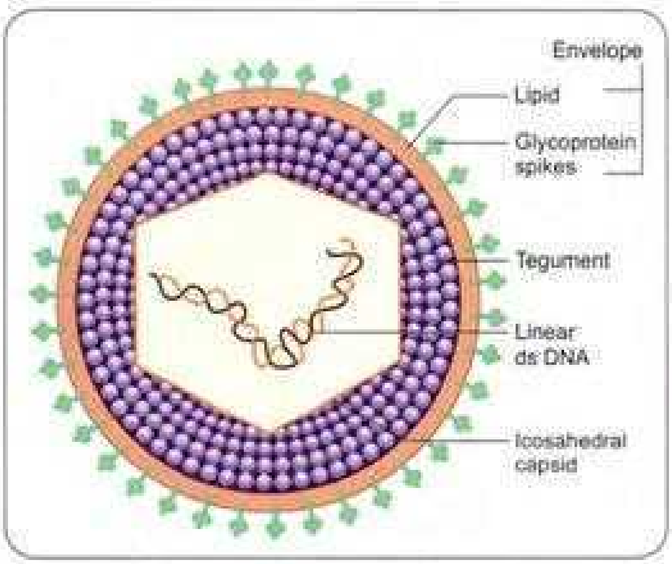


Fig. 42.1: Herpes simplex virus (schematic diagram)

- **Envelope:** The nucleocapsid is surrounded by an envelope which is lipoprotein in nature
  - Lipid part: It is derived from the nuclear membrane of the infected host cell
  - Protein part: Virus-coded glycoprotein spikes, about 8 nm long are inserted into the lipid part. They bind to the specific host cell receptors that help in viral entry.
- **Tegument:** Between the capsid and envelope, there is an amorphous, sometimes asymmetric structure present called tegument
- **Unique feature of herpesvirus DNA:** The genome contains several reiterated (or repeated) genes, which undergo sequence arrangement between the members
- **There is no DNA homology** between the members except:
  - Herpes simplex virus types 1 and 2 (exhibit 50% genomic sequence homology)
  - Human herpesviruses 6 and 7 (exhibit 30-50% sequence homology).
- **Replication** of herpesviruses takes place in the host cell nucleus and is similar to replication of any other dsDNA virus as described in Chapter 41. The only difference is that the linear dsDNA of herpesviruses becomes circular inside the host cell and then replicates by **rolling circle** mechanism.

Classification

Family Herpesviridae comprises of three subfamilies (α, β and γ)—classified based on the site of latency, duration of growth cycle and type of cytopathology they produce (Table 42.1).

- Each subfamily in turn has one or more genera and each genus consists of a few species

Table 42.1: Classification of human herpesviruses (Family-Herpesviridae)

Subfamily ("-herpesvirinae")	Duration of replication and cytopathology	Site of latency	Genus	Species	
				Official name	Common name
Alpha	Short (12–18 hours) Cytolytic	Neurons	*Simplexvirus*	Human herpesvirus 1	Herpes simplex virus type 1
				Human herpesvirus 2	Herpes simplex virus type 2
			Varicellovirus	Human herpesvirus 3	Varicella-zoster virus
Beta	Long (>24 hours) Cytomegalic	Glands, kidneys	*Cytomegalovirus*	Human herpesvirus 5	Cytomegalovirus
	Long (>24 hours) Lymphoproliferative	Lymphoid tissues (T cells)	*Roseolovirus*	Human herpesvirus 6	Human herpesvirus 6
				Human herpesvirus 7	Human herpesvirus 7
Gamma	Variable Lymphoproliferative	Lymphoid tissues (B Cells)	*Lymphocryptovirus*	Human herpesvirus 4	Epstein-Barr virus
			Rhadinovirus	Human herpesvirus 8	Kaposi's sarcoma-associated herpesvirus

- For general use, the common names are still popular, which are also followed in this textbook
- There are about 100 herpesviruses infecting different animals, out of which only eight are human herpesviruses which infect exclusively man
- Rarely, some herpesviruses infecting animals (e.g. herpes simian B virus of monkey) can infect man.

HERPES SIMPLEX VIRUS

Herpes simplex viruses belong to α-subfamily of Herpesviridae.

- They are extremely widespread and exhibit a broad host range; can infect many types of cells and different animals. However, the human herpesviruses infect exclusively man
- They replicate fast (12–18 hours cycle), spread fast and are cytolytic
- They can cause a spectrum of diseases, involving skin, mucosa and various organs
- They undergo **latency in nerve cells**; reactivate later causing recurrent lesions.

Herpes simplex viruses (HSV) are of two distinct types: HSV-1 and HSV-2. They differ from each other in many aspects (Table 42.2).

Pathogenesis

Primary Infection

- **Transmission** occurs through abraded skin or mucosa from any site, but more commonly by:
  - **HSV-1:** Oropharyngeal contact with infected saliva or direct skin contact
  - **HSV-2:** Sexual contact or rarely vertical mode (from mother to fetus).
- **Site of infection:** HSV replicates at the local site of infection and produces lesions anywhere, but more commonly in:
  - HSV-1 lesions are confined to areas above the waist (most common site—around mouth)
  - HSV-2 produces lesions below the waist (most common site—genital area).
- **Spread via nerve:** Virus then invades the local nerve endings and is transported by retrograde axonal flow to the dorsal root ganglia, where it replicates further, and then undergoes latency

Table 42.2: Differences between HSV-1 and HSV-2

Properties	Herpes simplex virus 1	Herpes simplex virus 2
Common modes of transmission	Direct contact with mucosa or abraded skin	Sexual mode or vertical mode
Latency in	Trigeminal ganglia	Sacral ganglia
Age affected	Young children	Young adults
Antibody distribution	Present in 70–90% of people (adult)	Present in 20% of people (adult)
Common manifestations	• Oral-facial mucosal lesions • Encephalitis and meningitis • Ocular lesions • Skin lesions—above the waist	• Genital lesions • Skin lesions—below the waist • Neonatal herpes
Egg (CAM*)	Forms smaller pocks	Forms larger pocks
Chick embryo fibroblast	Does not grow well	Replicates well
Neurovirulence	Less	More
Drug resistance	Less	More
Temperature sensitivity	Less temperature sensitive	More temperature sensitive
Antigenic homology	HSV-1 and 2 show >80% antigenic homology	
DNA homology	HSV-1 and 2 show >50% homology in the genomic sequence	

*CAM—Chorioallantoic membrane

- Primary HSV infections are usually mild; in fact, most are asymptomatic
- However in immunocompromized hosts, viremia occurs that leads to widespread organ involvement and systemic manifestations.

Latent Infection

- HSV has a tendency to undergo latency in neurons:
  - HSV-1: undergoes latency in trigeminal ganglia
  - HSV-2: undergoes latency in sacral ganglia.
- **Nonreplicating state:** HSV does not replicate in latent stage except for a small RNA, called **micro-RNA** (encoded by a latency-associated viral gene) which maintains the latent infection and prevents cell death
- The virus cannot be isolated during latency.

Recurrent Infections

Reactivation of the latent virus can occur following various provocative stimuli, such as fever, axonal injury, physical or emotional stress, and exposure to ultraviolet light.

- **Via the axonal spread**, virus goes back to the peripheral site and further replicates in skin or mucosa producing secondary lesions
- **Recurrent infections** are less extensive and less severe because of presence of pre-existing host immunity that limits the local viral replication
- Recurrent infections are usually asymptomatic; but the virus continues to shed in the secretions.

Clinical Manifestations

Both HSV-1 and 2 have been isolated from nearly all mucocutaneous sites and viscera; however, in general, oral-facial infections are common with HSV-1, whereas HSV-2 frequently causes genital infections and intrauterine infections. The incubation period ranges from 1 to 26 days (median, 6-8 days).

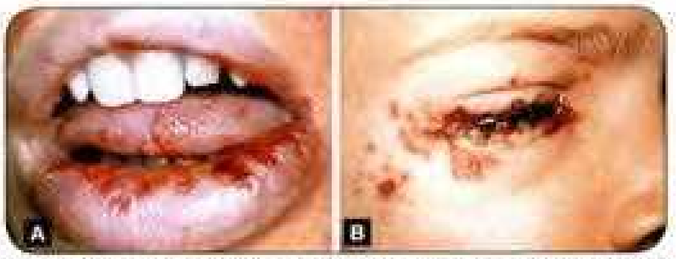
Oral-facial Mucosal Lesions

Oral-facial mucosal lesions are the most common manifestation of HSV infections.

- Most common affected site is buccal mucosa
- Most frequent primary lesions are gingivostomatitis and pharyngitis
- Most frequent recurrent lesion is herpes labialis (painful vesicles near lips) (Fig. 42.2A)
- Other lesions produced are—ulcerative stomatitis and tonsillitis
- Many cases are asymptomatic but can predispose to secondary bacterial infection.

Nervous System

HSV causes various neurological manifestations.



Figs 42.2A and B: A. Vesicular lesions on lips and tongue due to HSV-1 infection; **B.** Periocular vesicular lesions due to HSV-1 infection

Source: Public Health Image Library, A. ID# 12616 (Robert E Sumpter), B. ID# 6492 (Dr KL Hermann)/Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*).

- **Encephalitis:** HSV is the most common cause (10–20%) of acute sporadic viral encephalitis, most frequently involving temporal lobe. HSV-1 is more common (95%) than HSV-2
  - Children get primary infection: HSV is acquired exogenously and invades CNS via the olfactory bulb
  - Adults get recurrent infections due to reactivation of HSV in trigeminal nerve.
- **Meningitis:** HSV can cause recurrent lymphocytic meningitis called Mollaret's meningitis
- **Other neurological manifestations:**
  - Autonomous system involvement (sacral region)
  - Transverse myelitis
  - Guillain-Barré syndrome
  - Peripheral nervous system involvement
  - Cranial nerve involvement (e.g. facial nerve in Bell's palsy).

Cutaneous Lesions

HSV usually infects through abraded skin and causes various cutaneous lesions.

- **Herpetic whitlow:** Lesions present on the fingers of dentists and hospital personnel
- **Febrile blisters** (herpes febrilis): Fever due to any other cause can provocate HSV to cause recurrent blisters
- **Herpes gladiatorum:** Mucocutaneous lesions present on the body of wrestlers
- Skin lesions are often severe on underlying eczema or burns which permit extensive local viral replication and spread
- **Eczema herpeticum:** Caused by HSV-1 in patients with chronic eczema. Similar lesions are also produced by vaccinia virus or coxsackievirus A16 infection; these conditions together are designated as Kaposi's varicelliform eruptions
- **Erythema multiforme:** HSV is commonly associated with this condition. Herpes antigens have been detected in the immune complexes found in serum or skin biopsies.

Ocular Lesions

HSV-1 is more common than HSV-2 to infect eyes.

- Severe conjunctivitis is the most common manifestation
- Recurrent lesions develop into **dendritic ulcers** of cornea or vesicles on the eyelids
- Acute keratoconjunctivitis
- Follicular keratoconjunctivitis with vesicles on eyelid (Fig. 42.2B)
- **Corneal blindness:** Involvement of corneal stroma may cause opacity and blindness. HSV-1 infections are the second common cause of corneal blindness (next to trauma).

Genital Lesions

HSV-2 is more common than HSV-1 to cause primary as well as recurrent genital lesions.

- Genital lesions are described as bilateral, painful, multiple, tiny vesicular ulcers
- This may be associated with fever and inguinal lymphadenopathy.

Visceral and Disseminated Herpes

HSV viremia results in disseminated infection; involving multiple organs.

- **Risk factors:** Immunocompromized patients, underlying malnutrition or AIDS, pregnant women and transplant recipients are at a higher risk of disseminated infection
- **Common manifestations include:** Pneumonitis, tracheobronchitis and hepatitis.

Neonatal Herpes

HSV is one of the common causes of congenital infections, along with the other TORCH agents (refer Chapter 65, for detail).

- **Transmission:** Newborns acquire HSV infection most commonly during birth from the maternal genital tract. However, transmission can also occur *in utero* or after birth
- **Risk** of developing neonatal herpes is maximum (10 times more) if the mother recently acquires the virus (primary infection) than those who present with recurrent infection
- **HSV-2** is more common to cause neonatal herpes (50–70% of total cases) than HSV-1
- **Clinical features:** Babies are almost always symptomatic and present in one of the two forms:
  - *Local lesions* involving skin, eye and mouth are the most frequent manifestations
  - *Systemic:* Among all HSV-infected people, neonates are at highest risk of visceral and/or CNS (encephalitis) infection.
- *Mortality:* Neonatal herpes is associated with high mortality (65% without treatment).
- *Treatment:* Neonates with presumed herpes should be treated with antiviral drug acyclovir for 6–12 months.

Epidemiology

Herpes simplex viruses are worldwide in distribution. No animal reservoirs or vectors are involved with the human viruses. HSV-1 and 2 differ in their epidemiological pattern.

Epidemiological Pattern of HSV-1

- **Transmission:** HSV-1 infection is more common and is transmitted by contact with infected secretions (saliva)
- Primary infection occurs early in life and is either asymptomatic or remains confined to oropharyngeal disease
- **Age:** Children are commonly affected
- **Adults:** Antibodies develop in 70–90% of adults, but they fail to eliminate the virus from the body. Most adults become carriers throughout the life, occasionally get transient recurrent attacks.

Epidemiological Pattern of HSV-2

- HSV-2 is transmitted by sexual or vertical routes
- Primary infection occurs in adult life. Antibodies develop only in 20% of people particularly among black women than men and whites
- HSV-2 tends to recur more often than HSV-1, irrespective of the site of infection.

LABORATORY DIAGNOSIS — **Herpes simplex virus**

- Cytopathology (Tzanck preparation) by Wright's or Giemsa stain-detects inclusion bodies (Lipschultz body) and formation of multinucleated giant cells
- Virus isolation by:
  - Conventional cell lines—used to demonstrate diffuse rounding and ballooning of cell lines
  - Shell vial culture—detects antigens in cell line by IF.
- Viral antigen detection in specimen by direct IF
- HSV DNA detection by PCR and real-time PCR (detecting glycoprotein B and UL 30 genes)
- Antibody detection by ELISA or other formats-detecting antibodies to glycoprotein G.

Laboratory Diagnosis

The sensitivity of all the methods to diagnose HSV infection depends on the type of specimen, as well as the type of infection. The sensitivity is more for vesicular lesions and primary infection than for ulcerative lesions and recurrent infections.

Cytopathology

Scrapings obtained from the base of the lesion can be stained with Wright's or Giemsa (Tzanck preparation), or Papanicolaou stain. Sensitivity of staining is low (<30% for mucosal swabs). It cannot differentiate between HSV-1,

HSV-2, and varicella-zoster virus; as all of them produce similar but characteristic cytopathological changes such as:
- Production of Cowdry type A intranuclear inclusion bodies (Lipschultz body)
- Formation of multinucleated giant cells with faceted nuclei and ground glass chromatin (Tzanck cells). (Fig. 42.3A)
- Ballooning of infected cells, margination of chromatin.

Virus Isolation

It remains the most definitive tool for HSV diagnosis. Viral growth in cell lines can be detected in 2-4 days by:
- **Characteristic cytopathic effect:** Diffuse rounding and ballooning of the infected cells
- Viral antigen detection by neutralization test or immunofluorescence staining with specific antiserum
- Shell vial technique can be followed to decrease the detection time to <24 hours.

Viral Antigen Detection by Immunofluorescence

Viral antigen detection (targeting cell surface glycoprotein antigens) by direct IF is also a sensitive and specific assay. It can differentiate HSV-1 from HSV-2.

HSV DNA Detection

- Polymerase chain reaction (PCR) is the most sensitive test for detecting HSV infections and can be used to differentiate between HSV-1 and HSV-2
- Real-time PCR has been developed targeting genes such as glycoprotein B and UL 30; which are more sensitive; can quantitate the viral load in specimens and also can differentiate between HSV-1 and -2.

Antibody Detection

Antibodies appear in 4–7 days after the infection and peak in 2-4 weeks. IgM appears first and is replaced by IgG, which persists for life.
- Most available tests usually detect IgG or total antibodies, hence cannot differentiate between recent and past infections. Seroconversion or a rise in titer is more meaningful
- Serologic assays (e.g. ELISA) based on the type-specific antigens such as glycoprotein G antigens (gG1 and gG2) can differentiate between HSV-1 and HSV-2. Western blot is more accurate, with 98% sensitivity and specificity.
- Both ELISA and indirect IF formats are available (Fig. 42.3B).

TREATMENT **Herpes simplex virus**

Several specific antiviral drugs are effective for HSV infections. Acyclovir is the drug of choice. It acts by inhibiting viral DNA polymerase.
- **For mucocutaneous infections:** Acyclovir and its congeners famciclovir and valacyclovir have been the mainstay of treatment
- **Ocular infection:** Topical idoxuridine, trifluorothymidine, topical vidarabine, and cidofovir are used
- **For HSV encephalitis and neonatal herpes,** acyclovir is the treatment of choice.

Acyclovir resistance has been reported among few HSV strains which have altered substrate specificity for phosphorylating acyclovir. Resistance is more common in HSV-2 and among immunocompromized patients.

Foscarnet is the drug of choice to treat such cases.

Figs 42.3A and B: A. Tzanck smear of a tissue scraping showing multinucleated giant cell (Tzanck cell) in the center (arrow showing); **B.** Indirect IF for HSV1/2 antibody detection

Source: A. Public Health Image Library, ID# 14428/Centers for Disease Control and Prevention (CDC), Atlanta; B. Euroimmun (with permission).

Prevention

General measures can be taken such as:
- Use of condom to prevent genital herpes
- Neonatal herpes can be prevented by prior administration of acyclovir to mothers during third trimester of pregnancy or delivery by elective caesarean section
- No vaccine is currently licensed. Several vaccine trials are going on, such as recombinant HSV-2 glycoprotein vaccine.

Infection control measures: Patients with mucocutaneous herpes in hospitals, should be kept on airborne plus contact precautions until lesions are dry and crusted (Chapter 53).

VARICELLA-ZOSTER VIRUS

Varicella-zoster virus (VZV) produces vesicular eruptions (rashes) on the skin and mucous membranes in the form of two clinical entities:
1. **Chickenpox:** It is characterized by generalized diffuse bilateral vesicular rashes which occur following primary infection, usually affecting children (Fig. 42.4A).
2. **Zoster or shingles:** It occurs following reactivation of latent VZV, present in the trigeminal ganglia that occurs mainly in adult life. Vesicular rashes are unilateral and segmental (confined to the skin innervated by a single sensory ganglion) (Figs 42.4B and C).

Chickenpox

Pathogenesis

The pathogenesis of chickenpox involves the following steps.

- **Portal of entry:** VZV enters through the upper respiratory mucosa or the conjunctiva
- **Spread:** It replicates in the regional lymph nodes → spills over and enters the blood stream (primary viremia) → spreads to the liver and spleen, and multiplies → again enters bloodstream (secondary viremia) → VZV present in the infected mononuclear cells are transported to:
  - **Skin:** Virus replication in the epithelial cells leads to development of typical rashes. Swelling of epithelial cells, ballooning degeneration, and accumulation of tissue fluids result in the formation of vesicles
  - **Respiratory tract:** VZV is shed in the respiratory secretions of the infected individuals leading to the transmission of infection to other individuals
  - **Neurons:** VZV gains access to neurons of trigeminal ganglia and undergoes latency.

Clinical Manifestations

- Incubation period is about 10-21 days (2-3 weeks)
- Typical description of chickenpox rashes:
  - Rashes are vesicular (Fig. 42.4A)
  - Centripetal in distribution: Usually start on the face and trunk, spread rapidly to involve flexor surfaces; sparing distal part of the limbs
  - Bilateral and diffuse in distribution
  - Rashes appear in multiple crops: Lesions in various stages of evolution, such as maculopapules, vesicles, pustules, and scabs can be found in one area at the same time
  - Fever appears with each crop of rashes.
- Chickenpox is a disease of childhood
- When occurs in adults, it is more severe with bullous and hemorrhagic rashes leaving behind pitted scars on skin after recovery.

Complications

Complications are more common in adults and in immunocompromized individuals.

- **Most common infectious complication is:** Secondary bacterial infection of the skin
- **Most common extracutaneous complication is:** CNS involvement (cerebellar ataxia, encephalitis and aseptic meningitis), usually occurs in children
- **Most serious complication is:** Varicella pneumonia, which develops more commonly in adults (up to 20% of cases) than in children and is particularly severe in pregnant women
- **Reye's syndrome** can occur secondary to VZV infection. It is characterized by fatty degeneration of liver following salicylate (aspirin) intake
- **Other complications are:** Myocarditis, nephritis, corneal lesion and arthritis.

Chickenpox in Pregnancy

Chickenpox in pregnancy can affect both mother and the fetus.

- Mothers are at high risk of developing varicella pneumonia
- Fetus can develop two types of infection; depending upon the gestational period at which it acquires infection.

(1) Fetal or congenital varicella syndrome (early pregnancy):

- ***VZV is highly teratogenic. Risk is maximum when mother:***
  - Acquires primary infection during pregnancy
  - Acquires infection at early pregnancy (risk of transmission is 25%. However, the risk of developing into disease is 0.4% and 2% in infants whose mothers get chickenpox in <13 weeks and 13-20 weeks of gestation respectively).
- ***Characterized by:***
  - Cicatricial skin lesions and limb hypoplasia (most characteristic anomalies)
  - CNS defects: Microcephaly, mental retardation and seizures
  - Ocular defects: Chorioretinitis, microphthalmia, and cataract
  - Renal system defects: Hydroureter and hydronephrosis
  - Autonomic system defect: Neurogenic bladder
  - Low birth weight.

(2) Neonatal varicella (infection near delivery):

- If mother develops chickenpox more than 5 days before delivery—then baby is mostly asymptomatic due to protective maternal antibody which prevents the transmission of the virus during delivery
- If mother develops chickenpox 5 days before to 2 days after the delivery—maternal antibodies would not have produced in such a short time. This leads to dissemination of virus in the baby to cause neonatal varicella (a severe form of chickenpox with mortality rate exceeding 30%).

Epidemiology

Chickenpox is a highly contagious disease.

- **Period of infectivity:** Child is infectious from 2 days before the onset of rash to 5 days thereafter, until the vesicles are crusted
- One attack gives lifelong immunity
- **Reservoir:** Humans are the only known reservoir host
- **Source of infection:** Patients are the only source, there are no carriers
- Secondary attack rate is about 70-90%.

Zoster or Shingles or Zona

Zoster usually occurs due to reactivation of latent VZV in old age (>60 years of age), in immunocompromized individuals or occasionally in healthy adults.

Figs 42.4A to C: (A) Rashes of Chickenpox; (B and C) Segmental distribution of rashes of Zoster

Source: A. Public Health Image Library, ID# /2882/ JD Millar/ Centers for Disease Control and Prevention (CDC), Atlanta (with permission); B. CW Leung, Department of Paediatrics and Adolescent Medicine, Princess Margaret Hospital, Hong Kong (with permission); C. Recommendations of the Advisory Committee on Immunization Practices (ACIP). MMWR Morb Mortal Wkly Rep 2008;57(RR-5):1 (with permission).

- It usually starts with severe pain in the area of skin or mucosa supplied by one or more groups of sensory nerves and ganglia
- **Rashes:** They are unilateral and segmental, confined to the area of skin supplied by the affected nerves (Figs. 42.4B and C)
- **Most common nerve involved** is ophthalmic branch of trigeminal nerve. Head, neck and trunk are the most common affected sites.

Complications of Zoster

- **Post-herpetic neuralgia** (pain at the local site lasting for months): It is the most common complication in elderly patients
- **Zoster ophthalmicus:** Unilateral painful crops of skin rashes surrounding the eye
- **Ramsay Hunt syndrome** develops when geniculate ganglion of facial nerve is involved. It is characterized by tetrad of facial nerve palsy plus vesicle on tympanic membrane, external auditory meatus and the tongue
- **Visceral disease**, especially pneumonia can occur which is the most common cause of death (<1%) in zoster patients
- Recurrent or chronic zoster is common with HIV-infected patients.

Laboratory Diagnosis (VZV)

Laboratory diagnosis of VZV infection is as follows:

- **Cytopathology:** Giemsa staining of the scrapings from the ulcer base (Tzanck smear) reveals cytopathological changes similar to that of HSV infection, such as formation of multinucleated giant cells
- **Virus isolation:** Virus isolation in various cell lines can also produce HSV-like cytopathic effects such as diffuse rounding and ballooning of infected cells
- **VZV-specific methods:**
  - Specific antigen detection by direct immunofluorescence staining
  - PCR detecting VZV-specific genes.

TREATMENT — Varicella-zoster virus

Acyclovir, famciclovir or valacyclovir are the agents of choice. It can prevent the complications of chickenpox and can also halt the progression of zoster in adults, but cannot prevent post-herpetic neuralgia.

Vaccine

Live attenuated vaccine using **Oka strain** of VZV is available.

- It is given to children after 1 year of age; 2 doses, first dose is given at 12–15 months and second at 4–6 years
- In seronegative adults; 2 doses given at 1-month gap
- Transmission of the vaccine virus can occasionally cause mild rashes in the recipient
- Protectivity: The vaccine is >80% effective in preventing chickenpox in children but less so in adults (70%). However, it is 95% effective in preventing severe disease.

VZIG (Varicella-zoster Immunoglobulin)

- It is recommended for post-exposure prophylaxis. It is given within 96 hours (preferably within 72 hours) of exposure

- It is also indicated for neonates born to mothers suffering from chickenpox if the onset of chickenpox in mother is between <5 days before delivery till 48 hours after delivery. VZIG is not indicated for the neonate if the mother has zoster.

Infection Control Measures

Patients infected with VZV should be kept in isolation. Airborne precautions (e.g. negative air-flow rooms) plus contact precautions must be followed until lesions are dry and crusted (Chapter 53 for detail). For localized zoster in an immunocompetent host, contact precaution alone can be followed.

CYTOMEGALOVIRUS

Cytomegalovirus (CMV) is the largest virus in Herpesviridae family. It is so named because it causes massive enlargement of infected host cells.

Properties of CMV are similar to any other herpesvirus described earlier with some minor differences.

- CMV belongs to β-subfamily
- Its dsDNA is the largest among herpesviruses, which consists of 240 kbp nucleotides
- **Host specificity:** Cytomegaloviruses are strictly species-specific. Human CMV does not infect animals. Similarly, a number of animal CMVs exist, which do not infect humans
- **Cell-type specificity:**
  - In vivo, CMV infects kidney and salivary glands; where it undergoes latency
  - In vitro, CMV replicates only in human fibroblast cell line and produces a characteristic cytopathic effect (CPE) described as **Owl's eye** appearance (Fig. 42.5).

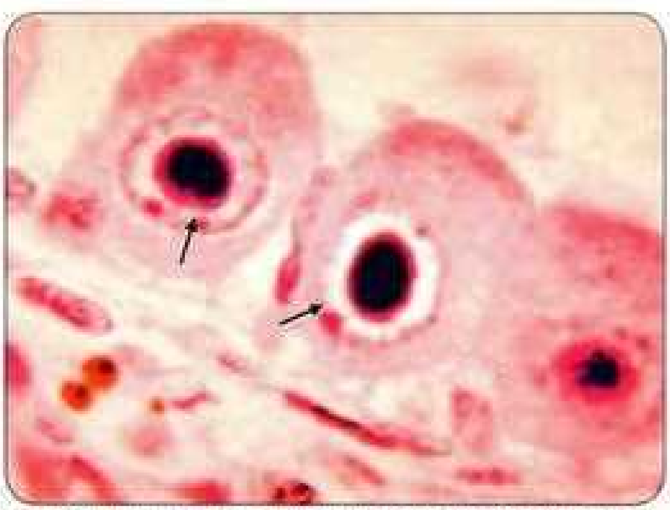


Fig. 42.5: Histopathology of kidney shows cytomegalic host cell containing characteristic Owl's eye inclusion (arrows showing)

Source: Public Health Image Library, ID# /1155 Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

- **Cell-to-cell spread:** CMV is almost always closely associated with the cells and spread primarily cell-to-cell, so that very little virus may be cell-free.

Clinical Manifestations

CMV causes an array of clinical syndromes such as congenital and perinatal infections, CMV mononucleosis in adults and severe infection in immunocompromized and transplant recipients.

Congenital CMV Infection

CMV is probably the most common intrauterine infection associated with congenital defects.

- Cytomegalic inclusion disease develops in about 5% of the infected fetus. The remaining are although asymptomatic at birth, 5–25% of them may develop significant psychomotor, hearing, ocular, or dental defects within 2 years
- **Congenital defects include:**
  - Most common defects are petechiae, hepatosplenomegaly, and jaundice (60–80% of cases)
  - Less common defects include: Microcephaly, cerebral calcifications, intrauterine growth retardation, and prematurity (30–50% of cases)
  - Occasional defects are inguinal hernias and chorioretinitis.
- **Risk is maximum** if the infection occurs in early pregnancy and if the mother is primarily infected during pregnancy (one-third of the primarily infected mothers transmit the virus to fetus in contrast to 1% of reactivated mothers)
- Mortality rate is very high (20%).

Perinatal CMV Infection

- Transmission to the newborn occurs either during:
  - Delivery—through infected birth canal or
  - Postnatal—through infected breast milk/secretions from mother.
- Most of the infected infants remain asymptomatic, but shed virus in urine from 8–12 weeks of age, up to several years
- Few infants, especially premature babies develop interstitial pneumonitis.

Immunocompetent Adults

In healthy adults, CMV produces an infection following blood transfusion called **mononucleosis-like syndrome**. This condition is similar to infectious mononucleosis caused by EBV (described later in this chapter) (Table 42.3):

Immunocompromized Host

CMV produces markedly severe infection in immunosuppressed individuals; most of which are due to reactivation of their own latent viruses.

Table 42.3: Comparison of infectious mononucleosis and mononucleosis-like syndrome

Features	Infectious mononucleosis	Mononucleosis-like syndrome
Agent	Epstein-Barr virus (EBV)	CMV (20–50%) HHV-6, *Toxoplasma Ehrlichia*, HIV
Atypical lymphocytosis	Seen	Seen
Clinical symptoms	Fever, myalgia, Hepatosplenomegaly, Exudative pharyngitis, Cervical lymphadenopathy, Rashes following ampicillin therapy	Similar presentation, except that exudative pharyngitis, cervical lymphadenopathy are absent
Heterophile antibodies	Elevated (detected by Paul-Bunnell test)	Negative
Specific antibodies	Antibodies to specific EBV antigens are elevated	Antibodies to CMV or other agents may be elevated

- **In AIDS patients** with CD4 T cell count <50/µL—CMV may cause chorioretinitis, gastroenteritis, dementia and other disseminated CMV infections
- **Organ transplant recipients:** CMV is probably the most common viral infection that occurs in transplant recipients. Infection occurs usually between 1 and 4 months following transplantation and presents in various forms such as:
  - Bilateral interstitial pneumonia is the most common form, seen in 15–20% of bone marrow transplant recipients
  - Febrile leukopenia is seen among solid organ transplant recipients
  - Obliterative bronchiolitis in lung transplants
  - Graft atherosclerosis in heart transplants
  - Rejection of renal allografts.

Epidemiology

- **Transmission:** Close person-to-person contact is required for transmission (unlike HSV). Various modes of transmission include:
  - Oral and respiratory spread is the predominant mode
  - Transplacental route (transmission from mother to fetus)
  - Blood transfusion: Risk of transmission is about 0.1–10% per blood unit transfused
  - Organ transplantation
  - Sexual contact (in young adults).
- **Reservoir:** Humans are the only known host for CMV
- **Source:** Virus may be shed in urine, saliva, semen, breast milk, and cervical secretions, and is carried in circulating white blood cells
- **Endemic:** CMV is endemic worldwide, present throughout the year without any seasonal variation
- **Risk factors** such as low socioeconomic status and poor personal hygiene facilitate the infection
- **Prevalence** is high in underdeveloped nations with 90% of people being seropositive in contrast to 40–70% seropositivity in developed nations.

LABORATORY DIAGNOSIS — Cytomegalovirus

- Cytopathology—detects inclusion bodies with owl's eye appearance
- Virus isolation:
  - Human fibroblasts cell line culture—detects CPE after 2–3 weeks
  - Shell vial culture—detects viral antigens in infected cell lines by IF (1–2 days).
- Antibody detection—by ELISA using pp150, pp65, pp52, glycoprotein antigens
- Antigen detection such as pp65 antigen
- Molecular methods—PCR and real-time PCR targeting UL54, UL55 and UL83 genes.

Laboratory Diagnosis

Detection of Inclusion Bodies

In urine, CMV produces characteristic perinuclear cytoplasmic inclusions in addition to the usual intranuclear inclusions seen in other herpesviruses (Owl's eye appearance) (see Fig. 42.5).

Virus Isolation

CMV can be isolated from throat washings and urine.

- Human fibroblasts are the most ideal cell lines, specific for CMV
- **Cytopathic effect:** After 2–3 weeks of incubation, the following CPE may be observed in the infected cell line:
  - Typical CMV inclusions (as described above)
  - Multinucleated giant cells are seen
  - Enlargement of infected host cells.
- **Shell vial technique** can be followed for early growth detection (1–2 days)
  - It involves centrifugation of cell culture (mixed with the specimen) to enhance the cell contact and viral replication, followed by detection of early CMV antigen in the infected cells by direct fluorescence technique
  - It is very useful in CMV mononucleosis where viral load is low and CPE takes several weeks to appear.

Antibody Detection

ELISA has been available for detecting serum antibodies against various antigens such as matrix phosphoproteins pp150 and pp65, glycoproteins gB and gH, and the major DNA binding protein (pp52).

- **CMV IgM antibodies** appear in 1–2 weeks after infection and indicates recent or on-going infection. It drops to below detectable levels within several

Table 42.4: Paul-Bunnell test and its confirmation by differential absorption test

Features	Paul-Bunnell test (performed first)	Treated with		Paul-Bunnell test (repeated)
		Guinea pig kidney cells	Ox red cells	
Infectious mononucleosis	Positive	Not absorbed	Absorbed	Serum prior treated with guinea pig kidney cells—test is positive Serum prior treated with ox red cells—Negative
Antibody after serum therapy	Positive	Absorbed	Absorbed	Negative for both sera
Normal serum	Positive	Absorbed	Not absorbed	Serum prior treated with ox red cells—test is positive Serum prior treated with guinea pig kidney cells—test is negative

- **Transmission:**
  - Intimate and prolonged oral contact is required for effective transmission. EBV is spread by direct contact with oral secretions, e.g. salivary contact during kissing
  - Other modes are blood transfusion and following bone marrow transplantation.
- **Source:** Asymptomatic seropositive individuals shed the virus in oropharyngeal secretions. Shedding is more in immunocompromized patients.

LABORATORY DIAGNOSIS — Epstein-Barr virus

- **Antibody detection:**
  - Nonspecific heterophile antibody detection:
    - Paul-Bunnell test
    - Differential absorption test
    - Monospot test.
  - EBV specific antibody detection—ELISA and indirect IF assay detect antibody to viral capsid antigen, EBNA and early antigen.
- **Molecular methods:**
  - Detects EBV DNA (by PCR)
  - Quantifies EBV DNA (by real-time PCR)-detecting genes BamH1W, EBNA1 and LMP.
  - Detects EBER RNA (by RT-PCR).
- **EBV antigen:** By direct IF assay
- **Virus isolation:** Not routinely performed.

Laboratory Diagnosis

Antibody Detection

Heterophile Agglutination Test (Paul-Bunnell test)

Paul-Bunnell test is a tube agglutination test that uses sheep RBCs to detect heterophile antibodies in patient's serum.

- **Procedure:** Serial dilutions of inactivated (56°C for 30 minutes) patient's serum are mixed with equal volumes of 1% sheep RBCs, and then the tubes are incubated at 37°C for four hours
- **Result:** Agglutination titer of >256 is considered as significant
- **False positive:** Heterophile antibodies are non-specific, may also be present following serum therapy or even in some normal individuals; hence confirmation is must
- **Differential absorption test** is done for confirmation. Patient's serum is first made to react with guinea pig kidney cells and ox red cells, following which Paul-Bunnell test is repeated (Table 42.4)
- **Monospot test** is modified heterophile agglutination test available commercially
  - It is a simple *slide agglutination test* that uses horse RBCs instead of sheep RBCs
  - Test serum is priorly treated with guinea pig kidney and ox red cells
  - It has largely replaced the differential absorption test, and has excellent sensitivity (75%) and specificity (90%).
- Heterophile antibodies appear early (40% in first week and 80-90% in third week of illness), then disappear within 3 months
- Heterophile antibodies are not detectable in children <5 years, in elderly or in patients with atypical symptoms.

EBV-specific Antibody Detection

Various formats such as ELISA and indirect immunofluorescence techniques are available to detect specific EBV antibodies. These tests have become more popular and are almost replacing the traditional heterophile antibody tests.

- **Antibody to viral capsid antigen** (VCA):
  - IgM type: Indicates current infection
  - IgG type: Is a marker of past infection and indicates immunity.
- **Antibodies to early antigen (EA):** These also indicate current viral infection. They are elevated in patients with Burkitt's lymphoma or nasopharyngeal carcinoma
  - EA-D antibody (antibody to early antigen that occurs in diffuse pattern in nucleus and cytoplasm of the infected cells) is elevated in acute infection and Burkitt's lymphoma
  - EA-R antibody (antibody to early antigen restricted to the cytoplasm) is elevated in nasopharyngeal carcinoma.
- **Antibodies to EBNA** (Epstein-Barr nuclear antigen) reveal past infection, but four fold rise of titer may suggest current infection.

Other Tests

- **Detection of EBV DNA** (by PCR), **EBER RNA** (EBV encoded small RNAs, by reverse transcriptase PCR), or **EBV antigens** (by direct-IF technique) have been useful for detecting the virus in various malignancies and in infectious mononucleosis
- **Real-time PCR** quantifying EBV DNA load in blood is extremely useful to monitor the treatment response in patients with lymphoproliferative disease. The various genes targeted are BamH1W, EBNA1 and LMP
- **Virus isolation:** It is laborious, time-consuming (6–8 weeks) and highly sophisticated, hence not routinely performed. EBV can be isolated from saliva, blood, or lymphoid tissue. It causes immortalization of normal human B cells, obtained from cord blood.

TREATMENT **Epstein-Barr virus**

- **Supportive measures** such as analgesics are used in the treatment of infectious mononucleosis
- **Acyclovir** is useful in the treatment of oral hairy leukoplakia, though relapse is common. It reduces EBV shedding from the oropharynx but it has no effect on the immortalized B cells, hence, it is not effective for infectious mononucleosis and other malignancies
- **Antibody to CD20** (rituximab) has been effective in some cases.

Prevention

The isolation of patients with infectious mononucleosis is not needed as temporary contact does not transmit the infection. No vaccine is currently available. A vaccine trial using EBV glycoprotein was found to be ineffective.

LESS COMMON HERPESVIRUSES

Human Herpesvirus 6

HHV-6 infects the T cells by binding to CD46 receptor. It has two variants 6A and 6B.

- **Transmission** is through infected oral secretions
- **Sixth disease:** In children, HHV-6 (usually the 6B variant) causes sixth disease, also called as **exanthem subitum or roseola infantum**. It is characterized by high grade fever and skin rashes
- In older age groups, HHV-6 has been associated with mononucleosis-like syndrome.

Human Herpesvirus 7 (HHV-7)

HHV-7 also shows tropism for T cells, transmitted by oral secretions, mainly in children.

- It shares 30–50% DNA homology with HHV-6
- It has been associated with fever, seizures, respiratory or gastrointestinal signs, and pityriasis rosea.

Human Herpesvirus 8 (HHV-8)

HHV-8 was first discovered in 1994 in patients with Kaposi's sarcoma, hence also called Kaposi's sarcoma-associated herpesvirus (KSHV).

- **Pathogenesis:**
  - HHV-8 belongs to γ subfamily of herpesviruses and infects the B cells
  - HHV-8 genome possesses several oncogenes that regulate certain host cell functions such as proliferation and apoptosis.
- **Epidemiology:**
  - **In high prevalence area:** HHV-8 is endemic in Africa, where it is transmitted by oral secretion
  - **In low prevalence areas** such as North America, Asia, northern Europe, it affects adults and it is transmitted by sexual route (homosexual men)
  - Other less common modes of transmission include: organ transplantation, injection drug abuse, and blood transfusion.
- **Disease association:** In immunocompromized individuals (e.g. HIV-infected people), HHV-8 is associated with:
  - Kaposi's sarcoma (Fig. 42.7): A soft tissue sarcoma of vascular origin; characterized by red to purple color growth under the skin, mouth, oral mucosa, lymph nodes, or in other organs.
  - Primary effusion lymphoma (body cavity-based lymphomas)
  - Castleman's disease (lymphoproliferative disorder of B cells)
  - In immunocompetent host, HHV-8 produces fever and rash.

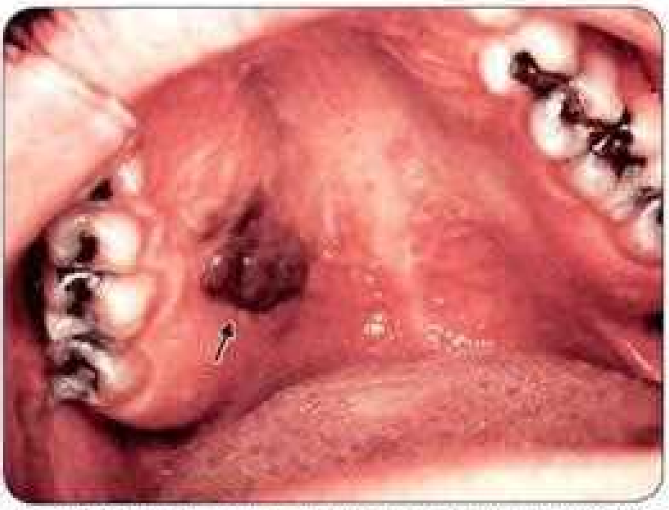


Fig. 42.7: Kaposi's sarcoma of the hard palate secondary to AIDS infection (arrow showing)

Source: Public Health Image Library, ID# 6070/Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*).

- **Diagnosis:** PCR detecting DNA is the confirmatory assay. Viral isolation is difficult. Antibody detection assays are available in various formats such as indirect-IF, Western blot, and ELISA.

TREATMENT **Human herpesvirus 8**

HHV-8 infections respond well to foscarnet, ganciclovir, and cidofovir. Effective antiretroviral therapy for HIV-infected individuals can reduce the risk of Kaposi's sarcoma in HHV-8 infected people.

Herpes Simian B Virus

Herpes simian B virus (formerly Cercopithecine herpesvirus-1, *Herpesvirus simiae or* B virus) is a zoonotic simplex virus infecting macaque monkeys.

- **Transmission:** Human infection is rare, occurs by (1) zoonotic spread by monkey bite (most common), or (2) rarely by respiratory route
- It is highly pathogenic for humans, causes acute ascending myelitis and encephalomyelitis with a high mortality rate (60%).

EXPECTED QUESTIONS

I. **Essay:**

1. A 7-year-old boy had developed multiple painful vesicles over the lips and buccal mucosa. His parents revealed that two children of his school had a similar presentation few days back. Scraping taken from the lesion demonstrated presence of multinucleated giant cell (Tzanck cell).
   a. What is the most probable diagnosis?
   b. List the other agents causing this type of infection.
   c. How is this infection diagnosed in the laboratory.
2. Describe the pathogenesis and laboratory diagnosis of cytomegalovirus infection.

II. **Write short notes on:**

1. Infectious mononucleosis.
2. Kaposi's sarcoma.
3. Chickenpox.

III. **Multiple Choice Questions (MCQs):**

1. **A Tzanck smear of a scraping obtained from a vesicle on the skin demonstrates multinucleated giant cells. Which of the following virus is associated with such finding:**
   a. HSV-1
   b. Variola major
   c. Coxsackie virus
   d. Molluscum contagiosum
2. **Which of the following virus causes a mononucleosis-like syndrome, and also a common cause of congenital infection?**
   a. Epstein-Barr virus b. Human herpesvirus-6
   c. Cytomegalovirus d. Varicella-zoster virus
3. **Which of the following tumor is not caused by Epstein-Barr virus?**
   a. Post-transplant lymphomas
   b. Hodgkin's disease
   c. Burkitt's lymphoma
   d. Kaposi's sarcoma
4. **A 25-year-old female has developed fever, sore throat, and lymphadenopathy accompanied with atypical lymphocytosis and an increase in sheep cell agglutinins. The diagnosis is most likely:**
   a. Hepatitis b. Infectious mononucleosis
   c. Chickenpox d. HSV infection
5. **A 48-year-old woman develops fever and focal neurological signs. MRI shows a left temporal lobe lesion. Most appropriate test that can be done to confirm the diagnosis of HSV encephalitis in this patient is:**
   a. Brain biopsy
   b. Tzanck smear
   c. PCR assay for viral DNA in CSF
   d. Serum IgM antibody detection
6. **Which of the following herpesvirus undergoes latency in kidney?**
   a. HSV b. VZV
   c. CMV d. EBV
7. **Which of the following herpesvirus undergoes latency in B cells?**
   a. HSV b. VZV
   c. CMV d. EBV
8. **HSV-1 differs from HSV-2 by all, *except*?**
   a. Most common site of latency—trigeminal neuron
   b. Most common site of lesion—around mouth
   c. Most common age effected—adults
   d. Most common mode of transmission—contact
9. **Most common cause of acute sporadic viral encephalitis?**
   a. HSV
   b. CMV
   c. Japanese B encephalitis
   d. Measles
10. **Which is NOT associated with Kaposi varicelliform eruption?**
    a. HSV b. HHV-8
    c. Vaccinia virus d. Coxsackievirus A16
11. **Which of the following is the agent of sixth disease?**
    a. HSV b. HHV-6
    c. HHV-7 d. HHV-8
12. **A neonate has hepatosplenomegaly. His urine was stained with Giemsa stain which revealed owl's eye appearance inclusions. Which will be the most probable cause?**
    a. HSV b. CMV
    c. EBV d. HHV-8

Answers

1. a **2.** c **3.** d **4.** b **5.** c **6.** c **7.** d **8.** c **9.** a **10.** b **11.** b **12.** b

Other DNA Viruses

43
CHAPTER

Chapter Preview

- Parvoviridae
- Papillomaviridae and polyomaviridae
- Poxviridae
- Adenoviridae
- Bacteriophages

PARVOVIRIDAE

PARVOVIRUSES

Morphology

Parvoviruses are the simplest animal viruses infecting humans, responsible for a common childhood exanthema called as **erythema infectiosum (fifth disease)**.

- They are the smallest viruses (18-26 nm size)
- Non-enveloped with icosahedral symmetry
- Possess linear single-stranded DNA, comprising of about 5000 nucleotides (the only DNA virus to have ssDNA)
- Capsid is made up of 32 capsomeres
- They depend upon the host cell enzymes for replication.

Classification

Parvoviridae family has two sub-families—Parvovirinae (infect vertebrates) and Densovirinae (infect insects).

- Parvovirinae contains three genera—*Parvovirus, Erythrovirus* and *Dependovirus*
- The most pathogenic human parvovirus; i.e. Parvovirus B19 belongs to the genus *Erythrovirus.*

PARVOVIRUS B19

Pathogenesis

- **Transmission:** Parvovirus B19 exclusively infects humans, most commonly by the respiratory route, followed by blood transfusion and transplacental route
- **Infects precursors of red blood cells (RBCs):** Parvovirus B19 has a special tropism for erythroid progenitor cells present in adult bone marrow and fetal liver as it binds to blood group P antigen as receptors; which are present on the red blood cell (RBC) surface
- **Virus-induced cytotoxicity:** Results in red cell destruction and inhibition of erythropoiesis, which is profound in the presence of underlying immunosuppression and hemolytic anemia.

Clinical Manifestations

Erythema Infectiosum

- **Fifth disease:** In children, the disease is called as fifth disease; characterized by rashes on the face, described as **slapped cheek** appearance (Fig. 43.1)
- Adult women present with symmetrical polyarthropathy which usually involves the hand joints and knee. Rashes may or may not be present.

Transient Aplastic Crisis

It can occur in infected patients with underlying hemolytic anemia-leads to severe acute anemia.

Fig. 43.1: Fifth disease or rashes with slapped cheek appearance

Source: Wikipedia/ Andrew Kerr (with permission).

Table 43.1: Clinical manifestations and laboratory diagnosis of parvoviral infections

Syndrome	Host or condition	Clinical feature	Antibody	Real time PCR
Erythema infectiosum	Children (fifth disease)	Rashes with slapped cheek appearance	IgM (+ve) IgG (+ve)	>10^5 DNA copies/mL
	Adults	Polyarthropathy		
Transient aplastic crisis	Underlying hemolytic anemia	Severe acute anemia	IgM (-/+) IgG (-/+)	Initially >10^{12} DNA copies/mL, but rapidly decreases later
Pure red cell aplasia	Underlying immunosuppression	Chronic anemia	IgM (-/+) IgG (-/+)	>10^8 DNA copies/mL
Hydrops fetalis	Fetus	Fatal anemia	IgM (-/+) IgG (+ve)	PCR is positive (amniotic fluid) Quantitation is not applicable
Papular purpuric gloves and socks syndrome	Young adults	Painful redness and swelling of wrists and ankles with a gloves-and-socks distribution	IgM (+ve after 7 days) IgG (+ve after 2-4 weeks)	PCR (+ve after 3 days)

Pure Red Cell Aplasia

It can occur in those with underlying immunosuppression due to persistent B19 infections, resulting in chronic anemia.

Non-immune Hydrops Fetalis

It can occur in fetus which results in fatal anemia and fetal death. Transplacental transmission occurs in 30% of cases and maximum risk is in the second trimester (Table 43.1).

Laboratory Diagnosis

Molecular Methods

The most sensitive assay for diagnosis is polymerase chain reaction (PCR), which detects viral DNA (e.g. genes coding for VP1 and VP2) from serum, tissue or respiratory secretions (Table 43.1).

- **Real time PCR** is used for quantification. During acute infections, viral load in the blood may reach 10^{12} DNA copies/mL.
- **Genotyping:** Parvovirus B19 has three genotypes but antigenically has single serotype. Genotype 1 is common worldwide, whereas genotype 3 is predominant in Western Africa.

Antibody Detection

ELISA has been available detecting antibodies against VP1 and VP2 antigens.

- IgM antibody appears early, indicates recent infection and remains elevated for 2-3 months
- IgG antibody appears late. IgG against conformational epitopes of parvovirus persists for years, but that against linear epitopes declines within months of infection. Antibody may or may not be found in immunodeficient patients.

Immunohistochemistry has been used to detect viral antigens in fetal tissues and bone marrow. Parvovirus is very difficult to grow in culture.

TREATMENT **Parvovirus**

- No antiviral drug is available.
- Treatment is symptomatic.
- Immunoglobulins containing neutralizing antibodies to human parvovirus are available commercially.

OTHER PARVOVIRUSES THAT INFECT HUMANS

- **Human bocaviruses:** They have been isolated from cases with respiratory infection and gastroenteritis in children but their pathogenic role remains unclear
- **Human dependoviruses:** They are also called as adeno-associated viruses; as they are defective and depend on adenovirus for replication. They are nonpathogenic to man.

PAPILLOMAVIRIDAE AND POLYOMAVIRIDAE

Formerly, the papillomaviruses and polyomaviruses were together grouped under Papovaviridae family, but now they are separated as two different families (Table 43.2).

- **Family Papillomaviridae:** This family has 16 genera, out of which Human papillomavirus infects man
- **Family Polyomaviridae:** This family has several genera infecting animals. Human infections are associated with John Cunningham (JC) virus, BK virus and SV40 virus (Simian vacuolating virus 40) (Table 43.2).

HUMAN PAPILLOMAVIRUS

Human papillomavirus (HPV) has selective tropism for epithelium of skin and mucous membranes and produces an array of infections ranging from benign warts, to malignant neoplasia of cervix.

Morphology

Papillomaviruses are non-enveloped, measure 50-55 nm in size, have icosahedral capsids composed of 72 capsomeres. It contains a double-stranded circular DNA genome of 7900-8000 base pairs.

Table 43.2: Differences between Polyomaviridae and Papillomaviridae

Characteristic	Polyomaviridae	Papillomaviridae
Structure	45 nm in size, icosahedral symmetry, no envelope	55 nm in size, icosahedral symmetry, no envelope
DNA	• Circular, dsDNA • Comprises of 5 kbp of nucleotides • Both the DNA strands code for proteins	• Circular, dsDNA • Comprises of 8 kbp of nucleotides • Coding information is present on one DNA strand
Oncogenic potential	• Transforms cells frequently in vitro, but rarely in vivo (natural infections)	• It can produce tumors in natural hosts • In vitro, rarely transforms cells
Target tissue	Internal organs	Epithelium (skin and mucosa)
Viruses infecting humans and disease produced	• **JC virus:** Causes Progressive Multifocal Leukoencephalopathy (PML) • **BK virus:** Causes nephropathy in transplant recipients. It differs from JC virus, by its ability to grow in a wide range of cell lines and is less oncogenic (JC and BK viruses are named after the initials of the patients in whom they were described first) • **Merkel cell virus:** Causes Merkel cell carcinoma of skin • **SV40 virus** (Simian vacuolating 40 virus): It is not pathogenic to man	Human papillomaviruses (>100 types) cause: • Warts (skin warts and genital warts) • Epidermodysplasia verruciformis • Cervical carcinoma and cervical intraepithelial neoplasia (CIN) • Laryngeal papillomas and carcinoma

Viral Genome

Viral genome consists of an early (E) region, a late (L) region, and a noncoding regulatory region.

- **Early region genes (E1-E7):** They code for early non-structural proteins. The E1 and E2 proteins modulate viral DNA replication. Products of early genes E6 and E7 have oncogenic potential by following ways:
  - *E6 protein* facilitates the degradation of the p53 tumor-suppressor protein
  - *E7 protein* binds to the retinoblastoma gene product and related proteins.
- **Late region genes (L1 and L2):** They code for structural proteins such as capsid
  - L1—codes for major capsid proteins
  - L2—codes for minor capsid proteins.
- **Types:** More than 100 types of HPV are recognized based on DNA sequences of L1 region. Types differ from each other at least by more than 10% in the sequence of their L1 genes.

Pathogenesis

Human papillomaviruses typically infect skin (squamous epithelium) and mucous membranes and produce various benign and malignant lesions (Table 43.3).

Benign Warts

- Common warts (verruca vulgaris)—seen among young children
- Flat warts (verruca plana)—are common in children
- Plantar warts (verruca plantaris)—widely prevalent among adolescents and young adults
- Anogenital warts (condyloma acuminatum): It is a sexually transmitted disease seen among adults (Fig. 43.2).

Table 43.3: Clinical features of human papillomavirus (HPV) infection and the HPV types associated

Clinical Lesion	Oncogenic potential	HPV types
Plantar warts	Benign	1
Common skin warts	Benign	2, 4, 27, 57
Epidermodysplasia verruciformis	Mostly benign, Rarely progress to malignancy	5, 8, 9, 12, 17, 20, 36, 47
Anogenital warts Laryngeal papillomas Intraepithelial neoplasia	Low malignant potential	6, 11
Palmar warts seen in butchers	Low malignant potential	7
Carcinomas of cervix and other genital mucosa/larynx/esophagus	High malignant potential	Most common-16 and 18 Others- 30, 31, 33, 35, 39, 45, 51–53, 56, 58, 59, 66, 68, 73, 82

Epidermodysplasia Verruciformis

It is a rare autosomal recessive benign condition, has propensity to progress to squamous cell carcinoma particularly in sun-exposed areas. Malignant transformation is common when infected with unique HPV types 5 and 8 that do not cause any other disease.

Cervix Lesions

- CIN (cervical intraepithelial neoplasia)—is a benign condition, associated with low-risk HPV types 6 and 11

Figs 43.2A and B: Condyloma acuminatum. **A.** Penis; **B.** Vagina

Source: Public Health Image Library, A, ID# 3724, B, ID# 4097 /Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

- Carcinoma cervix (squamous cell)—is associated with high-risk HPV types such as 16 and 18 (most common); followed by 31, 33, 35, 39, 45, 51-53, 56, 58, 59, 66, 68, 73 and 82
  - High-risk serotypes are associated with squamous cell carcinoma involving other genital regions such as penis, anus, vagina and vulva.

Head and Neck Lesions

- Benign lesions such as recurrent laryngeal papillomas in children are associated with low-risk types 6 and 11
- Malignant lesions such as laryngeal and esophageal carcinomas (associated with high-risk types 16 and 18).

Pityriasis Versicolor Like Lesions

These type of lesions can be seen in immunosuppressed patients, particularly those who have undergone organ transplantation.

Laboratory Diagnosis

- **Molecular methods:** PCR or the hybrid capture assay can be used to detect HPV DNA and to identify specific virus types by targeting genes coding for E6 and E7 regions
- Most lesions are visible to the naked eye. Solutions of **5% acetic acid** can be applied to improve visibility
- **Cytologic evidence** of HPV infection is detected by:
  - Papanicolaou smears prepared from cervical or anal scrapings
  - Histopathological staining of biopsies.
- **Antibody** detection is not much useful.

TREATMENT **Human papillomavirus**

- **Removal of the lesions:** Frequently used procedures for removal of lesions include cryosurgery, electrodesiccation, surgical excision and laser therapy.
- **Topical preparations** of podophyllum, interferon or imiquimod (interferon inducer) can be used for genital warts.
- Recurrence is common.

Prevention (HPV Vaccine)

Recently developed HPV vaccines have shown dramatic reduction in rates of all HPV infections including cervical cancers. It is recommended to adolescent young females.

- Subunit vaccine consists of virus-like particles composed of HPV L1 proteins which are produced in yeast by DNA recombinant technology
- Both nine valent and bivalent vaccines are licensed
  - **Nine valent vaccine** (Gardasil 9, Merck): Includes seven common cancer-causing serotypes (16, 18, 31, 33, 45, 52 and 58) and two noncancer causing serotypes (6 and 11)
    - It is given IM; either as 2 doses (6 months gap) between 9-14 yrs or 3 doses (0, 2 and 6 months) between 14-26 yrs age
    - Immune response is better if given earlier (<14 yrs)
    - Protection appears to be long lasting.
  - Its earlier version was a quadrivalent vaccine which included type 6, 11, 16 and 18
  - **Bivalent vaccine** (Cervarix, GSK) includes only the high-risk serotypes 16 and 18. It is given as single dose, IM.

Barrier methods of contraception can block sexual transmission, thus prevent anogenital HPV infections.

ADENOVIRIDAE

Adenoviridae family consists of two genera:

- *Aviadenovirus:* Infects birds
- *Mastadenovirus:* Infects mammals including humans.

Human adenoviruses are further serotyped into 51 distinct antigenic types which are divided into six groups (I to VI) based on the following properties:

- Ability to agglutinate RBCs from either monkeys or rats
- Oncogenic potential in animals (rats) or cell lines (they are non-malignant to humans)
- Guanine-plus-cytosine (G+C) content of DNA.

Group I adenoviruses include serotypes 12, 18 and 31. They have maximum oncogenic potential, but are lowest in G+C content. It is also the only group that does not agglutinate monkey or rat RBCs.

ADENOVIRUS

Morphology

Adenoviruses have the following properties:

- They are non-enveloped, 70-90 nm in size, possess 252 capsomeres
- Icosahedral symmetry with fiber proteins projecting from each vertex (unique property): This gives a typical **space vehicle shaped** appearance (Fig. 43.3)
- They contain a linear dsDNA.

Pathogenesis and Clinical Manifestations

Adenoviruses infect and replicate in the epithelial cells of the respiratory tract, eye, gastrointestinal tract, urinary bladder and liver. Though one-third of the serotypes can cause human diseases, **types 1–7** are most common worldwide. Single serotype may cause different manifestations and conversely, more than one type may cause the same clinical illness.

- **Respiratory diseases**
  - Upper respiratory tract infection in children—mainly caused by serotypes 1, 2, 3 and 5
  - **Pneumonia:** Adenoviruses particularly types 3, 7, and 21 are responsible for about 10-20% of pneumonia in childhood
  - Acute respiratory disease syndrome outbreaks among military recruit—are commonly associated with type 4, 7 and occasionally type 3.
- **Ocular infections:**
  - **Pharyngoconjunctival fever:** It tends to occur in outbreaks, at children's summer camps (also called swimming pool conjunctivitis), and is associated with types 3 and 7

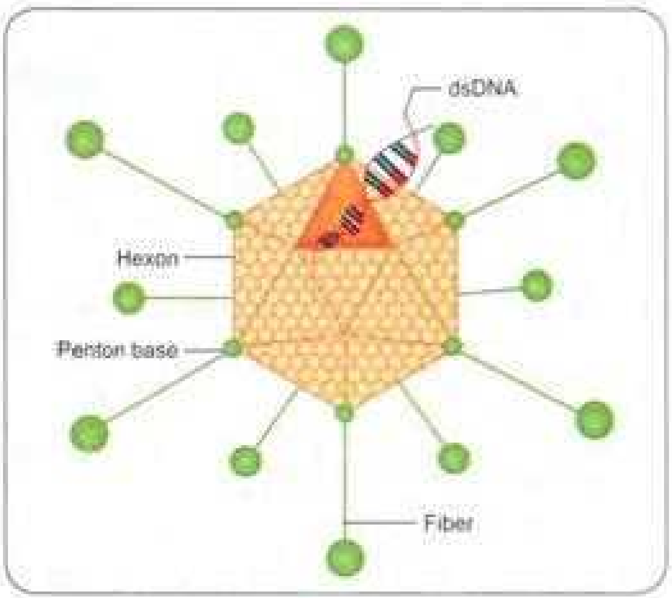


Fig. 43.3: Adenovirus (schematic diagram)

  - **Epidemic keratoconjunctivitis** or **shipyard eye:** It occurs mainly in adults and is highly contagious, caused by types 8, 19 and 37.
- **Infantile gastroenteritis:** Serotype 40 and 41 may account for 5–15% of cases of viral gastroenteritis in young children
- **Acute hemorrhagic cystitis** in children, especially in boys caused by serotypes 11 and 21
- **Immunocompromized** patients are at higher risk of developing serious pneumonia
- **Transplant recipients** may develop pneumonia, hepatitis, nephritis, colitis, encephalitis and hemorrhagic cystitis. Types 34 and 35 are isolated commonly from transplant recipients.

Laboratory Diagnosis

- **Specimen collection:** Depending on the manifestations, various specimens such as throat swab, conjunctival swab, stool or urine may be collected
- **Virus isolation:** Primary human embryonic kidney cell line and A 549 cell line are the most susceptible cell lines. Others such as HEp-2, HeLa, and KB cell lines can also be used
  - ***Viral growth*** can be detected by:
    - Characteristic cytopathic effect: Rounding and grape-like clustering of swollen cells
    - Antigen detection by direct-IF test.
  - ***Reporting:*** Reporting should be done cautiously when adenovirus is recovered from throat or stool as they can persist for long duration in the gut and in adenoids and shed intermittently in the setting of other infections
  - ***Shell vial technique*** can be performed prior to cell culture to enhance viral replication so that detection time can be reduced
  - ***Explant culture:*** Adenoviruses (especially group-C) can grow on adenoid explants, however, it is no longer in use now.
- **Serotyping:** Type specific antigens (viral capsid proteins) can be identified by hemagglutination test (targeting HA antigens) and neutralization test (targeting capsid proteins)
- **Direct-IF test:** It can be employed to detect adenoviral antigens from clinical samples such as throat or conjunctival secretions by using fluorescent tagged anti-hexon antibody
- **Fastidious enteric serotypes** such as type 40 and 41 from stool: They can be detected by electron microscopy or by antigen detection by ELISA
- **Molecular methods:** PCR has been available targeting group-specific conserved hexon or fiber genes. Multiplex PCR followed by sequencing is done for detection of adenovirus types. PCR is rapid and more sensitive than

conventional culture. Real-time PCR is used to monitor viral load, which is useful for immunocompromised and transplant recipients
- **Serum antibody detection:** It can be done by various tests such as CFT, neutralization test, ELISA, or rarely hemagglutination inhibition test (HAI) for few hemagglutinating serotypes.

Treatment and Control

There is no specific antiviral drug available. Only symptomatic treatment is given.

General Preventive Measures

- Effective hand washing: Use of paper towels is better than cloth towels for hand drying
- Sodium hypochlorite to disinfect environmental surfaces
- Chlorination of swimming pools and waste water should be followed to prevent waterborne conjunctivitis or gastroenteritis
- Strict asepsis during eye examinations.

Live Adenovirus Vaccine

Live adenovirus vaccine containing types 4 and 7 has been used in military recruits.
- It was available as gelatin coated capsules and given orally
- It was highly effective, but not in use since 1999 due to manufacturer issues. There are plans to develop this vaccine again.

Adenoviruses used for Gene Therapy

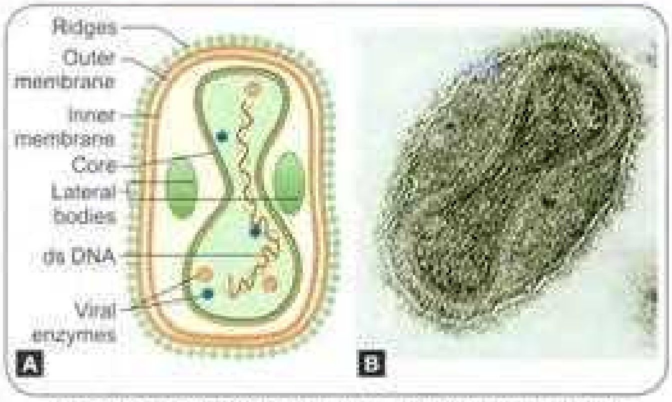
Replication defective adenoviruses can also be used as live-virus vectors for the delivery of vaccine antigens and for gene therapy; e.g. trials on adenovirus vectored *M. tuberculosis* (using 85A antigen) and HIV vaccines.

POXVIRIDAE

POXVIRUS

Morphology

Poxviruses are the largest (400 nm in length × 230 nm in diameter) among all the viruses, large enough to be seen under light microscope.
- Most complex viruses; their structure does not fit into either icosahedral or helical symmetry
- Brick-shaped or ellipsoid
- **Envelope:** Externally, there is an envelope, made up of two lipoprotein membranes (outer and inner) with ridges arising from the outer membrane. The envelope encloses a core and two structures of unknown function called lateral bodies (Figs 43.4A and B)



Figs 43.4A and B: A. Smallpox virus (schematic diagram); **B.** Smallpox virus (electron micrograph)

Source: B. ID# 1849 Dr Fred Murphy, Sylvia Whitfield/Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

- Core or the nucleocapsid is biconcave dumbbell shaped, surrounded by a corewall
- Capsid is 12 nm thick, made up of more than 1000 capsomers and encloses single linear dsDNA and many enzymes including transcriptases
- It is the only DNA virus that replicates in the cytoplasm.

Classification

Poxviruses are grouped into eight genera (Table 43.4). Species that cause human infections fall under four genera. Important members include:
- **Variola:** It was the causative agent of **smallpox**, the first disease to be eradicated from the world
- **Vaccinia:** It was used before as a vaccine for smallpox
- **Molluscum contagiosum virus:** It causes warty lesions called as molluscum contagiosum.

SMALLPOX VIRUS (VARIOLA)

Smallpox was the first infectious disease to be eradicated from the world. It was characterized by highly contagious severe exanthema (rashes). The agents of smallpox were variola major and variola minor.

Smallpox Time Line

- Last natural case of variola major was seen in a Bangladeshi woman in Assam in May 1975
- Last natural case of variola minor was seen in Merca, Somalia, 26th October 1977
- **Eradication** was declared by WHO nearly after three years of the last case, i.e. on 8th May 1980
- **Laboratory spread:** There was a small outbreak in Birmingham (1978), due to accidental spread of the virus from the virus laboratory, following which stocks from most laboratories have been destroyed

Table 43.4: Poxviruses and associated diseases in humans

Genus	Species	Host reservoir	Distribution	Human disease
Orthopoxvirus	Variola	Humans	Eradicated	Smallpox (eradicated)
	Vaccinia	Humans	–	Localized skin lesions; Used for smallpox vaccination
	Monkeypox	Rodents and monkeys	Africa	Rare; smallpox-like lesions, Systemic disease
	Cowpox	Cows	Europe	Rare; localized skin lesions, occasionally systemic
	Buffalopox	Water buffalo	Indian subcontinent	Rare; localized skin lesions
	Cantagalo and Araçatuba	Cattle	South America	Rare; localized skin lesions
Parapoxvirus	Orf	Sheep and goats	Worldwide	Rare; localized skin lesions called contagious pustular dermatitis
	Pseudocowpox (Paravaccinia)	Cattle	Worldwide	Rare; localized lesions called Milker's nodule
	Bovine papular stomatitis	Cattle	Worldwide	Rare; localized skin lesions
	Deerpox	Deer	Deer herds	Local pox like lesions
	Sealpox	Seals	Seal colonies	Local pox like lesions
Molluscipoxvirus	Molluscum contagiosum	Humans	Worldwide	Many benign skin nodules
Yatapoxvirus	Tanapox	Monkeys	Africa	Rare; localized skin lesion
	Yabapox	Monkeys	Unkown	Very rare and accidental; Localized skin tumors

- **Maintenance:** Currently, only two laboratories hold stocks of smallpox virus
  - CDC (Centers for Disease Control and Prevention) Atlanta (USA)
  - Center for Research on Virology and Biotechnology, Koltsova (Russia).
- **Agent of bioterrorism:** As vaccination was stopped following eradication, people borne after 1980 are not immunized. Hence, smallpox virus can be a potential agent of bioterrorism.

Reasons that Made Eradication Successful

- Variola was an exclusively human pathogen, no animal reservoir
- Source: Patients were the only source, there were no carriers
- Case detection was easy due to characteristic appearance of rashes (Table 43.5)
- Subclinical cases were not transmitting the disease
- Global smallpox eradication program was launched in 1967 by WHO (World Health Organization). With a strong international cooperation and intense effort; disease was wiped out nearly after 10 years
- Highly effective live vaccinia vaccine
  - Freeze dried form was used (↑stability)
  - Multiple puncture technique was followed to administer the vaccine by using a bifurcated needle, which was found to be simple, effective and economical.

Brief Description of Manifestations

- Portal of entry of the virus was via the mucous membranes of the upper respiratory tract

Table 43.5: Differences between smallpox and chickenpox

Smallpox	Chickenpox
Incubation period: 12 days (7–17 days)	**Incubation period:** 15 days (10–21 days)
Rash: Palm, sole and extensor surface	**Rash:** Axilla and flexor surface
Rash: Deep seated and appear in single stage, evolution is slow, centrifugal distribution	**Rash:** Superficial and pleomorphic (appear in crops) evolution is rapid, dew drop rashes, centripetal distribution
Fever subsides with appearance of rash	**Fever** rises with each crop of rash

- **Incubation period:** 12 days (7-17 days)
- **Description of Rashes:** Smallpox rashes were unique in appearance and could easily be differentiated from that of chickenpox (Table 43.5)
  - Rashes were deep seated and all rashes in an area appeared in one stage, evolution was slow (Fig. 43.5)
  - Centrifugal distribution-palm and sole and extensor surface were affected first
  - Fever subsided with appearance of rash.

Laboratory Diagnosis

- **Direct detection in scrapings from rashes:**
  - Intracytoplasmic inclusion bodies (Paschen bodies)
  - Electron microscopy: Brick-shaped appearance with biconcave DNA core.

Fig. 43.5: Smallpox rashes over the face

Source: Public Health Image Library, ID# 3/Centers for Disease Control and Prevention (CDC), Atlanta/Cheryl Tyron (*with permission*).

- **Egg inoculation:** Characteristic pock formation is seen on the chorioallantoic membrane (CAM) of a chick embryo (Fig. 43.6).

TREATMENT **Smallpox virus (variola)**

Cases used to be treated in the past with:
- Vaccinia Immunoglobulins
- Antiviral drugs such as methisazone and cidofovir.

Vaccination

- **Live vaccinia vaccine** was highly effective.
  - It was given as single dose between 1 and 2 years of age
  - As un-attenuated live virus was used, adverse reactions were common; such as mild vaccinia-induced rashes.

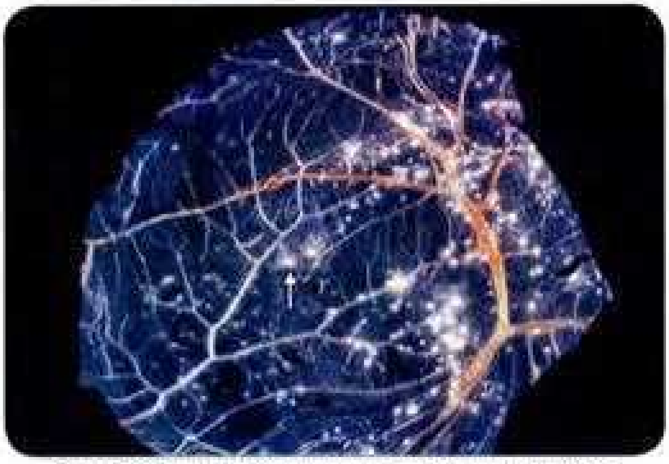


Fig. 43.6: Pocks of smallpox virus on the chorioallantoic membrane of a chick embryo

Source: Public Health Image Library, ID# 3274/Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*).

- **Cowpox vaccine** discovered by Edward Jenner (the father of vaccination) was in use before vaccinia vaccine was available
- **Variolation** was the first attempt of providing artificial immunity against smallpox. It was in use even before cowpox vaccine was available. Healthy people were inoculated with the skin scraping of a smallpox patient.

VACCINIA VIRUS

Vaccinia cross-reacts with variola and the antibodies produced against vaccinia are protective for variola. The antigenic cross reactivity was so much that vaccinia was able to eradicate variola globally.

However, vaccinia differs from variola in many ways as follows:

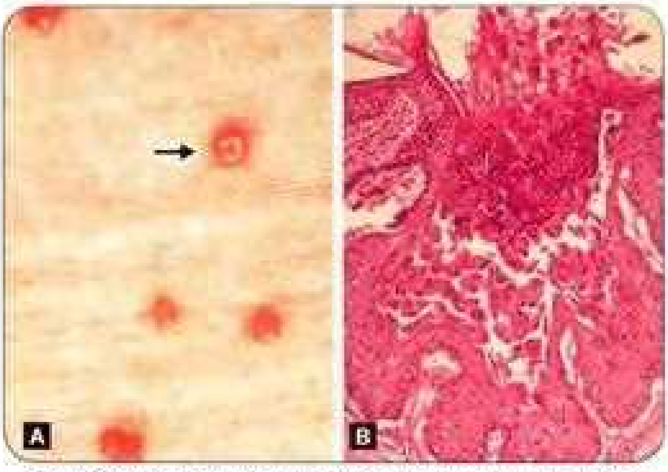
- It is non-pathogenic to humans or produces milder skin lesions
- Produces an inclusion body called Guarnieri body (variola produces Paschen body)
- On CAM, vaccinia virus produces larger and hemorrhagic and necrotic pock lesions than variola
- **Ceiling temperature:** It is the highest temperature beyond which the pock formation is inhibited on CAM. It is higher for vaccinia virus (41°C) than for variola virus (38°C)
- Vaccinia but not variola can produce plaques on chick embryo tissue cultures.

MOLLUSCUM CONTAGIOSUM VIRUS

Molluscum contagiosum virus is an obligate human poxvirus that produces characteristic skin lesions.

Clinical Manifestations

- **Lesions:** It produces pink pearly wart-like lesions (2–5 mm size), umbilicated, with a characteristic dimple at the center (Fig. 43.7A). Lesions are characterized by:
  - Lack of associated inflammation and necrosis
  - Found singly or in clusters
  - Distribution: Lesions are found anywhere on the body except on the palms and soles. Genital lesions are seen in adults.
- **Transmission:**
  - Children are commonly affected, acquire infection by direct and indirect contact (e.g. by barbers, common use of towels, swimming pools)
  - Rarely sexual transmission has been reported in young adults.
- **Self-limiting:** Lesions disappear in 3–4 months. There are no systemic complications, but sometimes; lesions may persist for 3–5 years
- **In HIV-infected patients:** Disease is more generalized, severe and persistent.



Figs 43.7A and B: A. Molluscum contagiosum lesions on skin; **B.** Histopathology of skin showing molluscum bodies

Source: A. CDC/ L Sperling, MD, Walter Reed Army Medical Center; B. Public Health Image Library, ID# 860/Centers for Disease Control and Prevention (CDC), Atlanta/Dr Edwin P Ewing, Jr (with permission).

Laboratory Diagnosis

- **Molluscum bodies** are the intracytoplasmic eosinophilic inclusions seen in skin scrapings stained with histopathological stains (Fig. 43.7B)
- Electron microscopy and PCR can be used for confirmation
- **Not cultivable:** It cannot be propagated in tissue culture, embryonated egg or in animals.

TREATMENT **Molluscum contagiosum**

Surgical removal of the lesions by ablation (by cryotherapy or laser therapy) is the mainstay of treatment. Cidofovir has shown to have some efficacy. As this virus does not cross-react with any other poxviruses, smallpox vaccine is not protective.

OTHER POXVIRUSES OF HUMAN IMPORTANCE

Monkeypox Virus

- **Reservoir:** Rodents are the primary reservoirs followed by monkeys
- **Distribution:** Cases typically occur in Africa
- **Transmission:** It is by direct contact with infected animals. Human-to-human propagation is rare
- **Manifestation:** It is characterized by a vesicular rash similar to those of smallpox but milder. Systemic illness like fever and lymphadenopathy may be seen
- The first outbreak occurred in USA, 2003 where more than 70 cases were reported. Transmission was linked to contact with pet prairie dogs who acquired the infection from rodents
- Smallpox vaccination can protect against monkeypox infection.

Orf Virus

This virus affects sheep and goats. Human infections are rare; characterized by localized skin lesions, called as contagious pustular dermatitis or mouth sore.

Pseudocowpox (Paravaccinia Virus)

This virus affects cattle, can rarely infect milk handlers to produce nodular skin lesions called **Milker's nodule.**

Cowpox and Buffalopox Virus

This virus can cause rare zoonotic infections in humans, characterized by pox-like lesions and mild systemic illness.

Tanapox Virus

This virus was named after its place of discovery (Tana river, Kenya). It causes nodular lesion on the exposed area after contact with infected monkeys.

BACTERIOPHAGE

Bacteriophages are the viruses that infect bacteria. It was first described by Twort and d'Herelle (1917).

MORPHOLOGY

Bacteriophages are typically tadpole-shaped possessing a hexagonal head and a tail attached with tail fibers (Fig. 43.8A).

- **Head** is hexagonal in shape, 28 nm to 100 nm in size and consists of a tightly packed nucleic acid core (containing a dsDNA) enclosed by a capsid protein coat
- **Tail** is composed of a hollow core surrounded by a contractile sheath ending in a base plate from which six tail fibers arise
- **Altered morphology may be seen in some phages:**
  - Shape-spherical or filamentous instead of hexagonal
  - Nucleic acid—may contain ssDNA or RNA instead of dsDNA.

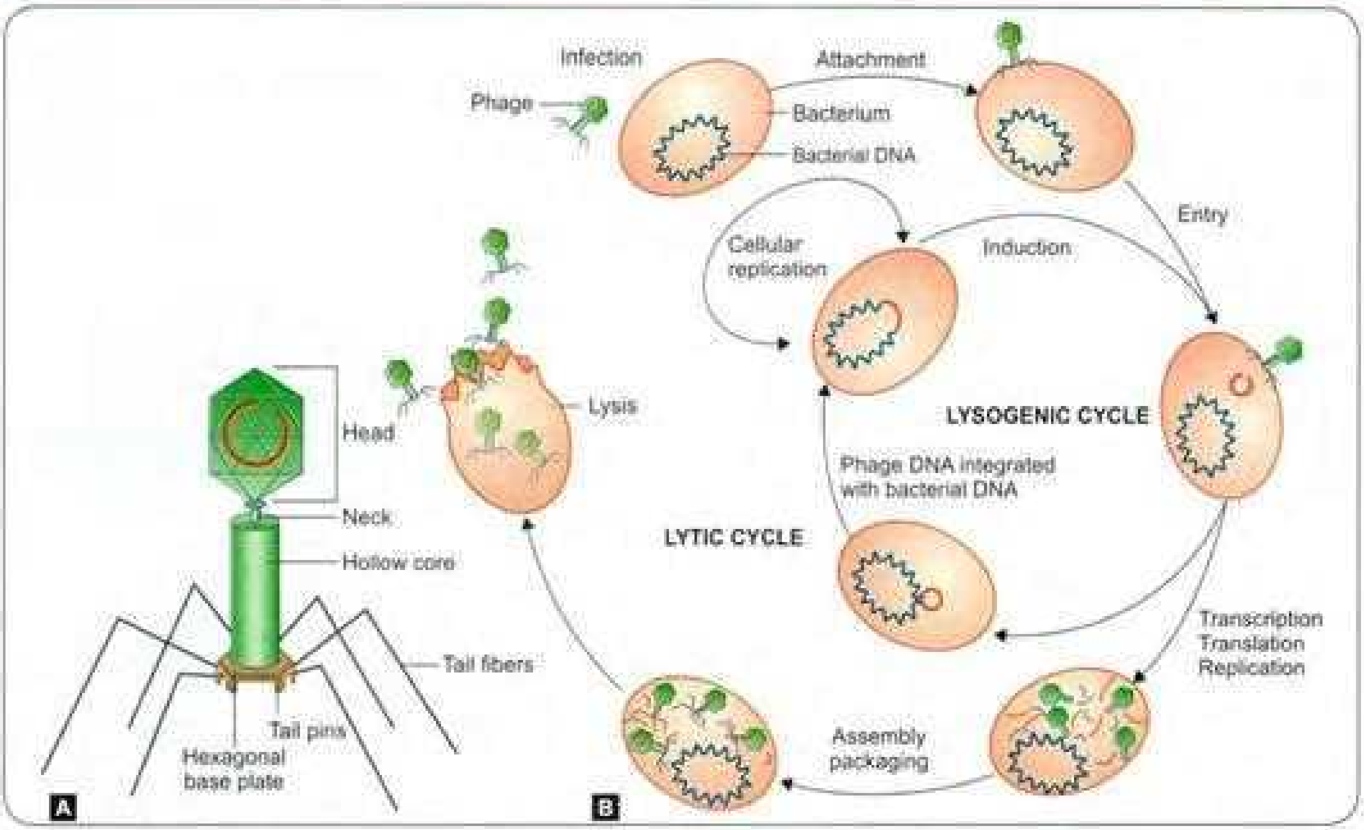
LIFE CYCLE

Bacteriophages exhibit two different types of life cycles—virulent or lytic cycle and temperate or lysogenic cycle.

Lytic Cycle (seen in Virulent Phages)

It resembles with the replication of other DNA viruses; consists of the following steps (Figs 43.8B).

- **Adsorption:** The phages come into contact with bacterial cells by random collision and attach to specific receptors on bacterial cell wall by means of tail fibers
  - Adsorption is an essential step to initiate the life cycle
  - Transfection: Experimentally, bacteria may be infected by direct injection of naked phages (nucleic acid without capsid).
- **Penetration:** Phage acts as piston or a syringe, injects the dsDNA through the hollow core which is helped by the contractile sheath present in tail region
  - Lysozyme present on tail tip makes a hole on the bacterial cell wall



Figs 43.8A and B: A. Morphology of bacteriophage; **B.** Life cycle of a bacteriophage (Lytic and lysogenic cycles)

- The empty phages remain outside, attached to bacterial cell wall as 'ghosts', hence there is no uncoating step needed, as seen with other viruses.

- **Biosynthesis:** Phage components such as dsDNA and capsid proteins are synthesized. Bacterial metabolism remains inhibited during the entire process
- **Maturation and assembly:** Phage DNA, head and tail proteins are assembled to form infective daughter virions
- **Release** of the daughter phages occurs by lysis of the bacterial cell which is mediated by phage enzymes
- **Duration of eclipse phase** is about 15–30 minutes. It is the interval between entry of phage DNA and the appearance of first infectious phage particle inside the host cell. During this period, phages are not detected.

Lysogenic Cycle (seen in Temperate Phages)

After the entry into bacteria, the temperate phage DNA gets integrated into the bacterial chromosome (Fig. 43.8B).

- The integrated phage genome is called the **prophage**
- The bacterium that carries a prophage is known as a **lysogenic bacterium**
- **Lysogeny:** The prophage behaves like a segment of the host chromosome and replicates synchronously with it. This phenomenon is called lysogeny
- **Lysogenic conversion:** During the integrated state, the phage DNA confers certain new properties to the bacteria (e.g. provides gene for toxin synthesis)
- **Super-infection immunity:** A lysogenic bacterium is resistant to reinfection by the same or related phages
- **Lysogenic to lytic interconversion:** Temperate phages remain integrated into the bacterial chromosome. But when they want to come out, they get excised from bacterial chromosome, then transform to lytic phages, multiply in the cytoplasm and are released by lysis.

Significance/Uses of Bacteriophages

Phage Typing

The virulent phages can be used for further classifying the bacteria beyond the species level.

- This helps in epidemiological investigations during outbreak to know the relatedness between the strains of the same species
- Phage typing is employed for typing the following bacteria
  - *Staphylococcus aureus*
  - Vi antigen typing of *Salmonella* Typhi
  - *Vibrio cholerae* (Basu Mukherjee phage typing)
  - *Brucella* (Tbilisi phage typing)
  - *Corynebacterium diphtheriae*.

Phage Assay

- When virulent phages are spread over a lawn culture of a susceptible bacterium, areas of clearing or lysis called **plaques** develop surrounding the growth of each phage

- A single phage is capable of producing one plaque. Plaque assay can be employed to estimate the number of viable phages in a preparation.

Used in Treatment (Phage Therapy)

- Lytic phages can kill the bacteria, hence may be used for treatment of bacterial infection
- Studies are under trial for using lytic phages to treat various bacterial infections such as post-burn infections and wound infections.

Used in Diagnosis

Mycobacteriophages are used for the identification of *Mycobacterium tuberculosis*.

Used as a Cloning Vector

Bacteriophages have been used as cloning vectors in recombinant DNA technology.

Transduction

Temperate phages can act as vehicles in transferring genes from one bacterium to another.

- This may be an important method to transfer drug resistant genes between bacteria
- Example: In *Staphylococcus aureus*, the plasmids coding for β lactamases are transferred between the strains by transduction.

Codes for Bacterial Toxins

The phage genomes code for the following bacterial toxins:

- Diphtheria toxin
- Cholera toxin
- Verocytotoxin of EHEC (Enterohemorrhagic *E. coli*)
- Botulinum toxin C and D
- Streptococcal pyrogenic exotoxin A and C.

Alter Antigenic Property of Bacteria

The temperate phages of some salmonellae can modify the antigenic properties of the somatic O antigen, e.g. *Salmonella* Anatum changes to *Salmonella* Newington after acquiring its bacteriophage which alters the antigenic property of O antigen.

EXPECTED QUESTIONS

I. Write short notes on:

1. Fifth disease.
3. Smallpox eradication.
3. Manifestations produced by Adenovirus.
4. HPV vaccine.
5. Uses of bacteriophages.

II. Multiple Choice Questions (MCQs):

1. All of the following are clinical manifestations of Parvovirus B19 infection, *except*:
   a. Erythema infectiosum
   b. Transient aplastic crisis
   c. Condyloma acuminata
   d. Hydrops fetalis

2. All of the following are clinical manifestations of HPV, *except*:
   a. Plantar and palmar warts
   b. Epidermodysplasia verruciformis
   c. Carcinoma of cervix
   d. Slapped cheek appearance

3. Which of the following adenovirus serotypes cause epidemic keratoconjunctivitis?
   a. Serotypes 3 and 7
   b. Serotypes 8, 19, and 37
   c. Serotypes 40 and 41
   d. Serotypes 11 and 21

4. All the following statements are true for the poxvirus, except:
   a. It is a large brick-shaped virus
   b. The genome consists of a large ds linear DNA
   c. Replication cycle of the virus occurs in the nucleus of the host cell
   d. It is large enough to be seen under light microscopy

5. Phage typing is employed for typing of following bacteria except:
   a. *Staphylococcus aureus*
   b. *Vibrio cholerae*
   c. *Streptococcus*
   d. *Brucella*

6. Which toxin is not coded by bacteriophage?
   a. Diphtheria toxin
   b. Cholera toxin
   c. Tetanospasmin
   d. Verocytotoxin

7. Molluscum contagiosum virus: All the statements are true, *except*?
   a. Produces pink pearly wart-like lesions
   b. Lesions are common in palm and sole
   c. Children are commonly affected
   d. Self-limiting: Lesions disappear in 3–4 months

8. Molluscum contagiosum virus: All the statements are true, *except*?
   a. Produces intracytoplasmic eosinophilic inclusions
   b. Not cultivable
   c. Acyclovir is effective
   d. Surgical removal is the only way of treatment

9. Smallpox rashes differ from chickenpox by?
   a. Superficial
   b. Pleomorphic
   c. Centrifugal distribution
   d. Fever rises with each crop of rash

Answers

1. c **2.** d **3.** b **4.** c **5.** c **6.** c **7.** b **8.** c **9.** c

Myxoviruses and Rubella Virus

44
CHAPTER

Chapter Preview

- Orthomyxoviridae
  - Influenza virus
- Paramyxoviridae
  - Parainfluenza viruses
  - Mumps virus
  - Measles virus
  - Nipah virus and hendra virus
  - Respiratory syncytial virus
  - Metapneumovirus
- Rubella virus

Myxoviruses are a group of viruses that bind to mucin receptors on the surface of RBCs (*myxo* in Greek meaning 'mucin'); resulting in clumping of RBCs together to cause hemagglutination.

CLASSIFICATION

Myxoviruses are divided into two families—(1) Orthomyxoviridae and (2) Paramyxoviridae. Both differ from each other in various aspects (Table 44.1); the most important difference is the presence of segmented RNA in Orthomyxoviridae family.

Table 44.1: Differences between Orthomyxoviridae and Paramyxoviridae

Properties	Orthomyxoviridae	Paramyxoviridae
Size	80–120 nm	100-300 nm
Shape	Spherical; Rarely filamentous	Pleomorphic
Nucleic acid	• Negative sense ssRNA, • Segmented; eight pieces	• Negative sense ssRNA • Non-segmented; single piece
Genetic recombination	Seen	Not seen
Antigenic variation	Seen	Not seen
Site for RNA Replication	Nucleus	Cytoplasm
Important human pathogens	Influenza virus	• Parainfluenza virus • Mumps virus • Measles virus • Respiratory syncytial virus • Metapneumovirus

Abbreviations: ss, single stranded; RNA, ribonucleic acid.

ORTHOMYXOVIRIDAE

Influenza viruses are the members of Orthomyxoviridae family. They are one of the major causes of morbidity and mortality and have been responsible for several epidemics and pandemics of respiratory diseases in the last two centuries.

INFLUENZA VIRUSES

Influenza viruses consist of three genera—influenza A, B, C and D.

Morphology (Fig. 44.1)

Influenza viruses are spherical in shape, measure about 80–120 nm in size.

- **Helical symmetry:** It comprises of a helical nucleocapsid (9 nm), surrounded by an envelope
- **Viral RNA** comprises of **multiple segments** of (-ve) sense single stranded RNA. Each segment codes for a specific viral protein (Table 44.2) having a specific function
  - Influenza A and B contain **eight segments** of RNA
  - Influenza C and D contain seven segments of RNA. The segment coding for neuraminidase is absent.
- **Site of replication:** RNA replication occurs typically in the nucleus (in contrast to most other RNA viruses which replicate in the cytoplasm)
- **Viral proteins:** Influenza virus contains eight structural proteins (PB1, PB2, PA, NP, HA, NA, M1 and M2) and two non-structural proteins (NS1 and NS2) (Table 44.2)
  - **PB1, PB2, and PA** are the polymerase proteins responsible for RNA transcription and replication
  - **Nucleoprotein (NP)** is the major capsid protein, associated with viral RNA to form a ribonucleoprotein (RNP) or nucleocapsid with a helical symmetry

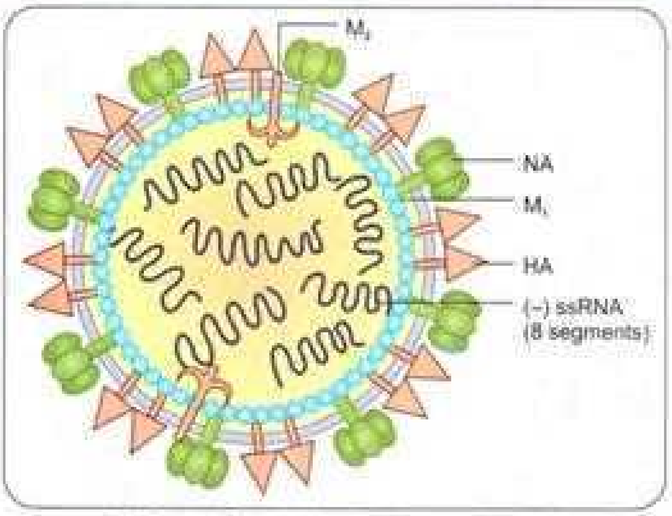


Fig 44.1: Influenza virus (schematic diagram)

Table 44.2: Influenza virus RNA segments and coded proteins

RNA segments	Coded protein(s)	Function of proteins
Segment-1	PB2	RNA transcription and replication
Segment-2	PB1	
Segment-3	PA	
Segment-4	HA (hemagglutinin)	Binds to receptors of RBC to cause hemagglutination
Segment-5	NP (nucleoprotein)	Associates with RNA to form helical nucleocapsid
Segment-6	NA (neuraminidase)	Replaces HA from RBCs to cause elution (reversal of hemagglutination)
Segment-7	M1 and M2	M1-forms a shell underneath the envelope M2-forms ion channels
Segment-8	NS1 and NS2	NS1-is interferon antagonist and inhibits pre-mRNA splicing NS2-is nuclear export factor

- **Matrix proteins: M1 protein** is the major viral protein (40% of total protein). It forms a shell (protein layer) underneath the envelope. **M2 proteins** form ion channels in the envelope, help in transport of molecules
- **Non-structural proteins: NS1** is an interferon antagonist and inhibits pre-mRNA splicing. **NS2** helps in export of molecules across the nucleus
- **Hemagglutinin (HA) and Neuraminidase (NA)** are the glycoproteins inserted into the lipid envelope.

❖ **Envelope:** Envelope is lipoprotein in nature. Lipid part is derived from the host cell membrane. Proteins or the peplomers are virus coded, 10 nm long glycoproteins that are inserted into the lipid envelope. Two peplomers are present:

1. **Hemagglutinin (HA):** It is triangular-shaped peplomer, binds to mucin or sialic acid receptors on RBCs, resulting in clumping of RBCs to cause **hemagglutination.** It also binds to the same receptors on the respiratory epithelial cells, thus facilitating viral entry.
2. **Neuraminidase (NA):** It is mushroom-shaped peplomer, present in fewer number than HA. It is a sialidase enzyme that degrades the sialic acid receptors on RBCs. It acts as receptor destroying enzyme; thus helps in:
   - It displaces HA from RBCs resulting in reversal of hemagglutination called **elution.**
   - It facilitates release of virus particles from infected cell surfaces during budding process by preventing self-aggregation of virions to the host cells.
   - NA helps the virus to pass through the mucin layer in the respiratory tract to reach the target epithelial cells.

Antigenic Subtypes and Nomenclature

Based on RNP and M proteins, influenza viruses are divided into four genera: A, B, C and D.

❖ **Subtypes:** Based on HA and NA antigens,
- Influenza A has distinct 18 H subtypes (H1 to H18) and 11 N subtypes (N1-N11)
  - Most of the subtypes infect animals and birds, but occasionally undergo genetic changes and infect humans to cause major epidemics and pandemics
  - For example, Six HA (H1, H2, H3, H5, H7 and H9) and two NA (N1 and N2) subtypes have been recovered from humans.
- Influenza B and C viruses though have subtypes; but are not designated.
- Influenza D virus primarily infects cattle and are not pathogenic to humans.

❖ **The standard nomenclature system for influenza virus:** Any influenza virus isolates should be designated based on the following information: Influenza virus type/host (indicated only for non-human origin)/geographical origin/strain number/year of isolation/(HA NA subtype). For examples:
- **Human strain:** Influenza A/Hong Kong/03/1968 (H3N2)
- **Non-human strain:** Influenza A/swine/Iowa/15/ 1930 (H1N1).

Antigenic Variation

Antigenic variation is the unique property of influenza viruses, which is due to the result of antigenic changes occurring in HA and NA peplomers. It is of two types:

1. Antigenic Drift

It is a minor change occurring due to **point mutations** in the HA/NA gene, resulting in alteration of amino acid sequence of the antigenic sites on HA/NA, such that virus can partially escape recognition by the host's immune system. The new variant must sustain two or more mutations to become epidemiologically significant.

- Seen in both influenza virus type—A and B
- Results in outbreaks and minor periodic epidemics
- Antigenic drift occurs more frequently, every 2–3 years.

2. Antigenic Shift

It is an abrupt, major drastic, discontinuous variation in the sequence of a viral surface protein (HA/NA), that occurs due to **genetic reassortment** between genomes of two or more influenza viruses infecting the same host cells; resulting in a new virus strain, unrelated antigenically to the predecessor strains. Thus, antibodies developed against previous strain (due to infection or vaccination) become ineffective.

- Occurs only in influenza A virus
- Results in pandemics and major epidemics, e.g. H1N1 pandemics of 2009
- Antigenic shift occurs less frequently every 10–20 years.

Pathogenesis

- **Transmission:** It is via infected aerosols generated by coughs and sneezes, rarely via contacts, or by fomites. Small-particle aerosols (<10 μm) are more efficient in the transmission
- **Target cell entry:** Viral HA attaches to specific sialic acid receptors on the host cell surface that leads to viral entry. Ciliated columnar epithelial cells are most commonly infected, but it may also infect other cells including alveolar cells, mucous gland cells, and alveolar macrophages
- **Multiply locally:** Virus replicates in the infected cells and infectious daughter virions spread to the adjacent cells to involve large number of respiratory epithelial cells over several hours
- **Spread:** Very rarely, virus spreads to the lower respiratory tract or spills over blood stream to involve extra-pulmonary sites
- **Local damage:** Influenza virus infection causes cellular destruction and desquamation of superficial mucosa of the respiratory tract
    - Edema and mononuclear cell infiltrations occur at local site leading to cytokine influx, which accounts for local symptoms
    - Local damage predisposes to secondary bacterial invasion.

Host Immune Response

Humoral immunity: It is the predominant immunity that provides resistance against influenza infections. Immunity developed is both type and subtype-specific and long lasting.

- Antibodies against HA and NA are protective in nature, and are subtype specific
- Antibodies to HA prevent initiation of infection by inhibiting viral entry; whereas antibodies to NA decrease the severity of the disease and prevent the transmission of virus to contacts
- Antibodies against other viral proteins are not protective
- Antibodies against the ribonucleoprotein are type-specific and are useful in typing viral isolates as influenza A or B or C
- All the three types of influenza viruses (i.e. A, B and C) are antigenically unrelated and there is no cross-protection
- Immunity may be incomplete, as reinfection with the same virus can occur
- **Original antigenic sin:** When a previously infected individual gets a repeated infection with a different antigenic variant of influenza virus, antibodies are produced against both the subtypes, but predominant response would be against the original strain, a phenomenon called "original antigenic sin."

Components of both cell mediated immunity (e.g. cytotoxic T cells) and innate immunity (NK cells, interferons) are also important in providing immunity against influenza infections.

Clinical Manifestations

Incubation Period

It is about 18–72 hours, which directly depends upon the inoculum size and the immune status of the host.

Uncomplicated Influenza (Flu Syndrome)

Majority of individuals are either asymptomatic or develop minor upper respiratory symptoms such as chills, headache, and dry cough, followed by high-grade fever, myalgia and anorexia. It is a self-limiting condition, indistinguishable from the infections caused by other upper respiratory tract pathogens.

Complications

- **Pneumonia:** Secondary bacterial pneumonia is the most common complication to occur in patients infected with influenza virus. Common agents are staphylococci, pneumococci and *Haemophilus influenzae*. Primary influenza pneumonia is rare but leads to more severe complication
- **Other pulmonary complications** include worsening of chronic obstructive pulmonary disease, exacerbation of chronic bronchitis and asthma
- **Reye's syndrome:** It is fatty degeneration of liver with acute encephalopathy occurring in children and adolescents (2 to 16 years of age) following aspirin or salicylate intake. Though the cause is unknown, this

condition is often seen following influenza B, varicella-zoster and rarely influenza A viral infections. The mortality rate is high (10–40%).

Epidemiology

Influenza outbreaks occur worldwide almost every year, however they differ widely in severity and the extent of spread.

- **Incidence:** It is estimated that annually about 3-5 million cases of severe illness and 2.5-5 lakhs of deaths occur due to influenza epidemics worldwide and is associated with significant economic impact
- **Seasonality:** Influenza outbreaks are common during winters. The most common seasonal flu strain varies from season to season and from place to place (e.g. H3N2 in Pondicherry in 2018)
- **Epidemiological pattern:** It depends upon the nature of antigenic variation that occurs in the influenza types (as described earlier).

History of Influenza Outbreaks

Till now several influenza pandemics and major epidemics have occurred worldwide (Table 44.3).

- **Seroarchaeology:** The outbreaks that occurred prior to influenza isolation (influenza isolated first in 1933 using ferrets) were detected later by retrospective serologic survey of individuals alive during those years
- **The severe most pandemic** (Spanish flu) recorded so far was the swine flu strain H1N1 in 1918-1919, where >50 million people died, mostly due to secondary bacterial pneumonia. This strain was not a reassortant, but believed to be derived entirely from an avian strain that had adapted to human conditions and pigs acted as a mixing vessel
- This was followed by series of several epidemics and pandemics as mentioned in Table 44.3.

Table 44.3: Major influenza outbreaks

Years	Subtype	Extent of outbreak
1889–1890	H2N8	Severe pandemic
1900–1903	H3N8	Moderate epidemic
1918–1919	H1N1[a] (HswN1) (Spanish flu)	Severe pandemic
1933–1935	H1N1[a] (H0N1)	Mild epidemic
1946–1947	H1N1	Mild epidemic
1957–1958	H2N2 (Asian flu)	Severe pandemic
1968–1969	H3N2 (Hong Kong flu)	Moderate pandemic
1977–1978[b]	H1N1 (Russian flu)	Mild pandemic
2009–2010	H1N1 pdm09	Pandemic

[a]Hemagglutinins formerly designated as Hsw and H0 are now classified as variants of H1.
[b]From this time until 2008–2009, viruses of the H1N1 and H3N2 subtypes circulated either in alternating years or concurrently.

Sialic Acid Receptors

Sialic acid receptors found on the host cell surfaces are specific for HA antigens of influenza virus, which in turn determines the different host specificities of influenza virus.

- **α 2-6 sialic acid receptors** are specific for human influenza strains and are found abundantly on human upper respiratory tract epithelium, but not on lower respiratory tract. This explains why most human flu strains cause mild upper respiratory tract infections but not pneumonia
- **α 2-3 sialic acid receptors** are specific for avian influenza strains and are found abundantly on bird's intestinal epithelium
  - In humans, they are present in very few numbers on upper respiratory tract, and also on some epithelial cells in the lower tract
  - This explains why avian flu strains cannot easily infect humans and need close contact. However, once infected, they can infect lower respiratory tract and cause pneumonia.

Why pigs are the most common mixing vessels?

- Both α 2-3 and α 2-6 sialic acid receptors are found on the same respiratory epithelial cells of pigs and swine flu strains have specificity for both the receptor types
- Hence pigs can be infected simultaneously by human, swine and avian strains, thus serving as a mixing vessel
- Reassortment between the segments of various strains can take place inside the same swine cell.

Avian Flu

Birds are the primary reservoir for influenza viruses.

- All influenza subtypes (18H types and 11N types) are found in birds and some of the subtypes can be transmitted to mammals (e.g. H1, H2, H3, H5, H7 and H9 to humans; H1 and H3 to swine; and H3 and H7 to horses)
- Usually the avian flu strains are highly virulent as they possess **PB1F2** protein, which targets host mitochondria and induces apoptosis.

Avian Flu Infection in Birds

- Bird flu strains are highly lethal to chickens and turkeys (but avirulent to ducks) and are the major cause of economic loss in poultry causing severe mortality in chickens
- Unlike in mammals, avian flu multiplies in intestinal tracts of birds and shed through feces into water (avian flu is a water-borne disease in birds)
- The influenza viruses do not undergo antigenic variation in birds, because of the short life span of birds.

Avian Flu Infection in Humans

It is believed that, to date, all human pandemic strains have originated by reassortment between avian and human influenza viruses and the mixing has occurred in pigs.

A/H5N1 is the most common avian flu strain that has been endemic in the world for the past 15 years.

- **Origin:** It was first reported from Hong Kong in 1997 and has spread to various countries including India within few years
- **Transmission** to man occurs only from birds, and requires close respiratory contact
- **Less morbidity:** As there is no human-human transmission, morbidity is less. Only 500 cases were reported between 1977 to 2010 from Asia and Middle East
- **More mortality:** The avian flu strains are highly virulent (due to presence of PB1F2 protein) and mortality rate is >60%
- **Clinical feature:** H5N1 avian flu strains are associated with higher rates of pneumonia (>50%) and extrapulmonary manifestations such as diarrhea and CNS involvement.

Other avian flu strains infecting humans are:

- A/H7N7(Netherlands)
- A/H9N2 (Hong Kong)
- A/H7N9 (caused an outbreak in China, 2013).

Laboratory Diagnosis

Avian flu strains can be identified by real time reverse transcriptase PCR detecting specific HA and NA genes.

Influenza A (H1N1) pdm09

It has caused the most recent pandemic of influenza, emerged in California in March 2009 and rapidly spread to the entire world including India over the next few months. WHO declared the pandemics on 11th June 2009.

Epidemiology

- **Origin:** H1N1 2009 flu originated by genetic reassortment of four strains (1 human strain + 2 swine strains + 1 avian strain) and the mixing had occurred in pigs (Fig. 44.2)
- Though people commonly use the word 'swine flu' to describe H1N1 2009 flu, but this is not the correct terminology as it is a **reassortant of four strains**
- **Transmission:** It can be transmitted from human to human, which has accounted for its rapid spread

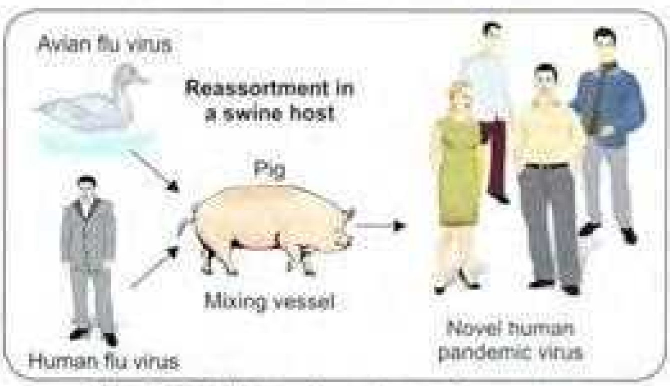


Fig. 44.2: Evolution of pandemic influenza virus

- However, it is less virulent (as it lacks the *PB1 F2* protein)
- Therefore in contrast to H5N1, the H1N1 2009 flu has caused more morbidity but less mortality
- Currently, **World** is in the post-pandemic period except in India and New Zealand where still local intense transmission is on-going.

H1N1 in India

Seasonal flu

After the pandemic in 2009, A/H1N1 has become a seasonal influenza strain circulating in India along with the other two seasonal strains (A/H3N2 and type B). Most cases in a year occur during the winter season (Dec-Jan).

Epidemiological Surveillance for Influenza

Integrated Disease Surveillance Program (IDSP) under NCDC (National center for disease control), Government of India has established a network (of 12 regional centers) for epidemiological surveillance for H1N1 and other influenza like illness (ILI). It also monitors the changes in the circulating influenza strain (if any).

Government of India (NCDC) report for H1N1, 2018

Between 2010–2017, about 1,15,630 cases and 8,681 deaths due to H1N1 were reported from India. The number of cases varied with time and geographical regions.

- **Varied with geographical regions:**
  - Maharashtra was the worst-hit state with 23,958 cases and 2,710 deaths, followed by Gujarat and Rajasthan
  - Sikkim and Lakshadweep are the only two states/UTs with no cases over the past seven years:
- **Varied with year:** Year 2015 (maximum), 2017 and 2010 recorded highest number of cases
  - The first peak occurred in 2010 (20,604 cases with 1,763 deaths)
  - Second peak was observed in 2015 (threatening outbreak, 42,592 cases with 2,990 deaths). Mortality rate was higher during this season than during pandemics of 2009
  - The third peak occurred in 2017 (38,811 cases with 2,266 deaths). Gujarat has the highest number of cases followed by Maharashtra; however, mortality was higher in Maharashtra.

Clinical Features

- **Uncomplicated influenza:** Most of the cases present with mild upper respiratory tract illness and diarrhea
- **Complicated/severe influenza** can occur very rarely in high-risk groups, is characterized by features such as secondary bacterial pneumonia, dehydration, CNS involvement, and multiorgan failure.

Categorization of Seasonal Influenza A/H1N1

Ministry of Health and Family Welfare, Government of India has published the guideline on categorization of seasonal influenza A/H1N1 cases (Table 44.4). While screening the patients with influenza like illness, this guideline helps in taking decision on performing laboratory test, initiating

Table 44.4: Guideline on categorization of seasonal influenza A/H1N1 cases during screening for home isolation, testing, treatment and hospitalization (issued by Ministry of Health and Family Welfare, Government of India)

Category	Definition	Guideline for laboratory testing for H1N1*, treatment** and isolation
Category A	Mild fever plus cough/sore throat with or without bodyache, headache, diarrhea and vomiting	*Laboratory testing for H1N1:* not required *Treatment:* only symptomatic, antiviral drugs not required. *Isolation:* Confine patients at home, avoid contact with public and high risk members in the family
Category B	Category A plus any one: i. High-grade fever and severe sore throat or ii. Presence of any of the risk factors: Children, age >65 years, pregnant women, patients with lung/ heart/liver/kidney/ neurological disorders, diabetes, cancer, HIV or on long term steroid therapy	*Laboratory testing for H1N1:* not required *Treatment:* Symptomatic treatment required. Antiviral drug (oseltamivir) may be required *Isolation:* Confine patients at home, avoid contact with public and high risk members in the family
Category C	Category B plus any one: i. Breathlessness, chest pain, fall in blood pressure, sputum mixed with blood, bluish discoloration of nails; ii. Children with influenza like illness who had a severe disease as manifested by the red flag signs (inability to feed well, convulsions, difficulty in breathing, etc.) iii. Worsening of underlying chronic conditions	*Laboratory testing for H1N1:* required Immediate hospitalization: required *Treatment:* start antiviral drug (oseltamivir) immediately without waiting for laboratory result *Isolation:* all components of droplet precaution to be followed. (refer 'prevention of influenza' section)

*Real time reverse transcriptase PCR is recommended to detect and quantify the specific HA and NA genes of H1N1.
** Oseltamivir (Tamiflu) tablet or Zanamivir (inhalational form).

antiviral treatment and putting the patient on home isolation or hospitalization.

GISRS: Influenza surveillance has been conducted globally through Global Influenza Surveillance and Response System (GISRS) under World health organization (WHO). It monitors the evolution of influenza viruses globally and serves as a global alert mechanism for the emergence of pandemic influenza viruses.

LABORATORY DIAGNOSIS — Influenza virus

- **Specimen:** Nasopharyngeal swab, kept at 4°C
- **Isolation of virus:**
  - Inoculation in embryonated eggs and primary monkey kidney cell lines
  - Growth is detected by hemadsorption, hemagglutination test.
- **Viral antigens** detection by direct IF test
- **Molecular methods:** Simultaneously detects seasonal flu strains such as A/H1N1, A/H3N2, Influenza B
  - RT PCR: detects viral RNA
  - Real time-RT PCR: quantifies viral RNA.
- **Antibody detection** by hemagglutination inhibition test, neutralization test and ELISA.

Laboratory Diagnosis

Specimen Collection

- **Ideal specimens** are nasopharyngeal swab or lavage fluid, nasal aspirate or to less extent throat swab
- **Swabs** with a synthetic tip (e.g. polyester or Dacron swabs) are best for specimen collection (Fig. 44.3). Cotton or alginate swabs are unsatisfactory

Fig. 44.3: Viral transport medium and swab

Source: Department of Microbiology, JIPMER, Puducherry (with permission).

- **Transport:** Swabs are immediately put inside the viral transport media, kept at 4°C during transport up to 4 days, thereafter at -70°C.

Isolation of Virus

Embryonated eggs (amniotic cavity) and primary monkey kidney cell lines have been the methods of choice for the isolation of influenza viruses in the past. The viral growth in cell line was detected by hemadsorption or hemagglutination test. Because of technical difficulty, isolation is not routinely followed for diagnostics.

Direct Immunofluorescence Test

Viral antigens coated onto epithelial cells can be directly detected in nasal aspirates by using fluorescent tagged antibodies. This is rapid, but less sensitive than viral isolation.

Molecular Methods

Molecular methods have revolutionized the diagnosis of influenza.

- **RT-PCR** (reverse transcriptase polymerase chain reaction): It is highly sensitive, specific and rapid (turnaround time of <1 day). It can also detect the specific type and subtype of influenza virus.

- **Real-time RT-PCR:** It is currently the gold standard method for influenza diagnosis and has been recommended by Government of India. It is quantitative, higher sensitivity and specificity than RT-PCR with turnaround time of 2–3 hours. It simultaneously detects the three common seasonal flu strains (A/H1N1, A/H3N2 and type B). The result is expressed as the emission of fluorescence during the cycles as described in Table 44.5 and Figure 44.4.

Antibody Detection (Serology)

Various assays are available to detect subtype specific serum antibodies by using specific influenza antigens. Four-fold rise in the antibody titer between acute and convalescent sera is more significant than a single high titer, as antibodies may be present in normal individuals. The tests available are:

- **HAI test** (Hemagglutination inhibition): It was used previously (now obsolete) to detect antibodies in serum against subtype specific HA antigen. Refer to author's first edition for detail
- **Neutralization test:** Though it is the most specific and best predictor of susceptibility to infection, is time-consuming and difficult to perform

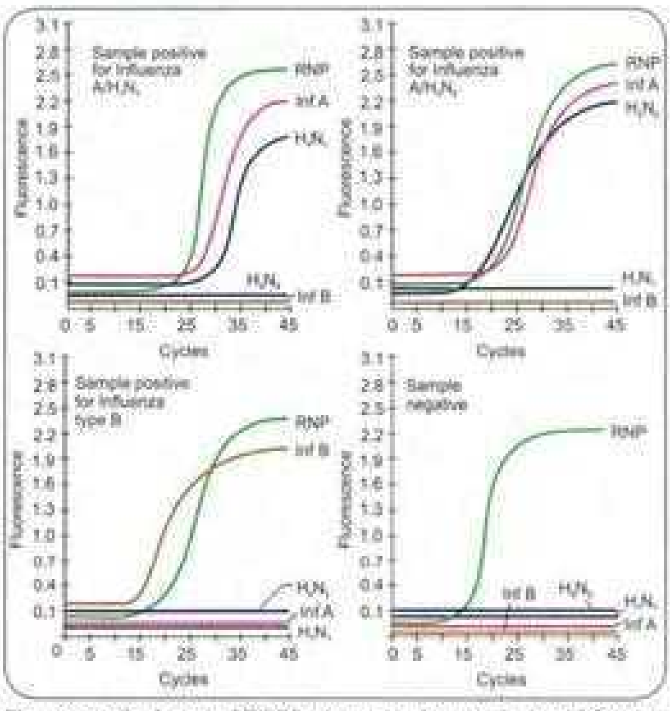


Fig. 44.4: Real time RT-PCR showing the result (amplification curves) of specimen(s) tested positive for Influenza type: A) A/H1N1, B) A/H3N2, C) type B and D) negative (refer Table 44.5)

Source: Department of Microbiology, JIPMER, Puducherry (with permission).

- **ELISA** is more sensitive than other assays; detects antibodies against HA and/or NA antigens.

TREATMENT — Influenza viruses

Specific antiviral therapy is available for influenza virus infection

- **Neuraminidase inhibitors** (such as zanamivir, oseltamivir, and peramivir) can be administered for influenza A and influenza B infections
  - It is the drug of choice for A/H1N1 2009 flu, A/H5N1 avian flu and influenza-B
  - Dosage:
    - Oseltamivir (Tamiflu 75 mg tablets)
    - Zanamivir (10 mg, inhalational form).
  - Schedule:
    - For treatment—given twice a day for 5 days
    - For chemoprophylaxis—given once daily. Duration depends on the clinical setting; usually 7 days.
- **Matrix protein M2 inhibitor** such as amantadine and rimantadine can be given for some strains of influenza A infection. However, strains of A/H1N1 2009 flu and A/H5N1 avian flu and influenza B virus have developed resistance.

Prevention

General Preventive Measures

Measures of droplet precaution (refer Chapter 53 for detail) should be followed:

- **Strict hand hygiene**
- **Isolation room:** Patients should be kept in isolation room or cohorting to be followed
- **Containment of coughs and sneezes**
  - Respiratory hygiene and cough etiquette
  - Use of personal protective equipment (PPE) such as gloves and mask for staff and patient.
- **Work restriction:** CDC recommends that people with influenza-like illness remain at home until at least 24 hours after they are free of fever (<100°F) without the use of fever-reducing medications.

Vaccination Prophylaxis

Vaccine Strains

Based on WHO recommendations, influenza vaccines are prepared every year.

Table 44.5: Real time RT-PCR for seasonal influenza types

Simultaneously detects five genes	Specimens positive with influenza type			Influenza negative
	A/H1N1	A/H3N2	Type B	
Influenza A (matrix gene)	+	+	–	–
H1N1 (HA gene)	+	–	–	–
H3N2 (HA gene)	–	+	–	–
Influenza B (HA gene)	–	–	+	–
RNP (ribonucleoprotein)*	+	+	+	+

*RNP (ribonucleoprotein) is an internal control: indicates presence of human DNA in specimen. If it is not detected, then the test is considered invalid.

- **Strains to be included** in the vaccine depend upon the strains isolated in the previous influenza seasons and strains that are anticipated to circulate in the upcoming season
- **Formulations:** Most of the influenza vaccines are cocktails containing two type A and one or two type B influenza strains
  - *Trivalent form:* This is the most common form available; comprises of three strains: A/H1N1, A/H3N2 and influenza B strain
  - *Quadrivalent form:* In addition to trivalent form, this contains another type B strain.
- **GISRS:** WHO's Global Influenza Surveillance and Response System (GISRS) reviews the vaccine composition annually and gives recommendations for both northern and southern hemispheres
- **For 2018–19 season:** The WHO recommended vaccine strains for 2018-19 season are as follows.
  - *For northern hemisphere:* Quadrivalent form vaccine:
    - Type A/H1N1(Michigan lineage, 2015)
    - Type A/H3N2 (Singapore lineage, 2016)
    - Type B (Yamagata lineage, 2013)
    - Type B (Victoria lineage, 2017).
  - *For southern hemisphere:* Trivalent vaccine, same as that for northern hemisphere except the Type B (Victoria lineage, 2017).
- **Types:** Both injectable (inactivated) and nasal spray (live attenuated) vaccines are available.

Injectable Vaccines

Injectable vaccines are the most widely used vaccines in immunization programs.

- **Types:** There are three types of injectable vaccines
  1. *Inactivated Influenza Vaccine (IIV)*, e.g. Fluzone: It is prepared by growing the vaccine strains in allantoic cavity of embryonated chick eggs and then harvested, purified, inactivated by formalin or beta propiolactone and then standardized based on hemagglutinin antigen content (15 µg of HA/dose).
  2. *Cell Culture-based Inactivated Influenza Vaccine (ccIIV3)*; e.g. Flucelvax: Same as IIV, but prepared in cell lines such as Madin-Darby Canine Kidney (MDCK) cell line.
  3. *Recombinant Influenza Vaccine (RIV)*; e.g. FluBlok: Contains recombinant influenza HA antigens in trivalent formulation. RIV does not contain any egg protein.
- **Schedule:** Single dose administered by intramuscular (IM) route; except for 6 months-8 years of age (2 doses are required ≥4 weeks apart)
- **Timing of vaccination:** Optimally before onset of influenza season, i.e. by end of October
- **Efficacy:** The vaccine efficacy varies from 25-67% (25% for H3N2, 42% against type B and 67% against H1N1). The efficacy is lower if vaccine virus does not match to currently circulating viruses in the locality. Immunity lasts for 6-12 months
- **Side effects:** Mild reactions can occur in 5% of cases such as redness at injection site, fever and aches. Serious side effects such as allergic reactions can occur very rarely
- **Indications:** Routine annual influenza vaccination is recommended for all persons aged ≥6 months who do not have contraindications. If facility are not feasible, then high-risk groups should be given first priority for vaccination
  - Age: ≥6 months to <5 years and ≥50 years
  - Persons with chronic pulmonary, cardiovascular, renal, hepatic, neurologic, hematologic, or metabolic disorders
  - Low immunity (e.g. HIV infection)
  - Pregnant women
  - Those receiving aspirin
  - Extremely obese (body mass index ≥40)
  - Caregivers and contacts of those at risk, e.g. health care workers and household contacts.
- **Contraindication:** IIV should not be administered to people who have allergy to eggs or have history of hypersensitivity to previous dose of vaccine
- **Travelers:** If traveling to an area of increased influenza activity; can consider vaccination, preferably ≥2 weeks before departure.

Live Attenuated Influenza Vaccine (LAIV)

This vaccine is generated by reassortment between currently circulating strains of influenza A and B virus with a cold-adapted attenuated master strain which is adapted to grow at 25-33°C.

- Such live attenuated strains can grow in upper respiratory tract (at 33°C) but not in lower respiratory tract (at 37°C); therefore they may cause mild flu like symptoms but never infect lower respiratory tract, hence never cause serious adverse effects
- It is a trivalent vaccine, administered by intranasal spray
- **Indication:** It can be given to all healthy persons of 2–49 years age (except in pregnancy), but is not given to high-risk groups
- However due to efficacy issues, LAIV is not recommended for use in any population for 2017–18.

Chemoprophylaxis

Antiviral drugs are not recommended for routine seasonal or pre-exposure prophylaxis. It is recommended only for post-exposure and during outbreak situations in hospitals.

- **Indications:** Following exposure to an influenza case, it is recommended to the following groups: (i) if not vaccinated or vaccinated recently (<2 weeks), (ii) HIV infected people

- **Duration:**
  - *Non-outbreak exposure* (e.g. in community): It should be started as soon as possible following exposure (within 48 hours) and continued for 7 days
  - *During outbreaks in hospitals* (for elderly persons and children and health care workers): duration for a minimum of 2 weeks, and to be continued up to 1 week after the last known case was identified.
- **Antiviral drugs** recommended are:
  - Oseltamivir is the drug of choice. It is given as 75 mg orally, once a day for 7 days
  - Zanamivir: 10 mg (two 5-mg inhalations) once daily for 7 days.
- **Efficacy:** The efficacy of chemoprophylaxis is about 70% to 90% in preventing influenza.

PARAMYXOVIRIDAE

Paramyxoviridae contains a group of viruses, which are transmitted via the respiratory route following which:

- They may cause localized respiratory infection in children (e.g. respiratory syncytial virus and parainfluenza viruses) or;
- They may disseminate throughout the body to cause highly contagious diseases of childhood such as mumps (parotid enlargement) and measles (rashes).

Rubella virus is though not a paramyxovirus, because of its clinical and epidemiological resemblance to measles virus; it has been discussed in this chapter.

MORPHOLOGY AND CLASSIFICATION

Paramyxoviruses resemble orthomyxoviruses in morphology, but are larger and more pleomorphic (Fig. 44.5).

- **Size:** Vary from 100-300 nm, rarely upto 800 nm. Long filaments and giant forms may be seen rarely
- Possess a **helical nucleocapsid** of 18 nm size

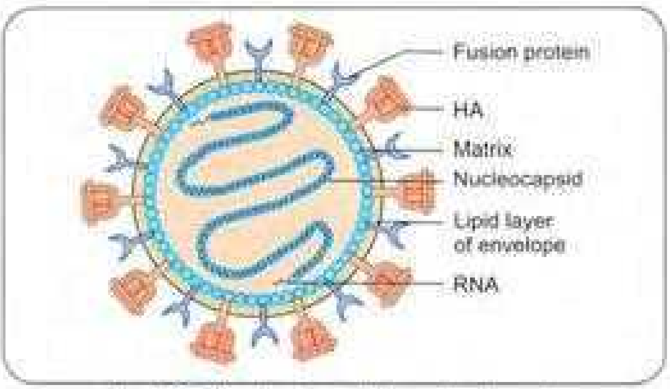


Fig. 44.5: Measles virus (Schematic diagram)

- **RNA:** Contains a ss RNA which is linear, non-segmented and negative-sense
- **Contains six structural proteins** which form capsid, polymerase, matrix protein (that underlies the viral envelope) and envelope glycoproteins
- **Envelope:** The nucleocapsid is surrounded by a host derived lipid envelope in which the following virus-coded peplomers (glycoproteins) are inserted (see box below).

Glycoproteins of paramyxoviruses

- **F-glycoproteins** are present in all paramyxoviruses and mediate membrane fusion. They also have hemolysin activity (except in pneumoviruses)
- **Larger glycoproteins** help in attachment to the host cells. They may be either H or HN or G types
  - HN glycoproteins have both hemagglutinin and neuraminidase activities, e.g. in parainfluenza and mumps viruses
  - H glycoproteins have only hemagglutinin activity, e.g. in measles G glycoproteins do not have hemagglutinin and neuraminidase activities, but help in attachment, e.g. in respiratory syncytial virus.

Paramyxoviridae family is divided into two subfamilies and seven genera, out of which six contain human pathogens (Table 44.6).

PARAINFLUENZA VIRUSES

Human parainfluenza viruses are one of the major causes of lower respiratory tract disease in young children. It has five serotypes:

- Types 1 and 3 belong to the genus *Respirovirus*
- Types 2, 4 a and 4b belong to the genus *Rubulavirus*.

Pathogenesis and Clinical Manifestations

- Transmission is by respiratory route (by direct salivary contact or by large-droplet aerosols)
- The incubation period appears to be 5-6 days
- Virus multiplies locally and causes various respiratory manifestations such as:
  - Mild common cold syndrome like rhinitis and pharyngitis are the most common presentation, seen with all serotypes
  - Croup (laryngotracheobronchitis):
    - Occurs in 2-3% of cases
    - Typically seen with type 1 and 2
    - Involves children (between 6 to 18 months of age).
  - Pneumonia or bronchiolitis:
    - Occurs very rarely
    - Seen especially with serotype 3
    - Involves infants below 6 months of age.
  - Otitis media: It is the most common complication of parainfluenza virus infection.

Table 44.6: Characteristics of family Paramyxoviridae

Subfamilies	Paramyxovirinae				Pneumovirinae	
Genera	*Respirovirus*	*Rubulavirus*	*Morbillivirus*	*Henipavirus*	*Pneumovirus*	*Metapneumovirus*
Human viruses	Parainfluenza 1, 3	Mumps, Parainfluenza 2, 4a, 4b	Measles	Hendra, Nipah (Zoonotic)	Respiratory syncytial virus	Human metapneumovirus
Nucleocapsid	18 nm	18 nm	18 nm	*	13 nm	13 nm
Large Glycoproteins	HN type	HN type	H type	G type	G type	G type
Hemagglutinin	+	+	+	–	–	–
Neuraminidase	+	+	–	–	–	–
Fusion protein	+	+	+	+	+	+
Hemolysin	+	+	+	*	–	–
Inclusions	Cytoplasm	Cytoplasm	Cytoplasm and Nucleus	Cytoplasm	Cytoplasm	Cytoplasm

* Not yet identified

Abbreviations: HN, have both hemagglutinin and neuraminidase activities; H, have only hemagglutinin activity; G, do not have both hemagglutinin and neuraminidase activities.

- Reinfections are common, but less severe. There is no cross protection between the serotypes
- Immunocompromized people are susceptible to severe infections. In older children and adults, disease is milder.

Epidemiology

Parainfluenza viruses are worldwide in distribution.

- Type 3 is the most prevalent serotype. It exists as endemic throughout the year with annual epidemics occur during spring
- Types 1 and 2 infections are less common and seasonal, and tend to cause epidemics during the rainfall or winter, cyclically every alternate year
- Type 4a and 4b cause much milder illness and these serotypes are the most difficult to be isolated
- Parainfluenza viruses are important cause of outbreaks in pediatric wards, day care centers and in schools.

Laboratory Diagnosis

- **Antigen detection:** Viral antigens in the infected exfoliated epithelial cells of the nasopharynx can be detected by direct-IF test by using specific monoclonal antibodies. It is rapid, but less sensitive than viral isolation
- **Viral isolation:**
  - **Specimens** such as nasal washes, bronchoalveolar lavage fluid and lung tissue can be used. Specimen should be inoculated as early as possible to obtain best results
  - **Primary monkey kidney cell line** is most sensitive and alternatively, a continuous monkey kidney cell line-LLC-MK2 can be used
  - They produce little or no cytopathic effect
  - Viral growth can be detected by performing hemadsorption using guinea pig erythrocytes or antigen detection by direct-IF test
  - Shell vial technique is followed to enhance viral replication.
- **Serum antibodies** can be measured by neutralization test, hemagglutination inhibition test or ELISA. Presence of IgM or fourfold rise of IgG titer is indicative of active infection
- **Reverse transcriptase PCR** assays are highly specific and sensitive but available only in limited settings.

Animal Parainfluenza Viruses

Certain animal parainfluenza viruses are related to the human strains.

- Sendai virus of mice is a subtype of human parainfluenza virus type 1
- SV5, a common contaminant of primary monkey kidney cell lines, is related to parainfluenza virus type 2
- Shipping fever virus of cattle and sheep (SF4) is a subtype of parainfluenza virus type 3.

Avian Parainfluenza Viruses (Newcastle Disease Virus or NDV)

NDV (also called Ranikhet virus in India) produces pneumoencephalitis in young chickens and mild flu like illness in older birds.

Human infection is rare and occupational; characterized by mild self-limiting conjunctivitis that may occur in workers handling infected birds.

MUMPS VIRUS

Mumps virus is the most common cause of parotid gland enlargement in children. In severe cases, it can also cause orchitis and aseptic meningitis.

Pathogenesis

- **Transmission** is through the respiratory route via droplets, saliva, and fomites

- **Primary replication** occurs in the nasal mucosa or upper respiratory mucosa → infects mononuclear cells and regional lymph nodes → spills over to blood stream resulting in viremia → dissemination
- **Target sites:** Mumps virus has a special affinity for glandular epithelium. The classic sites include salivary glands, testes, pancreas, ovaries, mammary glands and central nervous system.

Clinical Manifestation

- **Incubation period** is about 19 days (range, 7–23 days)
- **Inapparent infection:** Up to half of the infected people are either asymptomatic or present with non-specific symptoms such as fever, myalgia and anorexia. This is more common in adults than in children
- **Bilateral parotitis:** Acute non-suppurative parotid gland enlargement is the most common specific manifestation, present in 70–90% of the cases (Fig. 44.6)
  - Rarely, parotitis may be unilateral
  - In some cases, other salivary glands may also be involved.
- **Epididymo-orchitis** is the next most common manifestation of mumps, developing in 15–30% of cases in postpubertal males. Orchitis is unilateral in most of the cases, hence infertility following mumps orchitis is rare
- **Aseptic meningitis** occurs in less than 10% of cases, with a male predominance. It is self-limiting condition except the deafness (due to cranial nerve palsy) which may be permanent
- **Oophoritis** occurs in about 5% of women
- **Pancreatitis** occurs in 4% of infections and may lead to diabetes
- **Atypical mumps:** Parotitis may be absent in 10% of cases and patients directly present with aseptic meningitis.

Epidemiology

Mumps is endemic worldwide, sporadic cases occurring throughout the year, with a peak in cases typically in winter and spring. Epidemics occur every 3–5 years; typically associated with unvaccinated people living in overcrowded areas.

- **Period of communicability:** Patients are infectious from 1 week before to 1 week after the onset of symptoms
  - Most contagious period is within 1–2 days before the onset of symptoms
  - Infective material: Mumps virus is shed in saliva, respiratory droplets, and urine.
- **Source:** Cases (both clinical and subclinical cases) are the source of infection
  - There is no carrier state
  - Subclinical cases (30–40% of all cases)are responsible for maintaining the cycle of infection.
- **Reservoir:** Humans are the only reservoir of infection
- **Incidence:** In most part of the world, the annual incidence of mumps is about 100–1,000 cases per 10,000 populations. However, cases are much reduced after the start of immunization, except for the 2006 outbreak of USA which had witnessed over 5700 cases
- **Age:** Children of **5–9 years** of age are most commonly affected; however, no age is exempt if there is no previous immunity. Disease tends to be more severe in adults
- **Immunity:** One attack (either by vaccine or infection) gives lifelong immunity
- Secondary attack rate is high (86%).

LABORATORY DIAGNOSIS — Mumps virus

- **Specimen:** Buccal or oral swab
- **Antigen detection** by direct IF test
- **Viral isolation**
  - Primary monkey kidney cell lines
  - Shell vial technique.
- **Serum antibodies** by ELISA, neutralization test, hemagglutination inhibition test
- **RT PCR:** detects viral RNA.

Laboratory Diagnosis

- **Specimens:** The buccal or oral swab specimens are the most ideal specimens, although mumps can also be detected in saliva, CSF, urine (shed up to two weeks), seminal fluid and rarely blood. Massaging the parotid gland area for 30 seconds prior to swabbing is recommended
- **Direct viral antigen detection** can be done by using mumps-specific immunofluorescent staining (direct-IF) of clinical specimens
- **Virus isolation:** Monkey kidney cells are the preferred cell lines. Specimens should be inoculated immediately

Fig. 44.6: Parotitis in a mumps virus-infected patient (arrow showing)

Source: Public Health Image Library, ID# 1861/Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*).

- Viral growth after 1-2 weeks can be detected by demonstration of cytopathic effect (cell rounding and giant cell formation) or hemadsorption
- Shell vial technique is followed for rapid detection in 1-2 days.

❖ **Serum antibodies detection:** ELISA is the most widely used assay. Separate ELISA formats are available for detecting IgM and IgG antibodies; against mumps specific whole-virus antigen, nucleoprotein and hemagglutinin antigens. Mumps ELISA is highly specific, does not cross-react with parainfluenza antibodies
- Detection of IgM antibodies (present up to 60 days of infection), or a rise in IgG titer indicates active infection
- The traditional tests such as neutralization test, hemagglutination inhibition test and complement fixation tests are seldom used now.

❖ **Reverse-transcription PCR** is available to detect mumps specific RNA such as N gene(nucleoprotein) in clinical specimen. It is highly sensitive and specific.

❖ **Genotyping** is done targeting small hydrophobic (SH) gene; the most variable region of mumps genome.

TREATMENT **Mumps virus**

❑ There is no specific antiviral drug available. Treatment is mostly symptomatic

❑ Mumps immunoglobulin is available; but not effective, hence not recommended for treatment or post-exposure prophylaxis.

Prevention (Live Attenuated Vaccine)

❖ **Vaccine Strain:** Live attenuated Jeryl Lynn strain is the recommended strain used worldwide. Other strains available are RIT 4385, Urabe strain and L-Zagreb strain

❖ Mumps vaccine is prepared in chick embryo cell line

❖ **Mumps vaccine is available as**
- Trivalent MMR vaccine (live attenuated measles-mumps-rubella vaccine) or
- Quadrivalent MMR-V vaccine (contains additional live attenuated varicella vaccine)
- Monovalent mumps vaccine (not commonly used).

❖ **Schedule:** Two doses of MMR is given by subcutaneous route at 1 year (12-15 months) and 4-6 years (before starting of school)

❖ **Efficacy** is about 88% after the second dose. Neutralizing antibodies appear in 95% of the recipients. Duration of long term immunity is unknown.

MEASLES (RUBEOLA) VIRUS

Measles is an acute, highly contagious childhood disease, characterized by fever and respiratory symptoms, followed by typical maculopapular rash (see Fig. 44.7B).

Pathogenesis

❖ **Transmission** occurs predominantly via the respiratory route either by—
- Droplets inhalation over short distances (common)
- Small-particle aerosols that remain suspended especially in schools, hospitals, and enclosed public places in the air for longer period (less common).

❖ **Spread:** The virus multiplies locally in the respiratory tract; then spreads to the regional lymph nodes → enters into the bloodstream in infected monocytes (primary viremia) → further multiplies in reticuloendothelial system → spills over to blood (secondary viremia) → disseminates to various sites

❖ **Target sites:** The virus is predominantly seeded in the epithelial surfaces of the body, including the skin, respiratory tract, and conjunctiva.

Clinical Manifestations

Incubation period is about 10 days which may be shorter in infants and longer (up to 3 weeks) in adults. Disease can be divided into three stages.

1. Prodromal Stage

This stage lasts for 4 days (i.e. from 10th to 14th day of infection) and is characterized by manifestations such as:

❖ **Fever** is the first manifestation, occurs on day 1 (i.e. on 10th day of infection)

❖ **Koplik's spots** are pathognomonic of measles, appear after two days following fever (i.e. on 12th day of infection) and are characterized by:
- White to bluish spot (1 mm size) surrounded by an erythema
- Appear first on buccal mucosa near second lower molars (Fig. 44.7A)
- Rapidly spread to involve the entire buccal mucosa and then fade with the onset of rash.

❖ **Non-specific symptoms** may be present such as cough, coryza, nasal discharge, redness of eye, diarrhea or vomiting.

2. Eruptive Stage

Maculopapular dusky red rashes appear after 4 days of fever (i.e. on 14th day of infection).

❖ Rashes typically appear first **behind the ears** → then spread to face, arm, trunk and legs → then fade in the same order after 4 days of onset (Fig. 44.7B)

❖ Rashes are typically absent in HIV infected people.

Fever (10th day) → Koplik's spot (12th day) → rash (14th day)

Figs 44.7A to C: A. Koplik spot in buccal mucosa (measles) (arrow showing); **B.** Measles rashes (on face); **C.** Multinucleated giant cell of measles infected cell lines (Arrow showing)

Source: A. Public Health Image Library, ID# 6111, B. ID# 17980, C. ID# 859/Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*).

3. Post-Measles Stage

It is characterized by weight loss and weakness. There may be failure to recover and gradual deterioration into chronic illness.

Complications

Secondary Bacterial Infections

Following measles, there is profound immune suppression and fall of cell mediated immunity which in turn predisposes to various secondary bacterial infections:

- Otitis media and bronchopneumonia are most common
- Recurrence of fever or failure of fever to subside with the rash
- Worsening of underlying tuberculosis with a false negative Mantoux test.

Complications Due to Measles Virus Itself

- Giant-cell pneumonitis (Hecht's pneumonia) in immunocompromized children, and in HIV infected people
- Acute laryngotracheobronchitis (croup)
- Diarrhea, leads to malnutrition including vitamin A deficiency.

Central Nervous System Complications

CNS complications are rare, but most severe.

- **Post-measles encephalomyelitis:** It develops within 2 weeks of onset of rash. It represents an autoimmune response against the myelin basic protein. Its occurrence is about 1 in 1000 cases, affecting mainly older children and adults
- **Measles inclusion body encephalitis** occurs months after rashes, typically affecting people with defective cell-mediated immunity (CMI)
- **Subacute sclerosing panencephalitis (SSPE):** It is a slowly progressive disease characterized by seizures and progressive deterioration of cognitive and motor functions
    - SSPE belongs to group C slow virus infection, caused by a defective measles virus
    - Occurrence is 1 in 300,000 measles cases
    - Age: SSPE typically develops if the primary measles virus infection occurs in children less than 2 years of age
    - SSPE usually develops after 7–13 years after primary measles infection. It is fatal within 1–3 years of onset with mortality rate of 10–20%
    - High titers of antibody to measles virus in CSF is diagnostic.

LABORATORY DIAGNOSIS — Measles virus

- **Specimen:** Nasopharyngeal swab
- **Antigen detection:** By using anti-nucleoprotein antibodies
- **Virus isolation:**
    - Monkey or human kidney cells or Vero/hSLAM cell line-produces CPE as multinucleated giant cells (Warthin-Finkeldey cells)
    - Shell vial culture.
- **Antibody detection in serum:** Against nucleoprotein antigen by ELISA or neutralization tests
- **Reverse-transcription PCR:** detects viral RNA.

Laboratory Diagnosis

Specimens

Nasopharyngeal swab, conjunctival swab, blood, respiratory secretions, and urine are the ideal specimens. Synthetic swabs are recommended.

Antigen Detection

Measles antigens in the infected cells can be detected directly by using anti-nucleoprotein antibodies (direct immunofluorescence test).

Virus Isolation

- **Cell lines:** Monkey or human kidney cells or a lymphoblastoid cell line (B95-a) are optimal cell

lines used for isolation of measles virus. Vero/hSLAM cell line is the CDC recommended cell line. This is a Vero cell line coated with measles specific hSLAM receptors (human signaling lymphocytic activation molecule)
- Cytopathic effect may be observed after 7–10 days of inoculation into cell lines characterized by multinucleated giant cells (**Warthin-Finkeldey cells**) containing both intranuclear and intracytoplasmic inclusion bodies (see Fig. 44.7C)
- Shell vial culture (centrifugation of culture followed by immunofluorescence detection of measles antigens in the inoculated cultures) is recommended for early detection within 2–3 days.

Antibody Detection

- Detection of measles-specific IgM antibody in serum or oral fluid or four-fold rise of IgG antibody titer between acute and convalescent-phase sera is taken as significant
- Demonstration of raised titers of anti-measles antibody in the CSF is diagnostic of SSPE
- ELISA is the most recommended test that uses recombinant measles nucleoprotein (NP) antigen. IgM antibodies are detected by capture ELISA whereas IgG antibodies are detected by indirect ELISA
- In the past, other tests such as neutralization test, hemagglutination inhibition test and complement fixation tests were used
- Neutralization tests are sensitive and specific, and the results are highly correlated with protective immunity; however, require maintenance of virus in cell lines and thus are expensive and labor intensive.

Reverse-transcription PCR

RT-PCR is available targeting measles specific RNA such as N gene(nucleoprotein) in clinical specimen.
- It is extremely sensitive and specific
- It may also permit characterization of measles virus genotypes for molecular epidemiologic studies
- It can distinguish wild-type from vaccine virus strains
- RNA can be detected in specimens up to 10–14 days post rashes, in contrast to virus isolation, which often becomes negative after 3 days of rash.

Measles genotypes

There are 8 clades of measles which are further grouped into 23 recognized genotypes (WHO).
Genotype D8 followed by B3 and D4 are the commonly reported globally as well as from India.

TREATMENT — Measles virus

- There is no specific antiviral therapy available for measles
- Treatment is symptomatic and consists of general supportive measures
- Vitamin A has been effective in reducing the morbidity and mortality due to measles.

Prevention

General Preventive Measures

Airborne precaution such as isolation in negative pressure room, use of PPEs such as N95 mask, etc. must be followed while handling measles cases (Chapter 53 for detail).

Measles Vaccine

Live attenuated vaccine is available for measles:
- **Strains:** Most attenuated strains that are in use currently are derived from the original Edmonston strain isolated in 1954, which includes:
  - Schwartz strain (currently serves as the standard in much of the world)
  - Edmonston-Zagreb strain
  - Moraten strain.
- Vaccine is prepared in chick embryo cell line
- **Reconstitution:** Vaccine is available in lyophilized form and it has to be reconstituted with distilled water and then should be used within 4 hours
- Vaccine is thermolabile, hence, it must be stored at -20°C
- One dose (0.5 mL) containing more than 1000 infective viral units is administered subcutaneously
- **Combined vaccines:** Measles vaccine is available in combined form with rubella (MR vaccine), with mumps and rubella vaccine (MMR vaccine) and with varicella (MMR-V vaccine)
- **Indication:** Under national immunization schedule of India, measles-rubella (MR) vaccine is given at 9 completed months to 12 months along with vitamin A supplements and second dose of MR vaccine at 16–24 months. The second dose is initiated only in selected states, namely Karnataka, Tamil Nadu, Goa, Lakshadweep and Puducherry
- **Side effects include:**
  - Mild measles like illness may develop in 15–20% of vaccinees. There is no spread of the vaccine virus in the community
  - Toxic shock syndrome (due to contamination of vial with *S. aureus* toxins).
- **Contacts:** Susceptible contacts over 9–12 months may be protected against measles if the measles vaccine is given within 3 days of exposure. This is because incubation period of measles induced by the vaccine strain is about

7 days, compared to 10 days for the naturally occurring measles

- Measles immunoglobulin (Ig) can also be given within 3 days, at a WHO recommended dose of 0.25 mg/kg of body weight
- However, both vaccine and Ig should not be given together. At least 8-12 weeks of gap must be maintained.

Epidemiology

Measles is endemic throughout the world with epidemics which recur regularly every 2-3 years, typically in late winter and early spring.

- **Source:** Cases are the only source of infection. Carriers are not known to occur. In-apparent or sub-clinical infections are rare
- **Reservoir:** Humans are the only reservoir of infection. There is no animal reservoir
- **Infective material:** Virus is shed in the secretions of nose, throat and respiratory tract of cases of measles, especially during the prodromal stage and early stage of rash
- **Period of communicability:** Patients are infectious from four days before to four days after the onset of rash. Patients are highly contagious, isolation is recommended from the onset of prodromal stage until third day of rash
- **Secondary attack rate** is very high (>90%)
- **Age:** Measles is a childhood disease
  - Children (6 months to 3 years) are the most susceptible group in developing countries
  - Older children (>5 years) are commonly affected in developed countries or in vaccinated population.
- **Immunity:** No age is immune if there is no previous immunity
  - There is single serotype hence one attack (vaccine or infection) gives lifelong immunity
  - Infants are protected up to 6 months due to pre-existing maternal antibodies.
- **Epidemic** of measles occurs if proportion of susceptible children exceeds 40%. Though disease burden has much decreased after the vaccine is made available, measles is still a leading cause of death of young children in many developing countries
- **World:** The measles cases and deaths dramatically reduced in recent days due to widespread vaccination
  - In 2017, there were 1,37,860 confirmed cases of measles occurred worldwide; South east Asian region (SEAR) accounted for maximum cases (69,719)
  - Globally, the number of deaths reduced below 1 Lakh/year (89,780 in 2016)
  - According to WHO, outbreaks of measles during 2016-17 have been reported from various countries such as Italy, Romania, China, Ethiopia, India, Indonesia, etc. The largest recent outbreak was from Philippines (2014, >50,000 cases).
- **India:** Though reduced, still India accounts for the maximum burden of measles cases. In 2017, India reported 55,226 cases (40% of total cases globally) with annual incidence of 41.7 cases per million population.

Measles Elimination

Measles is amenable to eradication. With the efficient and widespread immunization program, it is possible to eradicate measles from the world.

WHO has introduced **'The Strategic Plan for Measles Elimination and Rubella Control'**. The target timeline for South East Asia Region (including India) is set by 2020. The following objectives are set to achieve this target:

1. ≥95% coverage with two doses of MR vaccine.
2. Develop and sustain a case-based surveillance system
3. Develop and maintain an accredited measles and rubella laboratory network
4. Strengthen support and linkages to achieve the above three strategic objectives.

Measles is said to be eliminated if the confirmed cases are <1 per million population. The American region and some of the western pacific region (Australia, Hong Kong, Japan, Korea , New Zealand, etc.) have recently achieved the measles elimination status.

NIPAH VIRUS AND HENDRA VIRUS

They are zoonotic paramyxoviruses. Hendra virus was first isolated in 1994 in Hendra (Australia) and Nipah virus was discovered in 1999 in Malaysia.

- **Reservoir:** Fruit bats (flying foxes) are the natural host for both Nipah and Hendra viruses
- **Geographical distribution:**
  - Hendra virus infections are confined to horses in Australia, whereas Nipah viruses cause infection of pigs in Malaysia
  - Human infection of Nipah virus is emerging especially in Southeast Asia including Bangladesh (mainly), India, Thailand and Malaysia. 477 cases were reported so far with 52% mortality.

Nipah virus in India

Two outbreaks of Nipah virus in humans were reported from West Bengal (Siliguri in 2001 and Nadia in 2007); together accounted for 71 cases with 50 deaths. In 2018, cluster of 18 cases of Nipah encephalitis (with 16 deaths) have been reported from Kozhikode, Kerala.

- **Transmission:**
  - Hendra virus is transmitted by exposure to infected body fluids and excretions of horses
  - Transmission of Nipah virus to humans may occur after direct contact with infected bats, pigs, or persons. Consumption of infected raw date palm sap is thought to be another mode of transmission.

- **Clinical manifestations:** Both the viruses can produce of encephalitis in humans
- **Laboratory diagnosis:**
  - Real-time PCR from throat and nasal swabs, CSF, urine, and blood should be performed in the early stages of disease.
  - Antibody detection by ELISA (IgG and IgM) can be used later stage.
  - Immunohistochemistry is performed on tissues collected during autopsy, which confirm the postmortem diagnosis.
  - They are also prone to cause laboratory acquired infections and are classified as biosafety level 4 pathogens.
- **Treatment:** No antiviral drug is available.
- **Vaccine:** A subunit vaccine, using the Hendra G protein, produces cross-protective antibodies against Hendra and Nipah viruses. It has been recently used in Australia to protect horses against Hendra virus. It can be used in humans as well.

RESPIRATORY SYNCYTIAL VIRUS

Respiratory syncytial virus (RSV) is a major respiratory pathogen of young children and is the most common cause of lower respiratory disease (bronchiolitis and pneumonia) in infants.

Pathogenesis

- **Transmission:** RSV is spread by (i) direct contact (contaminated fingers or fomites and by self-inoculation onto the conjunctiva or anterior nares or (ii) by large droplets inhalation
- **Spread:** RSV replicates locally in the epithelial cells of the nasopharynx and may spread to the lower respiratory tract to cause bronchiolitis and pneumonia
- **Pathology:** Lymphocytes in large numbers migrate to the site of infection and secrete several cytokines which cause the following changes:
  - Peribronchiolar infiltration of inflammatory cells
  - Submucosal edema
  - Necrosis of the bronchiolar epithelium and
  - Formation of plugs consisting of mucus, cellular debris, and fibrin which occlude the smaller bronchioles.

Clinical Manifestations

RSV causes a wide spectrum of respiratory illnesses.

- **Incubation period** is about 3–5 days
- **Infants:** RSV is the most common cause of lower respiratory tract infection below 1 year of age, causing bronchiolitis, pneumonia, and tracheobronchitis in 25–40% of infected infants
- **Symptoms:** It begins with running nose, fever and accompanied by cough, wheezing and dyspnea
  - Chest X-ray shows peribronchial thickening, diffuse interstitial infiltration and occasionally lobar consolidation
  - Infection is severe in premature infants and underlying congenital cardiac disease, bronchopulmonary dysplasia, nephrotic syndrome, or immunosuppression.
- **Adults:** RSV produces influenza-like upper respiratory symptoms such as common cold, running nose, sore throat, and cough. Infections are common in overcrowded communities (military recruits)
  - Lower respiratory tract infections can occasionally occur among transplant recipients or immunocompromized adults or elderly nursing-home residents
  - RSV can cause exacerbation and worsening of asthma or COPD (chronic obstructive pulmonary disease).
- **Recurrent infection** is common in both children and adults, but is much milder (common cold).

Laboratory Diagnosis

Antigen Detection

Direct identification of viral antigens in clinical samples is rapid and sensitive. Two methods are commonly used; both use monoclonal antibodies specific for RSV.

- Direct immunofluorescence test detecting antigens on exfoliated cells or
- ELISA detecting antigens in nasopharyngeal secretions.

Virus Isolation

HeLa and HEp-2 are the most sensitive cell lines for RSV isolation. As RSV is extremely labile, freezing should be avoided and specimens should be processed immediately.

- A characteristic cytopathic effect, **syncytium formation** (multinucleated giant cell)—appears after **10 days**. Hence, it is named as syncytial virus
- **Shell vial technique** is done to enhance the viral replication, which helps in early growth detection within 1–2 days
- Sensitivities of virus isolation or antigen detection are excellent in children (80–95%), but low in adults.

Reverse Transcriptase-PCR

RT-PCR amplifying viral RNA (such as nucleoprotein N gene) has shown higher sensitivity and specificity, particularly in adults.

Antibody Detection

Various formats such as immunofluorescence, ELISA, and neutralization tests are available for antibody detection.

- Antibodies are often found in older children and adults but may not be elevated in children <4 months of age
- Serum antibodies are of less diagnostic importance; rather they are the markers of prevalence of infection (epidemiological significance).

Epidemiology

Infection with RSV occurs worldwide.

- **Seasonality:** Annual epidemics tend to occur following rainfall, in winter and spring and last up to 5 months. Infection is not seen in summer
- **Age:** RSV is a leading respiratory pathogen in children. Infants between ages of 6 weeks to 6 months of age are commonly affected, with peak incidence at 2 months
- **Prevalence:** About 70% of infants are infected by 1 year of age and almost all by 2 years of age
- **Subgroups:** RSV can be typed into two subgroups; Subgroup A infections appear to cause more severe illness than subgroup B.

TREATMENT — Respiratory syncytial virus

Unlike other respiratory viruses, specific antiviral drug is available for treatment of RSV.

- **Ribavirin** is the drug of choice
  - It is indicated for severe infections in infants. However, its beneficial effect in older children and adults is doubtful
  - It is administered as aerosols for 3–6 days. Oral ribavirin is not recommended.
- Supportive care is also needed such as removal of secretions, administration of oxygen, bronchodilators and ventilatory support
- Immunoglobulin with high titers of antibody to RSV was used in the past, but was found ineffective and hence it is not recommended currently.

HUMAN METAPNEUMOVIRUS

Human metapneumovirus was first reported in 2001, though the avian strains were prevalent since 1970s.

- They cause both upper and lower respiratory tract illnesses similar to those caused by RSV but less severe and tend to affect slightly older children
- It may be the second most common cause (next to RSV) of lower respiratory infection in young children
- They also cause respiratory disease in adults with underlying hematologic malignancies
- **Diagnosis:** RT-PCR is available to amplify the RNA extracted from respiratory specimens. Specific antigens in nasopharyngeal secretions can be detected by direct-IF test.

RUBELLA

Rubella is not a myxovirus, but it has been discussed in this chapter because it produces a childhood exanthema similar to that of measles (hence rubella is also known as **German measles**). However, unlike measles, it is highly teratogenic; can cause congenital rubella syndrome.

MORPHOLOGY

Rubella belongs to Togaviridae family, and is the only member under genus *Rubivirus*.

- It is enveloped, single-stranded RNA virus measuring 50–70 nm in size; surrounded by capsid (C) protein
- Its envelope contains a lipid layer from which two types of spike-like glycoproteins (E1 and E2) are projected
- There is only one serotype and humans are its only known reservoir.

TYPES OF RUBELLA INFECTIONS

Rubella may present as postnatal infection or congenital infection.

Postnatal Rubella

Postnatal rubella may occur during neonatal age, childhood, and adult life.

Transmission

Rubella virus spreads from person-to-person by respiratory droplets via upper respiratory mucosa.

Spread

Rubella virus replicates locally in the nasopharynx, and then spreads to the lymph nodes. Subsequently, viremia develops after 7–9 days, and lasts until 14th day by which time both antibody and rashes appear almost simultaneously suggesting an immunologic basis for the appearance of rash.

Clinical Manifestations

- Incubation period is about 14 days (range, 12–23 days)
- Subclinical infection may be seen in 20–50% of cases
- Rashes are often the first manifestations in children, but in older children and adults, 1 to 5-day prodrome often precedes the rash, which includes low-grade fever, malaise, and upper respiratory symptoms
- **Rashes** are generalized and maculopapular in nature, start on the face, extend to trunk and extremities, and disappear in 3 days (Fig. 44.8A)
- **Lymphadenopathy** (occipital and postauricular) is the most striking feature
- **Forchheimer spots** may be seen in some cases. They are pin-head sized petechiae; develop on the soft palate and uvula; usually start with the onset of rash.

Complications

Arthralgia and arthritis are common in adults, particularly in women. Thrombocytopenia and encephalitis are rarely encountered.

LABORATORY DIAGNOSIS — **Rubella virus**

- **Specimen:** Nasopharyngeal or throat swabs
- **Virus Isolation:**
  - In monkey or rabbit origin cell lines and then growth is detected by viral interference
  - Shell vial technique.
- **Antibody detection:** By HAI or ELISA.

Laboratory Diagnosis

Isolation of Virus

- **Ideal specimen:** Nasopharyngeal or throat swabs taken 6 days before and after onset of rash
- **Ideal cell line:** Monkey or rabbit origin cell lines may be used
- Rubella is a non-cytopathic virus. Traditionally, it can be identified by demonstrating viral interference property by co-infecting the cell line with cytopathic echovirus
- It can also be identified more rapidly in cell line by shell vial technique.

Serology (Antibody Detection)

Hemagglutination inhibition test (HAI) was most widely used standard antibody detection test for rubella infection. However, it cannot differentiate recent and past infection and serum needs to be pretreated to remove nonspecific inhibitors before testing.

ELISA is the preferred method of rubella diagnosis in modern days because serum pre-treatment is not required and it detects both IgM and IgG separately. Various antigens employed are whole virus lysate or recombinant E1/E2 antigens.

- IgM appears on 15th day of infection (2–5 days after rash) and lasts for 1–3 months. IgM may give false positive result especially in rubella low-prevalence countries; where the result has to be confirmed by IgG avidity test.
- IgG is produced against three (E1, E2, and C) antigens.
  - Anti-E1-IgG appears 1 week after IgM and persists for life. It indicates either active (primary or recurrent) or past infection or post-vaccination. Active infection can be differentiated from past infection by four fold rise of titre or by performing IgG avidity test.
  - Anti-E2-IgG appears 3 months after infection and persists long; whereas anti-C-IgG appears early in acute phase and declines thereafter.

Molecular Test

RT-PCR is available detecting rubella specific RNA (nucleoprotein N gene) in clinical specimens.

Congenital Rubella Syndrome

The most serious consequence of rubella virus infection is congenital rubella syndrome. Rubella is highly teratogenic and is included in the list of the agents causing congenital infection known as 'TORCH complex [***To****xoplasma*, **O**thers (e.g. varicella, agent of syphilis), **R**ubella virus, **C**ytomegalovirus and **H**erpes simplex virus].

Figs 44.8A and B: A. Child with rubella rash; **B.** Cataract seen in congenital rubella infection (arrows showing)

Source: Public Health Image Library, A) ID# 10146, B)ID#4284 /Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

Transmission

- Both the risk of transmission to fetus and severity of congenital infection are maximum if the mother acquires the infection during first trimester of pregnancy
- Risk after 5th month of pregnancy is almost negligible (90% risk at 11 weeks vs 20% risk at 20 weeks of gestation).

Clinical Manifestations

Permanent Congenital Defects

- **Classical triad consists of:**
  - **Ear defect:** Sensory neural deafness (most common defect of congenital rubella syndrome)
  - **Ocular defects:** Salt-and-pepper retinopathy is the most common ocular defect followed by cataract (Fig. 44.8B)
  - **Cardiac defect:** Patent ductus arteriosus (PDA) is the most common cardiac defect followed by pulmonary artery stenosis and ventricular septal defect.
- **CNS defects** such as—microcephaly, mental retardation, motor delay and autism.

Transient Congenital Changes

The transient changes such as hepatosplenomegaly, bone lesion, intrauterine growth retardation (IUGR) and thrombocytopenia with petechiae (**Blueberry muffin syndrome**) may be seen.

Outcome in the fetus may be miscarriage, fetal death, or premature birth with congenital defects.

Laboratory Diagnosis

- IgM antibodies do not cross placenta; their presence in a neonate is diagnostic of congenital rubella infection
- IgG antibodies cannot differentiate between maternal transfer and a true congenital infection. However,

IgG antibodies persisting in baby's serum beyond the expected time of disappearance of maternal IgG (9 months of age) can also be used as a criterion to diagnose congenital rubella infection

- Isolation of virus can be done especially from the throat swab, to lesser extent from urine (excreted up to 1 year). and CSF. It is more likely to be positive in the first six months after the birth
- Reverse transcriptase PCR to detect viral RNA.

EPIDEMIOLOGY

- **Source:** The cases are the only source of infection. There is no known carrier state
- **Duration of protection:** Once infected, the person acquires lifelong immunity
- **In India,** still 40% females of reproductive age group are susceptible to rubella infection
- **Period of communicability** of rubella is about 1 week before to 1 week after the appearance of rash
- **Transmission** occurs via—airborne droplet, transplacental and rarely via contact, and sexual modes
- **Occurrence:** Rubella occurs worldwide and throughout the year with a peak in the spring. Epidemics occur every 6-8 years, with explosive pandemics every 20-25 years
- **The largest rubella epidemic** (postnatal) occurred globally in 1962-1965. However; with the onset of vaccine use, the epidemics are less encountered nowadays
- **World:** In 2017, 11,675 number of confirmed rubella cases reported globally. Among WHO regions, African region accounted for maximum cases
- **India:** India accounts for the maximum cases of the world (2,476 in 2017). There was an outbreak of rubella reported from Rajasthan in 2014
- **Genotype:** Based on E1 protein coding region, Rubella has been typed into 13 genotypes; four of which are commonly circulating in world: 1E, 1G, 1J, and 2B. Genotype 2B is the predominant type in World and also from India
- **Congenital rubella** remains an important public health problem globally
- WHO has launched the global strategic plan (2012-20) for control of rubella and congenital rubella syndrome along with measles elimination by 2020.

TREATMENT **Rubella**

Rubella is a mild, self-limited illness and no specific treatment is available.

PREVENTION

General Preventive Measures

Airborne precaution must be followed while handling rubella cases (refer Chapter 53 for detail).

Rubella Vaccine

RA 27/3 is a live attenuated vaccine for rubella, prepared from human diploid fibroblast cell line.

- It is available singly or in combination with vaccines of mumps and measles (MMR vaccine)
- **Schedule:** Single dose (0.5 mL) of vaccine is administered subcutaneously
- Following vaccination, seroconversion occurs in 90% of recipients and immunity persists for 14-16 years or probably lifelong
- **Indication:** In India, rubella vaccine is indicated in all women of reproductive age (first priority group) followed by all children (1-14 years). Under national immunization schedule, rubella vaccine is given along with measles (MR vaccine) at 9-12 months of age and second dose at 16-24 months at selected states
- **Precautions:**
  - Vaccine is contraindicated in pregnancy
  - As it is teratogenic, pregnancy should be avoided at least for 4 weeks (28 days) following vaccination
  - However, if a woman conceives <4 weeks following vaccination: wait and watch policy should be followed. No immediate termination of pregnancy is required
  - Infants below 1 year should not be vaccinated due to possible interference from persisting maternal antibody.

EXPECTED QUESTIONS

I. **Essay:**

1. In early 2018, a 62-year-old debilitated man from Maharashtra presented with symptoms of severe upper respiratory tract infection. He had a history of exposure to a patient having similar condition. Throat swab collected was sent to the reference laboratory for real time PCR which revealed that causative agent could be a segmented RNA virus.
   a. What is the most probable etiological diagnosis and the mechanism of emergence of this particular strain of the virus?
   b. Describe the pathogenesis, mode of transmission and laboratory diagnosis of the causative agent.
   c. Add a note on the epidemiological impact of the recent 2017 epidemic in India produced by this causative agent.

d. What are the preventive measures available for this condition?

2. Describe the pathogenesis and laboratory diagnosis of measles virus infection.

II. Write short notes on:

a. Mumps.
b. H1N1 2009 pandemic flu.
c. Avian flu.
d. Congenital rubella syndrome.

III. Multiple Choice Questions (MCQs):

1. **The type-specific antigen (A, B, or C) of influenza viruses is found on which viral constituent?**
   a. Hemagglutinin
   b. Neuraminidase
   c. Nucleocapsid
   d. Lipid in the viral envelope
2. **Which of the following statements concerning antigenic drift in influenza viruses is correct?**
   a. It results in major antigenic changes
   b. It is exhibited only by influenza A viruses
   c. It is due to frame-shift mutations in viral genes
   d. It occurs frequently than antigenic shift
3. **Which of the following statements about congenital rubella syndrome is correct?**
   a. Following vaccination, seroconversion occurs in 40% of recipients
   b. Congenital abnormalities occur when a nonimmune pregnant woman is infected at any time during pregnancy
   c. Deafness is a common defect associated with congenital rubella syndrome
   d. Only rare strains of rubella virus are teratogenic
4. **Which of the following paramyxoviruses has a surface glycoprotein lacking hemagglutinin activity?**
   a. Measles virus
   b. Mumps virus
   c. Parainfluenza virus type 1
   d. Respiratory syncytial virus
5. **Most common cause of secondary bacterial pneumonia occur in patients infected with influenza virus:**
   a. *Staphylococcus* b. *E.coli*
   c. Pneumococci
   d. *Haemophilus influenzae*
6. **Reye's syndrome is a complication seen after all the following viral infections, *except*:**
   a. Influenza B b. Varicella-zoster
   c. Influenza A d. Measles
7. **Which of the following statement is not correct, for the management of patient with category A influenza like illness?**
   a. Laboratory testing for H1N1 is not required
   b. Treatment with oseltamivir is required
   c. Confine the patients at home
   d. Avoid contact with public and high-risk members in the family
8. **About Global Influenza Surveillance and Response System (GISRS), which statement(s) is/are correct?**
   a. It monitors the evolution of influenza viruses
   b. Provides recommendations in areas including laboratory diagnostics, vaccines and treatment.
   c. Serves as a global alert mechanism for the emergence of influenza viruses with pandemic potential
   d. All of the above
9. **According to CDC recommendation, the people with influenza-like illness remain at home until how much time after they are free of fever (<100°F) without the use of fever-reducing medications.**
   a. at least 24 hours b. at least 48 hours
   c. at least 7 days d. at least 10 days
10. **The trivalent vaccine for influenza includes all, *except*:**
    a. A/H1N1 b. A/H5N1
    c. A/H3N2 d. Influenza B strain
11. **Chemoprophylaxis for influenza is recommended for all the following situations, *except*:**
    a. Routine seasonal pre-exposure prophylaxis
    b. During outbreak situations in hospitals
    c. Following exposure to an influenza case if not vaccinated or vaccinated recently (<2 weeks)
    d. Following exposure to an influenza case if the individual is HIV infected
12. **Which of the following statement about mumps is not correct?**
    a. Bilateral parotitis is the most common presentation
    b. Other salivary glands are never involved.
    c. Atypical mumps presents as meningitis
    d. Incubation period is about 19 days
13. **The correct sequence of manifestation seen in measles is:**
    a. Fever → Koplik's spot → rash
    b. Koplik's spot → fever → rash
    c. Rash → Fever → Koplik's spot
    d. Rash → Koplik's spot → Fever
14. **Subacute sclerosing panencephalitis (SSPE) is a complication following which viral infection?**
    a. Mumps b. Measles
    c. Rubella d. Influenza
15. **Which virus-vaccine strain combination is not correct?**
    a. Mumps—Jeryl-Lynn strain
    b. Measles—Edmonston-Zagreb strain
    c. Rubella—Schwarz strain
    d. Chickenpox—Oka strain
16. **Following rubella vaccination, pregnancy should be avoided for at least how many days?**
    a. 1 month b. 2 months
    c. 3 months d. 6 months

Answers

1. c **2.** d **3.** c **4.** d **5.** a **6.** d **7.** b **8.** d **9.** a **10.** b **11.** a **12.** b **13.** a **14.** b **15.** c **16.** a

Picornaviruses

45
CHAPTER

Chapter Preview

- Morphology
- Enteroviruses
  - Poliovirus
  - Coxsackievirus
  - Echovirus
  - Parechovirus
  - Enteroviruses 68–116
- Rhinoviruses

CLASSIFICATION

Picornaviruses belong to the family Picornaviridae; which include two major groups of human pathogens: enteroviruses and rhinoviruses.

Enteroviruses: They are transmitted by feco-oral route.

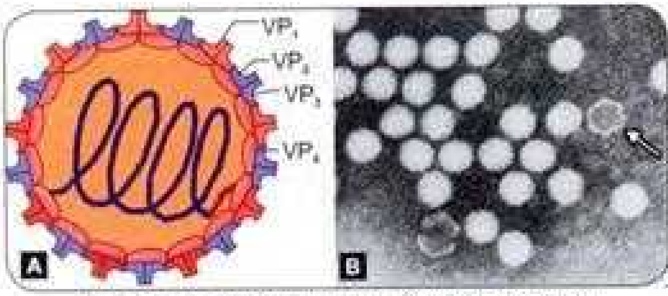
- Though they multiply in the intestine, they do not cause any intestinal manifestations
- They are associated with various systemic manifestations including *poliomyelitis* (a childhood flaccid paralysis), which is in the verge of eradication globally
- Enteroviruses comprise of 96 human serotypes:
  - Poliovirus (3 serotypes)
  - Coxsackievirus—Coxsackie A (1-24 serotypes), Coxsackie B (1-6 serotypes)
  - Echovirus (1-33 serotypes)
  - Parechovirus (1-3 serotypes)
  - Enteroviruses 68-116 (serotype 72 is reclassified as Hepatitis A virus).

Rhinoviruses: They are transmitted by respiratory route and cause common cold.

MORPHOLOGY

Picornaviruses are simple in structure, very small (28-30 nm size) and nonenveloped.

- They are spherical shaped and have icosahedral symmetry (Figs 45.1A and B)
- Capsid is composed of 60 subunits, each consisting of four viral proteins (VP1-VP4), except parechoviruses (have three proteins)
- Possess single-stranded positive sense linear RNA
- **Resistance:**
  - Enteroviruses are stable at acidic pH, whereas rhinoviruses are acid-labile



Figs 45.1A and B: Poliovirus. **A.** Schematic diagram; **B.** Transmission electron micrograph (arrow showing)

Source: B. Public Health Image Library, ID#235/Dr Joseph J Esposito, Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

  - *Heat labile:* All enteroviruses and some rhinoviruses are inactivated by heating at 55°C for 30 minutes, which can be prevented by magnesium chloride.

ENTEROVIRUS GROUP

POLIOVIRUSES

Antigenic Types

There are three antigenic types of polioviruses. Immunity (antibody response) is type-specific.

1. **Type-1** (Brunhilde and Mahoney strains): It is the most common serotype to cause epidemics of poliomyelitis. This serotype is responsible for all the natural cases of poliomyelitis occurring globally at present.
2. **Type-2** (Lansing and MEF-1 strain): It is the most antigenic serotype and hence is the easiest serotype to be eradicated.
   - No natural case of poliomyelitis caused by serotype 2 has been reported since 1999

  - However, the vaccine strains can cause poliomyelitis and it is the most common serotype found among the VDPV strains (vaccine derived poliovirus).
3. **Type-3** (Leon and Saukett strain)
  - No natural case caused by serotype-3 has been reported since 2013
  - However, the vaccine strains have the potential to cause paralytic poliomyelitis
  - It is considered as the most common serotype to cause VAPP (vaccine-associated paralytic poliomyelitis).

Poliovirus has two antigens: C and D

- The *D antigen* (dense): It is associated with the whole virion and is type-specific. Anti-D antibodies are protective and therefore the potency of the injectable polio vaccine can be measured in terms of D antigen units
- The *C antigen* (capsid): It is associated with the noninfectious virus and is less specific. Anti-C antibody does not neutralize virus infectivity.

Pathogenesis

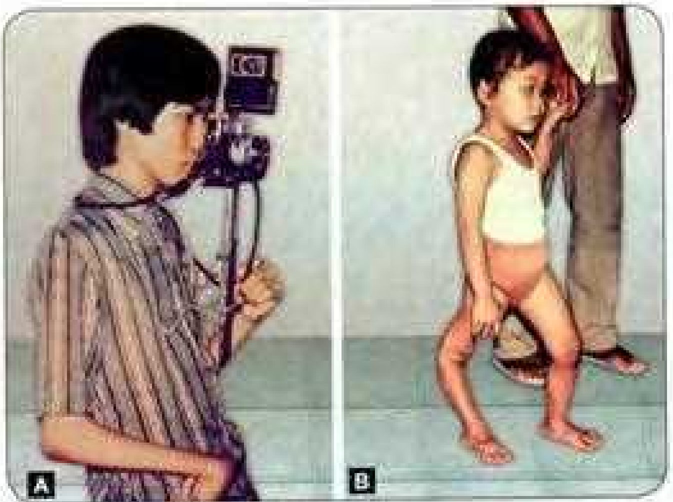
Polioviruses are transmitted by feco-oral route (most common), followed by respiratory droplets via inhalation or rarely by conjunctival contact

- **Multiply locally:** They multiply in intestinal epithelial cells, submucosal lymphoid tissues, tonsils and Peyer's patches
- **Receptor:** Viral entry into the host cells is mediated by binding to *CD155* receptors present on the host cell surface
- **Spread to CNS/spinal cord:**
  - **Hematogenous spread** (most common): Virus spreads to the regional lymph nodes and spills over to the bloodstream (primary viremia). After further multiplying in the reticuloendothelial system, the virus enters the bloodstream again, causing secondary viremia. Then it is carried to the spinal cord and brain
  - **Neural spread:** Virus may also spread directly through nerves. This occurs especially following tonsillectomy where the virus may spread via glossopharyngeal nerve present in the tonsillar fossa.
- **Site of action:** The final target site for poliovirus is the motor nerve ending, i.e. anterior horn cells of the spinal cord which leads to muscle weakness and flaccid paralysis
- **Neuron degeneration:** Virus-infected neurons undergo degeneration. Earliest change in neuron is the degeneration of **Nissl body** (aggregated ribosomes, normally found in the cytoplasm of neurons)
- Pathological changes are always more extensive than the distribution of paralysis.

Clinical Manifestations

The incubation period is usually 7–14 days. The manifestations may range from asymptomatic stage to the most severe paralytic stage.

- **Inapparent infection:** Following infection, the majority (91–96%) of cases are asymptomatic
- **Abortive infection:** About 5% of patients develop minor symptoms such as fever, malaise, sore throat, anorexia, myalgia, and headache
- **Nonparalytic poliomyelitis:** It is seen in 1% of patients, presented as aseptic meningitis
- **Paralytic poliomyelitis** is the least common form (<1%) among all the stages:
  - It is characterized by descending asymmetric acute flaccid paralysis (AFP)
  - Proximal muscles are affected earlier than the distal muscles; paralysis starts at hip → proceeds towards extremities; which leads to the characteristic tripod sign (child sits with flexed hip, both arms are extended towards the back for support) (Fig. 45.2)
  - **Sites involved** can be spinal, bulbospinal and bulbar. Accordingly, the nature of paralysis varies (e.g. respiratory insufficiency or dysphagia are common in bulbar involvement)
  - **Biphasic course:** In children, the disease progression is typically biphasic; aseptic meningitis occurs first → recovery → return of fever with paralytic features 1–2 days later
  - Cranial nerves are also involved occasionally
  - However, there is no sensory loss.
- **Risk factors:** Paralytic disease is more common among:
  - Older children and adults
  - Pregnant women



Figs 45.2A and B: Deformities seen in poliomyelitis

Source: Public Health Image Library, A. ID#: 5579; B. ID#5578, Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

- Following heavy muscular exercise
- Persons undergoing trauma at the time of CNS symptoms
- Tonsillectomy: It predisposes to bulbar poliomyelitis
- IM injections: They increase the risk of paralysis in the involved limb.

❖ **Postpolio muscle atrophy syndrome:** A recrudescence of paralysis and muscle wasting has been observed in individuals, usually decades (20–40 years) after the episode of paralytic poliomyelitis.

LABORATORY DIAGNOSIS	Polioviruses
❑ Virus isolation in primary monkey kidney cell line from throat swabs, rectal swabs or stool samples—viral growth is detected by CPE, viral antigen or viral gene in cell line	
❑ Antibody detection—by neutralization test and CFT	

Laboratory Diagnosis

Virus Isolation

❖ **Specimen:** Poliovirus may be recovered from the throat swabs (up to 1 week of illness) and from rectal swabs or stool samples (up to 6–8 weeks). However, long-term excretion has been observed in immunodeficient persons. Virus isolation from CSF or blood is very rare

❖ **Sewage testing:** Screening of sewage for detection of poliovirus (wild or vaccine virus) is routinely conducted under polio eradication program. This is to verify whether the transmission is on-going or interrupted

❖ **Transport:** Specimens should be kept frozen during transport to the laboratory

❖ **Cell line:** Primary monkey kidney cells are the most recommended cell lines. Virus growth can be identified by various methods
- Cytopathogenic effects appear in 3–6 days; described as crenation and degeneration of the entire cell sheet
- Antigen detection: Isolated virus can be identified and serotyped by neutralization with specific antiserum or by immunofluorescence test
- Polymerase chain reaction (PCR) assay is available targeting VP1 region of poliovirus.

Antibody Detection

A rise in antibody titer in paired sera collected 1–2 weeks interval is suggestive of poliomyelitis

❖ Both neutralizing antibodies (neutralization test) and complement fixing antibodies (complement fixation test) can be detected

❖ Only first infection with poliovirus produces strictly type-specific responses

❖ Subsequent infections induce antibodies against group-specific antigen common to all the three serotypes.

Molecular Method

Real-time multiplex reverse-transcriptase PCR has been developed using primers from VP1 region, which can detect and differentiate between various types of wild and vaccine polioviruses (VAPP and VDPV strains) directly from stool specimen.

Vaccine

Both inactivated and live attenuated polio vaccines are available; both have their own unique useful properties as well as drawbacks (Table 45.1).

Injectable Polio Vaccine (IPV, Salk Vaccine)

❖ **Discovery:** Jonas Salk had prepared IPV in HeLa cells in 1952. It was announced to the world by Dr Thomas Francis in 1955

❖ **Cutter incident** (named after the manufacturer): An outbreak of vaccine induced paralytic poliomyelitis had occurred in America (1955) that had killed more than 100 people. It was due to improper inactivation of IPV. Vaccine was modified later, after which it has been completely safe

❖ **Preparation:** Virus is grown in **monkey kidney cell line** and inactivated by formalin. Each dose (0.5 mL) of vaccine contains total 80 units of D-antigen of all the three poliovirus serotypes (40 units of type 1, 8 units of type 2, and 32 units of type 3)

❖ **Dose:** IPV can be given either as (i) full dose (0.5 mL/dose), intramuscular (IM) route at thigh; or (ii) as fractional dose (f-IPV): 1/5th of full dose (0.1 mL), intradermal (ID) route at upper arm

❖ **National immunization Schedule (India):**
- In 2015, IPV was introduced in national immunization program as single full dose (IM route) at 14th week along with bivalent OPV
- Since 2017, f-IPV is administered by ID route scheduled at 6th and 14th weeks of age along with bivalent OPV
- This change was made because (i) 2 f-IPV, given by ID route at 6 and 14 weeks had shown to provide higher seroconversion rates than a single full dose (IM) given at 14 weeks and (ii) cost saving (0.2 mL/2 doses of f-IPV vs 0.5 mL of full dose IPV).

❖ **Efficacy** of 80–90% is achieved after the full course of vaccination

❖ **Advantages:**
- IPV is much safer than oral polio vaccine (OPV), safer even in immunocompromized people
- It does not cause vaccine-associated paralytic polio (VAPP)
- It is more stable, does not require stringent storage conditions.

❖ **Disadvantages:**
- It does not provide herd immunity: Being inactivated vaccine, it cannot spread by feco-oral route
- It is not useful during epidemics; as there is no community protection. Instead, it can precipitate paralysis

Table 45.1: Differences between injectable and oral polio vaccines

Polio vaccine	Salk (injectable)	Sabin (oral)
Preparation	Formalin killed preparation in MKC (Monkey kidney cell line) **Total 80 units of D-antigen of:** • Type 1 (40 units) • Type 2 (8 units) • Type 3 (32 units)	**OPV is available as :** • Trivalent OPV (serotypes 1,2 and 3)- Each dose contains TCID50 of: Type 1: 3 lakhs, Type 2: 1 lakh, Type 3: 3 lakhs. • Bivalent OPV (serotype 1 and 3) • Monovalent OPV (any one serotype)
National immunization Schedule (India, 2018)	Two fractional doses (intra-dermal route at upper arm, 0.1 mL/dose): Given at 6th and 14th weeks of age along with OPV	Total five doses (2 drops/dose, oral) of bivalent OPV (serotype 1 and 3): Zero dose—given at birth 1st, 2nd and 3rd dose —given at 6th, 10th, 14th week of age, Booster: 16–24 months
Safety	Relatively safer than OPV	Safe except in immunocompromized patients, pregnancy, old age
Efficacy	80–90% by full course of IPV Immune response is slower than OPV	90–100% efficacy is achieved even by 1 or 2 doses of OPV Efficacy decreases by: • Interference by other enteroviruses • Diarrheal diseases • Breastfeeding
Economy	Relatively expensive	Economical
Duration of protection	Short, need booster doses periodically	Long-lasting
In epidemics	Can precipitate paralysis	Can be used safely
Herd immunity	Not provided	Provided due to feco-oral spread of vaccine virus
Local immunity	Weakly stimulated	Strongly stimulated (due to IgA antibody)
Can prevent	Only paralysis	Paralysis and intestinal re-infection
Storage condition	Relatively stable Does not require stringent condition	Should be stored at (–20°C) Stabilized in $MgCl_2$, pH<7
VAPP and VDPV	Zero chance	Relatively more chance

Abbreviations: OPV, oral polio vaccine; IPV, injectable polio vaccine; VAPP, vaccine-associated paralytic poliomyelitis; VDPV, vaccine-derived polioviruses; MKC, monkey kidney cell lines; TCID, tissue culture infective dose.

- It does not induce mucosal IgA production, hence, the local immunity is absent
- It is relatively expensive than OPV.

Oral Polio Vaccine (OPV, Sabin Vaccine)

- **Discovery:** OPV was developed by Albert Sabin, Koprowski and Cox who prepared OPV independently almost around the same time (1955)
- **Formulations:** OPV is available as (i) trivalent OPV (contains serotypes 1,2 and 3); (ii) bivalent OPV (contains serotype 1 and 3) and (iii) monovalent OPV (contains any one serotype)
- **Preparation:** Each dose (0.5 mL) contains type 1 virus (3 lakh), type 2 virus (1 lakh, absent in bivalent OPV), type 3 virus (3 lakh) of TCID50 (tissue culture infective dose-50)
- **Markers of attenuation:**
  - **Phenotypic markers** were used in the past to confirm attenuation. Vaccine virus will not grow in presence of low levels of bicarbonate, at 40°C, or in monkey kidney cell lines and it should be inactivated by specific antisera (McBride's marker)
  - **Genotypic markers:** Currently, attenuation is confirmed by detecting specific genes of attenuated virus, which are absent in wild virus.
- **National immunization schedule:** OPV is the vaccine recommended under national immunization schedule of India and most other countries of the world
  - Earlier trivalent OPV was used. As serotype 2 is eradicated from the world since 1999, and there is a high risk of causing vaccine virus induced paralysis, trivalent OPV is stopped and is replaced by bivalent OPV (1 and 3) since 2015
  - It is administered orally (two drops/dose). Total five doses are given
    - Zero dose: Given at birth
    - 1st, 2nd, 3rd doses: Given at 6,10 and 14th weeks. (at 6th and 14th week, IPV is given along with bivalent OPV)
    - Booster dose: Given at 16–24 months of birth.
- **Efficacy** is about 90–100%, which is achieved much faster (with one or two doses than IPV)
- **Advantages:**
  - **Herd immunity:** OPV strains being live, can shed in the feces and spread in the community by feco-oral

route, hence, it can induce herd immunity. It can provide both individual and community protection

- OPV is the vaccine of choice during epidemics
- **Local immunity:** OPV induces mucosal IgA production, hence provides local or mucosal immunity
- Cheaper than IPV
- Easy to administer (given by oral route).

❖ **Disadvantages:**

- **Safety:** OPV is otherwise safe, but it is risky to give in immunocompromized people, during pregnancy, and in old age
- **Stability:** OPV is unstable vaccine, requires stringent conditions such as:
  - Storage at (–20°C)
  - Stabilized in $MgCl_2$
  - Maintenance of pH <7.
- **Efficacy of OPV decreases by:**
  - **Interference** by other enteroviruses
  - **Diarrhea:** OPV gets washed away in diarrheal stool. However, OPV can be given during diarrhea for community protection, but such a dose should not be counted. After recovery from diarrhea, again a repeat dose should be given
  - **Breastfeeding:** OPV gets washed away in stool if given immediately before or after breastfeeding. Hence, breastfeeding should be avoided for 30 min before or after administration of OPV.
- OPV can cause vaccine-associated paralytic poliomyelitis (VAPP) and vaccine-derived polioviruses (VDPV) (described below).

VAPP and VDPV

Vaccine-associated Paralytic Poliomyelitis (VAPP)

VAPP denotes all the cases of paralytic poliomyelitis that occur following OPV administration.

- VAPP strains are **OPV-like isolates**, which show limited genetic divergence from their parental OPV strains (<1% divergence)
- VAPP cases are ubiquitous in places where OPV is used extensively
- VAPP can occur among OPV recipients as well as to their close contacts due to feco-oral spread
- However, VAPP strains are not capable of circulating in the community and do not cause secondary cases or outbreaks. This is largely because the spread of OPV-related virus is largely limited by high population immunity
- **VAPP rate:** VAPP occurs approximately at a rate of one case per 2.7 million doses of OPV
- **VAPP occurs more frequently**
  - Following the first dose of OPV than the subsequent doses
  - Among people with primary immunodeficiency disorder (↑ risk by 3000 fold)

Contd...

Contd...

- **Most common serotype** associated with VAPP is Sabin type 3 (60%) followed by Sabin type 2(40%).

Vaccine-derived polioviruses (VDPVs)

VDPV isolates exhibit a higher level of genetic divergence from their parental OPV strains at VP1 sequence, which helps in their prolonged replication, and transmission.

- The genetic divergence of VDPVs from parental OPV strains is about:
  - More than 1% for Sabin types 1 and 3
  - More than 0.6% for Sabin type 2.
- Isolates showing genetic divergence lower than this cutoff are considered as **OPV-like isolates**
- VDPV isolates are indistinguishable from wild polioviruses both clinically (due to regain of neurovirulence) and phenotypically (due to reversal of markers of attenuation)
- Most VDPV isolates belong to Sabin type 2 (90%) followed by type 1. This is because wild type-2 strains are already eradicated and not circulating in the community since 1999.

VDPVs can be categorized into three types:

1. **Circulating VDPVs (cVDPVs):** These strains are capable of person-to-person transmission in the community and can cause outbreaks in areas with low OPV coverage.
   - They pose the same threat to the community as that of wild polioviruses
   - Most cVDPV isolates have vaccine/non-vaccine recombinant genomes, i.e. genetic recombination occurs between vaccine strains and other enteroviruses that facilitates person-to-person transmission
   - Since 2000, cVDPV outbreaks have occurred in several countries, with majority (90%) of reported cases are associated with **type 2** followed by type-1. All cVDPV reported in 2017 are type 2
   - In 2013, over 700 strains of VDPVs have been isolated worldwide including India. Nigeria was worst affected, accounting for half of those strains.
2. **Immunodeficiency-associated VDPVs (iVDPVs):** They are isolated from persons with primary immunodeficiency disorder.
   - Such patients do not develop disease, but excrete the iVDPVs for many years
   - Contacts infected with iVDPV strains are at ↑ risk of developing paralytic poliomyelitis
   - iVDPVs exhibit greater genetic diversity than cVDPVs-some strains may be diverse by >10%
   - The extent of sequence divergence is proportional to the duration of the infection
   - Unlike cVDPV, infections due to iVDPV cannot be prevented by high OPV coverage
   - iVDPV strains show heterogeneity within a serotype; leading to mixed virus populations.
3. **Ambiguous VDPVs (aVDPVs):** They are heterogeneous; They are either cVDPVs for which only 1 case isolate had yet been detected, or they may be sewage isolates obtained from developed countries with unknown source (probably iVDPV).

Epidemiology

- **Reservoir:** Man is the only known reservoir. Most cases are subclinical
- **Clinical-subclinical ratio:** For every clinical case, there may be 1,000 children and 75 adults of subclinical cases
- There are **no chronic carriers**. However, immunodeficient individuals may excrete the virus for longer periods
- **Source:** Infective materials such as stool and oropharyngeal secretions are the sources of infection
- **Age:** Younger children and infants are more susceptible to infection than adults. However in developed countries, there is shift of age; affecting older children
- **Period of communicability:** Patients are infectious, shedding the virus in the feces from 7–10 days before the onset of symptoms up to 2–3 weeks thereafter, sometimes as long as 3–4 months.

Polio Eradication

Poliomyelitis is now at the verge of eradication. This is attributed to the extensive immunization program being conducted globally.

Pulse Polio Immunization (PPI) was initiated globally to eradicate poliomyelitis. In India, it is in operation since 1995–96.

- Two rounds of PPI (6 weeks apart) are scheduled every year during the winter season (for e.g. 18th January and 11th March in 2018), where all children under the age of five years are vaccinated with OPV irrespective of their OPV vaccination status
- PPI doses of bivalent-OPV are considered as extra doses and they do not replace the OPV doses received under the routine national immunization schedule
- AFP Surveillance: Acute flaccid paralysis surveillance has been conducted to identify all remaining infected areas and to monitor progress towards eradication.

Polio Situation in the World

Based on the status of polio transmission, the countries are classified into: (i) endemic country, (ii) outbreak country, (iii) key at-risk country and (iv) polio free country (Table 45.2).

- **Endemic (PAN) countries:** Currently, wild polio is endemic only in three countries—Pakistan, Afghanistan and Nigeria (abbreviated as PAN countries). Nigeria reported the last wild case on August 2016
- **Wild poliovirus (wPV) cases:**
  - In 2017, 22 wild cases were reported globally (Afghanistan-14, Pakistan-8). However, there were 95 vaccine derived cases (cVDPV-2) reported from non-endemic countries such as Democratic Republic of the Congo (21) and Syrian Arab Republic (74).
- Currently, all natural cases due to wPV are caused by type-1. No natural cases due to Type-2 and 3 have been reported since 1999 and 2013 respectively

Table 45.2: Classification of countries based on their polio transmission status as of July 2018*

Endemic country	Ongoing transmission of indigenous wild poliovirus	PAN countries • Pakistan • Afghanistan • Nigeria
Outbreak country	Stopped indigenous wild poliovirus but are experiencing re-infection either by: • importation of wild or VDPV from another country, or • emergence and circulation of VDPV To stop these outbreaks, it is necessary to implement outbreak response guidelines	Democratic Republic of the Congo Syrian Arab Republic Somalia
Key At-Risk Countries	Low levels of immunity and surveillance leave countries at risk of polio returning	17 countries Cameroon, Central African Republic, Chad, Equatorial Guinea, Ethiopia, Guinea, Iraq, Kenya, Lao People's Democratic Republic, Liberia, Madagascar, Myanmar, Niger, Sierra Leone, Somalia, South Sudan, and Ukraine
Polio free countries	No transmission of indigenous wild poliovirus. No wild case for ≥3 years	India and rest 194 countries

*Source: Global Polio Eradication Initiative (GPEI).
Abbreviation: VDPV, vaccine-derived poliovirus.

- **India** has been declared polio-free since March 2014, the last natural case was detected three years back (January 2011)
- **The Global Polio Eradication Initiative (GPEI)** had launched 'Eradication and Endgame Strategic Plan' (2013–2018) aiming to wipeout polio from the entire world by 2018-2020 (see the box below).

Endgame Strategic Plan (2013–2018)

GPEI had initiated an end game strategic plan for polio eradication, which has four objectives.

1. **Interruption of wild poliovirus transmission.**
2. **Strengthening immunization systems** by step wise withdrawal of OPV along with switching over to IPV.
3. **Implementing containment of polioviruses** and to certify complete interruption of transmission and polio free by 2018.
4. **Legacy planning:** The infrastructure, fund, man-power, knowledge and experience that have been created through the global polio eradication program will be utilized to support other health programs following postpolio eradication.

Though eradication may not be achieved within the target time line (2018); but it is expected that world is soon going to achieve this milestone in coming days.

COXSACKIEVIRUSES

Coxsackieviruses (named after the place of discovery; Coxsackie village in USA) can be divided into two groups, A and B, based on their pathogenic potentials for suckling mice.

Serotypes: Group A coxsackieviruses are typed into serotypes 1-24 (except 15, 18 and 23) and group B are typed into serotypes 1-6.

Clinical Manifestations

Coxsackieviruses produce a variety of clinical illnesses in humans associated with different serotypes. The incubation period ranges from 2 to 9 days.

- **Aseptic meningitis:** It is caused by all types of group B coxsackieviruses and by many group-A coxsackieviruses (most commonly A7 and A9)
- **Herpangina:** It is a severe febrile vesicular pharyngitis that is caused by certain group A viruses (type 2-6, 8, 10)
- **Hand-foot-and-mouth disease:** It is characterized by oral and pharyngeal ulcerations and vesicular rashes of the palms and soles which heal without crusting. It is particularly associated with coxsackievirus A16
- **Pleurodynia** (also known as **Bornholm disease** or epidemic myalgia): It is caused by coxsackie B viruses. It is characterized by fever and abrupt onset of stabbing chest pain
- **Cardiac:** Myocarditis and pericarditis are caused by coxsackievirus B types 1-5
- **Respiratory:** Coxsackieviruses A and B have been associated with common colds. Pneumonia may be caused by coxsackieviruses B4 and 5
- **Acute hemorrhagic conjunctivitis:** It is caused by coxsackie-A24 and enterovirus 70
  - It is a self-limiting subconjunctival hemorrhage. Incubation period is about 1 day. Complete recovery occurs within 8-10 days
  - It had caused explosive epidemics among adults, during 1969-71 in Africa and Southeast Asia.
- **Generalized disease of infants:** It is an extremely serious disease involving multiple organs, caused by group B coxsackieviruses
- **Pancreatitis** leading to juvenile diabetes mellitus is caused by coxsackie B4.

The differences between group A and B coxsackieviruses are given in Table 45.3.

Laboratory Diagnosis

- **Specimen collection** depends on the type of infection. Important specimens include throat swabs, stool and CSF
- **Isolation of the virus:** Coxsackieviruses can be recovered by:
  - Intracerebral inoculation into suckling mice:
    - Coxsackie-A produce flaccid paralysis
    - Coxsackie-B produce spastic paralysis.
  - **Inoculating into tissue culture:** Cytopathic effect can be observed within 5-14 days.
- **PCR** targeting specific genes (e.g. VP1) is highly useful as it is rapid, more sensitive and serotype-specific
- **Serology** is performed to detect neutralizing antibodies.

Table 45.3: Differences between group-A and group-B Coxsackieviruses

Group A coxsackieviruses	Group B coxsackieviruses
Suckling mouse intracerebral inoculation	
• Flaccid paralysis • Generalized myositis	• Spastic paralysis in mice • Focal myositis and necrosis of brown fat
Manifestations	
• Aseptic meningitis (A7,A9) • Herpangina (vesicular pharyngitis) • Hand-foot-and-mouth disease (also by enterovirus: 71) • Acute hemorrhagic conjunctivitis: Caused by coxsackie virus-A24 (and also by enterovirus 70)	More organ involvement seen • Aseptic meningitis (B1-6) • Pleurodynia (epidemic myalgia or Bornholm disease) • Myocarditis, pericarditis • Hepatitis • Pancreatitis leading to Juvenile diabetes mellitus: Coxsackie B4 • Pneumonia • Generalized disease of infants

OTHER ENTEROVIRUSES

Enteroviruses 68–116

- Enterovirus 68—causes pneumonia
- Enterovirus 70—causes **acute hemorrhagic conjunctivitis**. It uses CD55 as host cell receptor
- Enterovirus 71—causes aseptic meningitis, encephalitis, hand-foot-and-mouth disease, herpangina, pulmonary edema and paralysis resembling poliomyelitis
- Enterovirus 72 is reclassified as hepatitis A virus.

Echoviruses

Echoviruses (enteric cytopathogenic human orphan viruses) infect the human intestine and they can be isolated in certain tissue cultures. They were named 'orphan' viruses because at the time of their discovery, they were not attributed to any disease.

- Echoviruses are further typed into serotypes 1-33 (there are no types—10, 22, 23 or 28), but not all cause human illness
- They are associated with aseptic meningitis, encephalitis, rashes, common cold, and ocular disease
- They can cause outbreaks in summer especially among children
- Association with the disease is confirmed if the virus is isolated from body fluids (such as CSF) and antibodies are found in patient's sera.

Parechoviruses

Parechoviruses have three serotypes:

1. Serotype 1 and 2 were previously classified as echoviruses 22 and 23 respectively.

2. Their capsid consists of three viral proteins (in contrast to four proteins in most picornaviruses).
3. They have been rarely associated with aseptic meningitis, respiratory and neonatal diseases.

RHINOVIRUS GROUP

Rhinoviruses are the most common cause of common cold. Human rhinoviruses comprise of three species (A, B and C) and >150 antigen types.

- They use host cell intercellular adhesion molecule-1 (ICAM-1) as receptor
- More than 100 serotypes have been identified
- They are similar to enteroviruses in structure and properties except that:
  - Acid-labile (unstable below pH 6)
  - Transmission is by close respiratory contact via infected secretions
  - Optimal temperature for growth is 33°C (in contrast to 37°C for enteroviruses).
- **Clinical features:**
  - The incubation period is about 2–4 days
  - **Common cold syndrome:** Rhinoviral symptoms are similar to that of any other viruses causing common cold syndrome such as coronaviruses, adenoviruses, enteroviruses, parainfluenza viruses, and influenza viruses
  - Usual symptoms in adults include sneezing, nasal obstruction, nasal discharge, and sore throat, but no fever
  - Secondary bacterial infection may produce otitis media, sinusitis, bronchitis, or pneumonitis, especially in children.
- Relapse: The average adult gets 1–2 attacks each year
- Rhinoviruses are infectious only for humans, gibbons, and chimpanzees
- **Laboratory diagnosis:** Rhinoviruses can be grown in human diploid cell lines such as WI-38 and MRC-5 cell lines. Organ cultures of ferret and human tracheal epithelium may be necessary for some fastidious strains. Most of the strains grow better at 33°C (nasopharynx temperature) but not at 37°C
- **Treatment** is supportive (i.e. symptomatic treatment).

EXPECTED QUESTIONS

I. Essay:

1. A 9-week-old baby named Sweety was brought to the emergency room with weakness in her right leg. On examination, her right leg appeared flaccid and no deep tendon reflex or Babinski can be elicited, although sensation was intact. The tone, movement, sensation, and reflexes of her other limbs were normal. Her immunization records were up-to-date according to the national immunization schedule of India. CSF demonstrates elevated protein with normal glucose levels. Fecal sample was collected and then sent to the referral center where the poliovirus is identified as a vaccine strain (not the "wild-type" strain) of poliovirus type-1 was isolated.
   a. What is the probable clinical diagnosis of this condition?
   b. Add a note on the laboratory diagnosis.
   c. Mention the types of vaccines available against the etiological agent.

II. Write short notes on:

a. Polio vaccine.
b. Polio eradication.
c. Coxsackievirus.

III. Multiple Choice Questions (MCQs):

1. **Zero dose of OPV is given:**
   a. At one month
   b. At birth
   c. When child is having diarrhea
   d. When child is having polio
2. **Enterovirus 72 is:**
   a. Hepatitis A virus b. Hepatitis E virus
   c. Hepatitis B virus d. Hepatitis C virus
3. **Not true about salk vaccine:**
   a. Expensive than OPV
   b. Not useful in epidemics
   c. Contraindicated in low immunity
   d. Booster doses are required
4. **The most common viruses that can cause meningoencephalitis in children are:**
   a. Arboviruses b. Herpesviruses
   c. JE virus d. Enteroviruses
5. **As of 2018, Polio is endemic in all the following countries, *except*:**
   a. India b. Pakistan
   c. Afghanistan d. Nigeria
6. **Hand-foot-and-mouth disease is caused by:**
   a. HSV b. Coxsackie virus
   c. Measles d. Chickenpox
7. **Acute haemorrhagic conjunctivitis is caused by?**
   a. Adenovirus b. Coxsackie virus A24
   c. Coxsackie virus A16 d. Coxsackie virus B6
8. **Most common serotype associated with VAPP?**
   a. Type 1 b. Type 2
   c. Type 3 d. Any of the above
9. **Which is not a Vaccine-derived polioviruses (VDPVs)?**
   a. mVDPV b. cVDPV
   c. iVDPV d. aVDPV

Answers

1. b **2.** a **3.** c **4.** d **5.** a **6.** b **7.** b **8.** c **9.** a

Arboviruses

46

CHAPTER

Chapter Preview

- Togaviridae
  - Fever-arthritis group—Chikungunya virus and others
  - Encephalitis group
- Flaviviridae
  - Encephalitis viruses—Japanese B encephalitis virus and others
  - Hemorrhagic group—Dengue virus, yellow fever virus and others
  - Tick transmitted flaviviruses—Kyasanur Forest disease virus and others
- Bunyaviridae
- Reoviridae
- Rhabdoviridae

INTRODUCTION

Definition

Arboviruses (arthropod-borne viruses) are diverse group of RNA viruses that are transmitted by blood sucking arthropods (insect vectors) from one vertebrate host to another.

Viruses must multiply inside the insects and establish a lifelong harmless infection in them. Thus, the viruses that are just mechanically transmitted by insects are not included in this group.

Taxonomically Incorrect But Worldwide Accepted Group

The members of arboviruses have diverse physical and chemical properties. Taxonomically also, they belong to different families.

Still, the name 'arbovirus' is internationally accepted as the members under this group are comparable in many ecological and epidemiological factors—such as geographical distribution, mode of transmission (type of insect vector), clinical features and their control measures.

Classification

Members of arboviruses belong to five different families: Togaviridae, Flaviviridae, Bunyaviridae, Reoviridae and Rhabdoviridae.

Individual viruses under each family are named after various features such as:

- **Clinical features:** For example, yellow fever is named after its main clinical feature—jaundice
- **Place of discovery:** For example, Kyasanur Forest disease virus
- **Vector needed for transmission:** For example, sandfly fever virus
- **Peak season:** For example, Russian spring-summer encephalitis virus
- **Multiple features:** Japanese encephalitis virus is named after the place of discovery and clinical feature.

Clinical Manifestations

Arboviruses may also be arbitrarily divided based on the pattern of clinical syndromes they produce.

- Fever and/or rash, and/or arthralgia group
- Encephalitis group
- Hemorrhagic fever group.

However, some of them may be associated with more than one clinical syndromes, e.g. dengue virus.

Epidemiology

- **Zoonotic:** Several hundred arboviruses exist in the world and all are believed to be endemic in animals. However, only about 100 are human pathogens
- **Transmission cycle:** Arboviruses are maintained in the nature between animals and their insect vectors
- **Humans are the accidental hosts** and do not play any role in the maintenance or transmission cycle of the virus, except for urban yellow fever and dengue
- **Arthropod vector:** Most arboviruses are transmitted by mosquitoes (*Aedes, Culex* or *Anopheles*) followed by ticks, and rarely sandfly and other insects (Table 46.1)
- **Climatic variation:** Arboviruses are more prevalent in the tropics than temperate climate, due to abundance of appropriate animals and arthropods in the former
- **Geographical distribution:** Arboviruses vary greatly in their geographical distribution which in turn depends

Table 46.1: General features of arbovirus

Virus	Manifestation	Distribution	Vector	Reservoir
Family: Togaviridae				
Chikungunya virus	Fever and arthritis	Asia, Africa	*Aedes aegypti*	Monkeys *
O'Nyong-nyong virus	Fever and arthritis	Africa	*Anopheles*	*
Mayaro virus	Fever and arthritis	South America	*Aedes aegypti*	Monkeys
Ross River virus	Epidemic polyarthritis	Australia	*Aedes*	Small animals
Sindbis virus	Arthralgia, and rash	Africa, Europe, Australia	*Culex*	Birds, mammals
Semliki Forest virus	Fever and arthralgia	Africa	*Aedes*	Birds, rodents
Eastern equine encephalitis virus	Encephalitis	Eastern part of North America	*Aedes, Culex*	Birds
Western equine encephalitis virus	Encephalitis	Western part of North America	*Culex tarsalis, Aedes*	Birds
Venezuelan equine encephalitis virus	Encephalitis	South and Central America	*Aedes, Culex*	Horses
Family: Flaviviridae				
Japanese B encephalitis virus	Encephalitis	South East Asia	*Culex tritaeniorhynchus*	Pigs, Birds
St Louis encephalitis virus	Encephalitis	United States	*Culex*	Wild birds
West Nile encephalitis virus	Encephalitis	East Africa (Uganda), Algeria, Romania	*Culex, Aedes, Anopheles*	Birds
Murray Valley encephalitis virus	Encephalitis	America	*Culex annulirostris*	Birds
Rocio virus	Encephalitis	São Paulo, Brazil	*Culex*	*
Russian spring-summer encephalitis virus	Encephalitis	Central Europe, Russia	Tick	Rodents, other mammals, birds
Powassan virus	Encephalitis	America	Tick	Rodents
Louping-ill virus	Encephalitis	Europe	Tick	Sheep
Dengue virus	Hemorrhagic fever	India	*Aedes aegypti*	*
Yellow fever virus	Hemorrhagic fever	West Africa, Central South America	*Aedes aegypti*	Monkeys
Kyasanur Forest disease virus	Hemorrhagic fever	India (Karnataka)	Tick	Monkeys and rats
Omsk hemorrhagic fever virus	Hemorrhagic fever	Russia	Tick	Small mammals
Zika virus	Fever and arthritis	First occurred in Brazil, then spread to other countries	*Aedes aegypti*	Monkeys
Family: Bunyaviridae				
California encephalitis virus	Encephalitis	USA	*Aedes triseriatus*	Rodents
Oropouche virus	Rash and aseptic meningitis	Central and South America	*Culicoides paraensis*	Not known
Sandfly fever virus	Fever and myalgia	Southern Europe, North Africa, India	Sandfly	Small mammals
Rift Valley fever virus	Fever and myalgia	Africa	*Aedes*	Sheep, cattle
Crimean Congo hemorrhagic fever virus	Hemorrhagic fever	Africa	Tick	Small mammals
Ganjam virus	Fever	India	Tick	Small mammals
Severe fever with thrombocytopenia syndrome virus	Fever, thrombocytopenia	China, Korea	Tick	Sheep, goat, chicken
Family: Reoviridae				
Colorado tick fever virus	Fever, rarely encephalitis	America (mountains)	Tick	Rodents
Orungo virus	Fever	Sub-Saharan Africa	*Aedes*	*
Kemerovo virus	Fever, meningism	Russia	Tick	*
Family: Rhabdoviridae				
Vesicular stomatitis virus	Oral mucosal vesicles	Indiana	Sandfly	*
Chandipura virus	Encephalitis	India	Sandfly	*

* Not yet identified, though some studies have shown domestic dog can be infected with dengue virus.

on the various factors such as climatic condition and presence of vector. Viruses that are highly endemic in one place, may not be found in other areas (Table 46.1).

- Yellow fever is highly endemic in West Africa, but not found at all in India in spite of its vector *Aedes aegypti* being widely distributed in India

- **Encephalitic arboviruses:** Eastern, Western and Venezuelan equine encephalitis viruses are prevalent in North America where as in India, Japanese encephalitis virus is the most common arbovirus causing encephalitis.

❖ **Arboviruses found in India:** Over 40 arboviruses have been detected in India, of which three are highly endemic and produce several outbreaks every year
- **Common:** Dengue, chikungunya, and Japanese B encephalitis viruses are highly endemic in India
- **Rare:** Kyasanur Forest disease, West Nile, Sindbis, Crimean Congo hemorrhagic fever, Ganjam, Vellore, Chandipura, Bhanja, Umbre, Sathuperi, Chittoor, Minnal, Venkatapuram, Dhori, Kaisodi and sandfly fever viruses are among the rare arboviruses found in India, with limited geographical distribution.

LABORATORY DIAGNOSIS — **Arboviral infections**

- **Antibody detection:**
  - Older methods: Hemagglutination inhibition test (HAI), complement fixation test (CFT), and neutralization test
  - Newer methods (most widely used assays): ELISA (IgM and IgG specific) and Immunochromatographic test (ICT).
- **Virus isolation:**
  - Mosquito inoculation (adult or larval stage of *Toxorhynchites* (best), *A. aegypti* and *A. albopictus*)
  - Mosquito cell lines, such as C6/36 and AP61
  - Mammalian cell lines (such as Vero and LLC-MK2 cell lines)—least sensitive
  - Suckling mice (intracerebral inoculation).
- **Detection of antigen:**
  - In blood, e.g. dengue virus specific NS1 (nonstructural antigen 1) by ELISA and ICT
  - In fixed tissues by immunohistochemistry or direct-IF.
- **Molecular methods:** RT-PCR and Real time RT-PCR.

Laboratory diagnosis of arboviral infections has been summarized in the synopsis box above and described in detail (in text), under individual arboviruses. Treatment of all arboviral diseases is only by supportive measures; no specific antiviral drugs are available.

TOGAVIRIDAE

Classification: Family Togaviridae contains two genera—

1. *Genus Alphavirus:* Contains about 30 different mosquito transmitted viruses out of which about 13 are human pathogens.
2. *Genus Rubivirus:* Contains rubella virus, which is not arthropod transmitted and is not an arbovirus. Because of its overlapping clinical features with measles, it is discussed in detail in Chapter 44.

Morphology: Togaviruses have the following properties:

❖ Spherical, 50–70 nm in diameter

❖ Nucleocapsid:
- Capsid contains 42 capsomeres
- Genome: Positive-sense, ssRNA.

❖ **Enveloped virus:** Capsid is surrounded by a lipid envelope that contains two glycoproteins having hemagglutinating activity

❖ **Replication:** They replicate in the cytoplasm and are released by budding through host cell membranes

❖ All togaviruses are **serologically related** to each other

❖ Based on clinical manifestations, the pathogenic members of the genus *Alphavirus* can be categorized in to fever-arthritis group and encephalitic groups.

ALPHAVIRUS (FEVER-ARTHRITIS GROUP)

Chikungunya

Chikungunya fever is a re-emerging disease characterized by acute fever with severe arthralgia.

History

The name is derived from the *Makonde* word "kungunyala" meaning "that which bends up or gets folded" in reference to the stooped posture which develops as a result of the severe joint pain that occurs during the course of illness.

Transmission

❖ **Human Transmission occurs by:**
- *Aedes* mosquito, primarily *Aedes aegypti* which bites during day time
- Rarely, by vertical transmission from mother to fetus or by blood transfusion.

❖ **Transmission cycle:** Chikungunya virus is maintained in the environment through both urban and sylvan transmission cycle.
- **Urban cycle:** Human beings serve as reservoir during epidemic periods and the transmission occurs between humans and *Aedes aegypti* mosquito
- **Sylvatic cycle:** It occurs in African forests, where the virus is maintained between the wild primates, e.g. monkeys (which serve as reservoir) and forest species of *Aedes* such as *A. furcifer, A. taylori, A.africanus;* which serve as vector.

❖ **Other modes:** Rarely transmitted by: (i) mother to fetus, (ii) blood transfusion and (iii) organ transplantation.

Clinical Manifestations

❖ Incubation period is about 5 days (3–7 days)

❖ **Acute stage:** Most common symptoms are fever and severe joint pain (due to arthritis), worsened at morning
- **Arthritis** is polyarticular, migratory and edematous (joint swelling), predominantly affecting the small joints of wrists and ankles

- Other symptoms include headache, muscle pain, tenosynovitis or skin rashes
- Symptoms are often confusing with that of dengue. In general, Chikungunya is less severe, less acute and hemorrhages are rare compared to dengue (Table 46.2)
- **Chik sign** (also called brownie nose appearance): Rare presentation: characterized by hyper-pigmentation over centrofacial area; occurs due to increased intraepidermal melanin retention triggered by the chikungunya virus. Most patients recover within a week, except for the joint pain (lasts for months).

❖ **Chronic stage** (10-15%): Most patients recover within a week, except for the joint pain (lasts for months; rarely up to a year)

❖ **High-risk group:** This group includes newborns, older adults (≥65 years), and persons with underlying hypertension, diabetes, or heart disease.

Epidemiology

Chikungunya virus was first reported in Africa (Tanzania, 1952), was subsequently introduced into Asia and had caused several outbreaks in various African and Southeast Asian countries (Bangkok and India).

❖ **India (past):** Several outbreaks were reported during 1963-1973; e.g. Kolkata in 1963 and South India in 1964 (Puducherry, Chennai-Vellore region) and Barsi in Maharashtra in 1973

❖ Since then, it was **clinically quiescent** and no outbreaks were reported between 1973-2005 from most parts of the world, except for the few sporadic cases, which occurred in various places in the world including India (Maharashtra)

Table 46.2: *Manifestations of chikungunya and dengue*

Features	Chikungunya	Dengue
Fever (onset, duration)	Acute, 2-4 days	Gradual, 5-7 days
Polyarthritis	Frequent, May last longer (>1 month)	Less common Short duration
Tenosynovitis	Common	None
Rashes appear on	Day 1-4, maculopapular	Day 3-7, Petechiae or maculopapular
Myalgia	Possible	Common
Leukopenia	Common	Infrequent
Thrombocytopenia	Infrequent	Common
Retro-orbital pain	Rare	Common
Hypotension and shock	Possible	Common
Minor bleeding	Rare	Common
Hematocrit	Normal	Increased

❖ **Re-emergence** (Reunion Outbreak): In 2005, Chikungunya re-emerged in Reunion Island of Indian Ocean and affected 2,58,000 people (almost one-third of country's population)

Reasons for Re-emergence

Re-emergence in 2005 was believed to be due to a novel mutation in the virus and a change in vector.

- ❑ **New mutation (E1-A226V):** Chikungunya virus underwent an important mutation. Alanine in the 226 position of E1 glycoprotein gene is replaced by valine
- ❑ **New vector** (*Aedes albopictus*): This mutation led to a shift of vector preference. Mutated virus was found to be 100 times more infective to *A. albopictus* than to *A. aegypti*.

❖ **Spread:** Following the re-emergence, it has been associated with several outbreaks in India, other Southeast Asian and African countries and has also spread to some areas of America and Europe

❖ The most recent epidemic had occurred in Colombia during 2014-15; which witnessed 82,977 clinically confirmed cases by end of 2014

❖ **India (at present):** Chikungunya is endemic in several states
- **States:** Karnataka, Tamil Nadu, Andhra Pradesh and West Bengal have reported higher number of cases
- In 2017, nearly 62,268 cases were reported
- Karnataka accounted for the maximum number of cases (50% of total cases of India in 2017; followed by Maharashtra and Gujarat).

Genotypes

Chikungunya virus has three genotypes—(1) West African, (2) East African, (3) Asian genotypes and (4) Indian Ocean Lineage (IOL).

❖ Most Indian cases before 1973 were due to Asian genotypes

❖ However, Reunion outbreak was caused due to a mutated strain which was closely related to an African genotype from Kenya and is responsible for most of the current outbreaks in India as well as in other parts of the world

❖ The genotypes distribution is due to differences in their transmission cycles: for example, human cycle in Asia and forest cycle in Africa.

Laboratory Diagnosis

Laboratory diagnosis of chikungunya is similar to that of other arboviruses as described before.

❖ **Viral isolation** in mosquito cell lines (takes 1-2 weeks) is useful for early diagnosis (0-7 days)

❖ **Serum antibody detection:**
- IgM appears after 4 days of infection and lasts for 3 months; IgG appears late (after 2 weeks) and lasts for

years. So, detection of IgM or a fourfold rise in IgG titer is more significant
- **MAC** (IgM Antibody Capture) **ELISA** (using virus lysate) is the best format available showing excellent sensitivity (95%) and specificity (98%) with only little cross reactivity with other alphaviruses and dengue. In India, MAC ELISA kits are supplied by National Institute of Virology (NIV), Pune
- Several other rapid tests (e.g. ICT using envelope antigens) are also available.

❖ **Molecular method:** Reverse-transcriptase PCR has been developed to detect specific gene (e.g. nsP1, nsP4) in blood

❖ **Biological markers** like IL-1β and IL-6 are increased and RANTES (Regulated on activation, normal T cell expressed and secreted) levels are decreased in chikungunya virus infection

❖ **Hematological finding:** Such as leukopoenia with lymphocyte predominance, thrombocytopenia (rare), elevated ESR and C-reactive protein.

Vaccine

Recently, few vaccine trials are ongoing for chikungunya. In one of these trial, a live measles vaccine virus (Schwarz strain) is used as a vector: into the genome of which five structural genes from chikungunya virus have been incorporated.

O'Nyong-nyong (ONN) Virus

ONN virus is closely related to Chikungunya virus both clinically (rashes and arthritis) and antigenically. However, it is transmitted by the *Anopheles* species and infection is confined to Africa.

Mayaro Virus

It also causes a Chikungunya like illness and is endemic in tropical South America. *Aedes aegypti* may be involved in human transmission in an urban setting.

Ross River Virus

It has been associated with **epidemic polyarthritis** in Australia and New Guinea.

❖ It is transmitted by several *Aedes* species such as *Aedes vigilax*, *A. polynesiensis* and *A. aegypti*

❖ Focal cases were reported in India (Pune, 2010).

Sindbis Virus

Sindbis fever is a chikungunya like illness characterized by arthralgia, and rash.

❖ It is transmitted by *Culex* mosquito, first isolated from Egypt

❖ **Geographical distribution:** It has been reported from the rural areas of Africa, Europe and Australia

❖ Sindbis fever is called by different names in northern Europe (Pogosta disease in Finland, Karelian fever in Soviet Union, and Ockelbo disease in Sweden)

❖ In India, though the virus has been isolated, from birds, it is not associated with human disease.

Semliki Forest Virus

It was first isolated from *Aedes* mosquitoes in the Semliki Forest, Uganda (1942), and since then it is confined to Africa.

❖ It can cause lethal encephalitis in rodents. Its pathogenicity to humans is doubtful, rarely produces symptoms such as headache, fever, myalgia and arthralgia. It is transmitted by several mosquitoes

❖ Because of its broad host range and efficient replication, it has also been used as a vector for genes encoding various vaccines and anti-cancer agents and as a tool for gene therapy.

ALPHAVIRUS (ENCEPHALITIS GROUP)

Eastern, Western and Venezuelan Equine Encephalitis Viruses

These viruses cause encephalitis in horses and humans.

❖ Eastern equine encephalitis (EEE) and Western equine encephalitis (WEE) are confined to eastern and western parts of North America respectively

❖ **EEE** causes a rare but severe form of encephalitis (around 5 cases/year). Bird-mosquito cycle is maintained by *Culiseta melanura*, but other mosquitoes such as *Aedes vexans* are involved in transmission to mammals

❖ **WEE** occurs more frequently (around 20 cases/year), particularly involving infants, and is transmitted by *Culex tarsalis*

❖ **VEE** (Venezuelan equine encephalitis) is confined to South and Central America. It starts with an influenza-like illness but can cause serious encephalitis in people with low immunity
- A larger outbreak of VEE had occurred in Venezuela and Colombia in 1995
- The usual vector *Culex taenopius*, which has preference for rodents, which was replaced later by *Aedes taeniorhynchus* due to deforestation. The latter is more likely to bite humans and large equines
- VEE has been used as biological weapon.

❖ **Vaccine:** Inactivated vaccines have been developed for EEE and WEE whereas for VEE, there are both live attenuated (known as TC-83) and inactivated (known as C-84) vaccines available.

FLAVIVIRIDAE (MOSQUITO-TRANSMITTED FLAVIVIRUSES)

ENCEPHALITIS GROUP

Japanese B Encephalitis (JE) Virus

Japanese B encephalitis is the leading cause of viral encephalitis in Asia, including India.

History

JE virus was so named because the disease was first seen in Japan (1871) as "Summer encephalitis epidemics" (however, it is now uncommon in Japan) and named 'B' to distinguish it from encephalitis A (encephalitis lethargica/von

Economo disease), which was endemic in Japan during that time.

Epidemiology

- **Vector:** JE virus is transmitted by bite of *Culex* mosquito
  - *C. tritaeniorhynchus* is the major vector worldwide including India
  - *C. vishnui* is the next common vector found in India.
- **Transmission cycle:** JE virus infects several non-human hosts, e.g. animals and birds. Two transmission cycles are predominant
  - Pigs → *Culex* → Pigs.
  - Ardeid birds → *Culex* → Ardeid birds.
- **Animal hosts**
  - Pigs have been incriminated as the major vertebrate host for JE. JE virus multiplies exponentially in pigs without causing any manifestation. Pigs are considered as the **amplifier host** for JE
  - Cattle and buffaloes may also be infected with JE virus; although they are not the natural host. They may act as **mosquito attractants**
  - Horses are probably the only animal to be symptomatic and develop encephalitis following JE virus infection
  - Humans are considered as dead end; there is no man → mosquito → man cycle (unlike in dengue).
- **Bird hosts:** Ardeid (wading) birds such as herons, cattle egrets, and ducks can also be involved in the natural cycle of JE virus
- **Age:** About 85% of cases occur in children below 15 years (but infants are not affected) and 10% occur in the elderly
- **Seasonal variation:** Infection due to JE is common in rainy season which coincides with maximum mosquito activity
  - Temperate areas (summer-autumn)
  - Tropical areas including India (June-October).

Geographical Distribution

Currently, JE is endemic in Southeast Asian region.

- It is increasingly reported from India, Nepal, Pakistan, Thailand, Vietnam and Malaysia
- Because of immunization, its incidence has been declining from Japan and Korea
- It is estimated that nearly 50,000 cases occur every year globally with 10,000 deaths
- **In India:** JE has been reported since 1955
  - JE is endemic in 15 states; Uttar Pradesh (Gorakhpur district) accounted for the largest burden
  - Between 2010-17; total 10,710 number cases (average 1,340 cases/year) and 1,782 deaths (average 222 deaths/year) have been reported
  - In 2017, nearly 2,040 cases of JE were reported from India with 230 deaths. Maximum cases reported from UP followed by Assam, Manipur, West Bengal, Tamil Nadu, Tripura, Bihar, and Odisha.

Clinical Manifestations

JE is the most common cause of epidemic encephalitis.

- **Incubation period** is not exactly known, probably varies from 5-15 days
- **Subclinical infection is common:** JE typically shows iceberg phenomena
  - Cases are much less compared to subclinical/inapparent infection with a ratio of 1:300 to 1:1000
  - Even during an epidemic, the number of cases are just 1-2 per village.
- **Clinical course** of the disease can be divided into three stages:
  1. **Prodromal stage** is a febrile illness; the onset of which may be either abrupt (1-6 hours), acute (6-24 hours) or more commonly subacute (2-5 days).
  2. **Acute encephalitis stage:** JE is the most common cause of acute encephalitis syndrome (AES) in India; characterized by an acute onset of fever, mental confusion, disorientation, delirium, or coma.
  3. **Late stage and sequelae:** It is the convalescent stage in which the patient may be recovered fully or retain some neurological deficits permanently (up to 50%). Case fatality rate is about 20-40%.

Laboratory Diagnosis

- IgM Capture Antibody (MAC) ELISA supplied by NIV, Pune has been the recommended method for diagnosis of JE. It is a two-step sandwich ELISA, uses JERA (JE recombinant antigen) to detect JE-specific IgM antibody in serum
- Reverse-transcriptase PCR has also been developed to detect JE virus specific envelope (E) gene in blood.

Vaccine Prophylaxis for Japanese Encephalitis

- **Live attenuated SA 14-14-2 vaccine:**
  - It is prepared from SA 14-14-2 strain of JE virus
  - It is cell line derived; primary hamster kidney cell lines are commonly used
  - Single dose is given subcutaneously, followed by booster dose after 1 year
  - It is manufactured in China, but now licensed in India
  - Under National Immunization Program, it is given to children (1–15 years) targeting 181 endemic districts of four states—UP, Karnataka, West Bengal and Assam
  - Schedule: Two doses: 1st at 9 completed month-12 months of age and 2nd at 16–24 months
  - Administered: 05 mL/dose, subcutaneously at left upper arm.
- **Inactivated vaccine** (Nakayama strain and Beijing strain)
  - Both are mouse brain derived and formalin inactivated
  - Prepared in Central Research Institute, Kasauli (India).

Contd...

Contd...

- **Inactivated vaccine (Beijing P3 strain):** It is a cell line derived vaccine
- Combined vaccine: A genetically engineered JE vaccine that combines the attenuated SA14-14-2 strain and yellow fever vaccine strain 17D (YF 17D) virus as a vector for genes encoding the protective antigenic determinants, has been tested in several clinical trials.

Other Mosquito-borne Encephalitis Flaviviruses

West Nile Virus

It was first described in West Nile region of Uganda (Africa).

- Since then, it is being transmitted among wild birds by Culex mosquitoes in Africa, Middle East, Europe, Asia and recently in America
- Febrile illness is the most common manifestation (fever, myalgia with rashes on the trunk), but occasionally, it can also cause severe encephalitis.

Murray Valley Encephalitis Viruses

It is endemic in northern Australia and Papua New Guinea. Major mosquito vector is *Culex annulirostris*; responsible for human transmission as well as maintenance of bird-mosquito cycle.

St Louis Encephalitis Viruses

It is transmitted by mosquito (*Mansonia pseudotitillans*). It is related to Japanese B encephalitis virus. It has caused several outbreaks of encephalitis, mainly affecting the United States.

Rocio Encephalitis Viruses

It was first observed in São Paulo State, in 1975. It had caused several epidemics of meningoencephalitis in coastal communities in southern São Paulo, Brazil, during 1975. Transmission is believed to be by Culex.

HEMORRHAGIC FEVER GROUP

Dengue Viruses

Dengue virus (DENV) is the most common arbovirus found in India.

- It is named after the Swahili word "dinga" meaning fastidious or careful, which would describe the gait of a person suffering from the bone pain of dengue fever
- It has four serotypes (DEN-1, to DEN-4). Recently, the fifth serotype (DEN-5) was discovered in 2013 from Bangkok.

Vector

Aedes aegypti is the principal vector followed by *Aedes albopictus*. They bite during the day time.

- *A. aegypti* is a nervous feeder (so, it bites repeatedly to more than one person to complete a blood meal) and resides in domestic places, hence is the most efficient vector
- *Aedes albopictus* is found in peripheral urban areas. It is an aggressive and concordant feeder, i.e. can complete its blood meal in one go; hence is less efficient in transmission
- *Aedes* becomes infective only when it feeds on viremic patients (generally from a day before to the end of the febrile period, i.e. 5 days.)
- Extrinsic incubation period of 8–10 days is needed before *Aedes* to become infective. However, once infected, it remains infective for life
- *Aedes* can pass the dengue virus to its offsprings by transovarial transmission
- **Transmission cycle:** Man and *Aedes* are the principal reservoirs. Transmission cycle of four serotypes do not involve other animals; in contrast the fifth serotype follows the sylvatic cycle.

Pathogenesis

Primary dengue infection occurs when a person is infected with dengue virus for the first time with any one serotype. Months to years later, a more severe form of dengue illness may appear (called **secondary dengue infection**) due to infection with another second serotype which is different from the first serotype causing primary infection.

Antibody Response Against Dengue Virus

Infection with dengue virus induces the production of both neutralizing and non-neutralizing antibodies.

- The **neutralizing antibodies** are protective in nature. Such antibodies are produced against the infective serotype (which last lifelong) as well as against other serotypes (which last for some time). Hence, protection to infective serotype stays lifelong but cross protection to other serotypes diminishes over few months
- The **non-neutralizing antibodies** last lifelong and are heterotypic in nature; i.e they are produced against other serotypes but not against the infective serotype
  - Such antibodies produced following the first serotype infection, can bind to a second serotype during secondary dengue infection; but instead of neutralizing the second serotype, it protects it from host immune system by inhibiting the bystander B cell activation against the second serotype
  - **ADE:** The above phenomena is called **antibody dependent enhancement (ADE).** Non-neutralizing antibodies promote recruitment of mononuclear cells followed by release of cytokines which explains the reason behind the severity of secondary dengue infection
  - Among all the serotypes combinations, ADE is remarkably observed when serotype 1 infection is followed by serotype 2, which also claims to be the most severe form of dengue infection.

Clinical Classifications

The Traditional (1997) WHO Classification

This classification divides dengue into three clinical stages:

1. **Dengue fever (DF):** It is characterized by:
   - Abrupt onset of high fever (also called biphasic fever, break bone fever or saddle back fever)
   - Maculopapular rashes over the chest and upper limbs
   - Severe frontal headache
   - Muscle and joint pains
   - Lymphadenopathy
   - Retro-orbital pain
   - Loss of appetite, nausea and vomiting.
2. **Dengue hemorrhagic fever (DHF):** It is characterized by:
   - High-grade continuous fever
   - Hepatomegaly
   - Thrombocytopenia (platelet count < 1 Lakh/mm^3)
   - Raised hematocrit (packed cell volume) by 20%
   - Evidence of hemorrhages which can be detected by:
     - Positive tourniquet test (>20 petechial spots per square inch area in cubital fossa
     - Spontaneous bleeding from skin, nose, mouth and gums.
3. **Dengue shock syndrome (DSS):** Here, all the above criteria of DHF are present, and in addition manifestations of shock are present, such as:
   - Rapid and weak pulse
   - Narrow pulse pressure (<20 mm Hg) or hypotension
   - Presence of cold and clammy skin
   - Restlessness.

2009 WHO Classification

This is the most recently described classification by WHO which grades dengue into two stages based on the severity of infection (Fig. 46.1):

1. Dengue with or without warning signs.
2. Severe dengue.

Factors Determining the Outcome

- **Infecting serotype:** Type 2 is apparently more dangerous than other serotypes
- **Sequence of infection:** Serotype 1 followed by serotype 2 seems to be more dangerous and can develop into dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) more often than others
- **Age:** Though all age groups are affected equally, children less than 12 years are more prone to develop DHF and DSS.

Geographical Distribution

Global Scenario

Dengue is endemic in more than 100 countries with 2.5 billion people at risk.

- Tropical countries of Southeast Asia and Western pacific are at highest risk

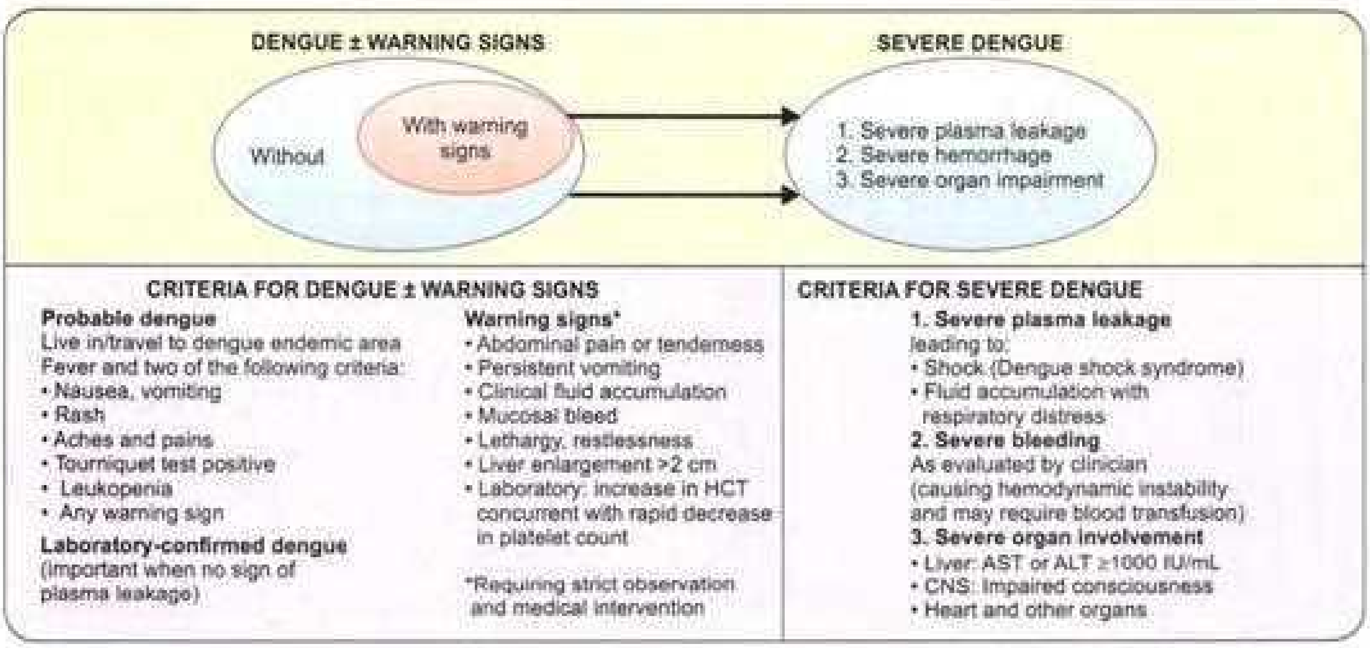


Fig. 46.1: Dengue case classification based on the severity (WHO 2009)

Abbreviations: HCT, hematocrit; DSS, dengue shock syndrome; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CNS, central nervous system.

- About 50 million of dengue cases occur every year worldwide, out of which 5 lakh cases (mostly children) proceed to DHF.

Situation in India

Disease is prevalent throughout India in most of the **urban cities/towns** affecting almost 31 states/Union territories.

- Between 2010-17, >6 Lakh cases with >1560 deaths have been reported from India. Maximum cases have been reported (in descending order) from West Bengal, Tamil Nadu, Punjab, Kerala, Delhi, Karnataka, and Maharashtra
- In 2017, nearly 1,57,220 cases were reported; maximum of cases were reported from Tamil Nadu followed by West Bengal
- All four dengue serotypes have been isolated from India. Serotype prevalence varies between seasons and places, but DEN-1 and DEN-2 are widespread. DEN-5 has not been reported yet.

Laboratory Diagnosis

The outline of laboratory diagnosis of dengue is similar to that of other arboviruses as described earlier.

NS1 Antigen Detection

ELISA and ICT formats are available for detecting NS1 antigen in serum. They gained recent popularity because of the early detection of the infection.

- NS1 antigen becomes detectable from day 1 of fever and remains positive up to 18 days
- **Highly specific:** It differentiates between flaviviruses. It can also be specific to different dengue serotypes.

Antibody Detection

- **In primary infection:** Antibody response is slow and of low titer. IgM appears first after 5 days of fever and disappears within 90 days. IgG is detectable at low titer in 14-21 days of illness, and then it slowly increases
- **In secondary infection:** IgG antibody titers rise rapidly. IgG is often cross reactive with many flaviviruses and may give false positive result after recent infection or vaccination with yellow fever virus or JE. In contrast, IgM titer is significantly low and may be undetectable
- **In past infection:** Low levels of IgG remain detectable for over 60 years and, in the absence of symptoms; is a useful indicator of past infection
- **MAC-ELISA** (IgM antibody capture ELISA): This is the recommended serological testing in India. Kits are supplied by NIV, Pune
  - Principle: It is a double sandwich ELISA; which captures human IgM antibodies on a microtiter plate using anti-human-IgM antibody followed by the addition of dengue virus four serotypes specific envelope protein antigens (this step makes the test specific) . There is a signal enhancement due to use of avidin-biotin complex (ABC) which makes the test more sensitive
  - Cross-reactivity with other flaviviruses is a limitation of this test.
- IgG specific ELISA format is also available separately
- Rapid tests such as dipstick assays are also available
- **Other antibody detection assays** used previously are:
  - HAI (Hemagglutination inhibition test)
  - CFT (Complement fixation test)
  - Neutralization tests such as plaque reduction test, and microneutralization tests: They are cumberson; but are most specific serologic tests.

Rapid diagnostic tests (RDT) for dengue

Rapid diagnostic tests (e.g. ICT) for dengue IgM antibodies or NS1 antigen are available, but have poor sensitivity and specificity. Government of India had passed an order in 2016, that a positive RDT for dengue NS1 or IgM should be considered as probable diagnosis; must be confirmed by ELISA.

Virus Isolation

Dengue virus can be detected in blood from -1 to +5 days of onset of symptoms. Virus isolation can be done by inoculation into mosquito cell line or in mouse.

Molecular Method

- Detection of specific genes of viral RNA (3'-UTR region) by real time RT-PCR: It is the most sensitive (80-90%) and specific assay (95%), can be used for detection of serotypes and quantification of viral load in blood. Viral RNA can be detected in blood from -1 to +5 days of onset of symptoms. A negative PCR result is interpreted as "indeterminate"; which has to be sent for serological confirmation after the 5th day of illness
- **Genotype detection:** Each serotypes of dengue virus comprises of several genotypes which can be detected by molecular typing.
  - A total of 13 genotypes have been detected so far; three for DENV-1, two for DENV-2, four each for DENV-3 and 4 serotypes respectively.
  - Dengue virus keep undergoing genetic alterations leading to introduction of new genotypes and also a shift between the existing genotype lineages within a serotype; which may attribute to rapid increase in the clinical severity (DHF and DSS) in many parts of the world. Hence, there is need for close molecular monitoring of dengue virus.

TREATMENT **Dengue**

There is no specific antiviral therapy. Treatment is symptomatic and supportive such as:

- Replacement of plasma losses
- Correction of electrolyte and metabolic disturbances
- Platelet transfusion if needed.

Prevention

- **Vaccine:** Vaccine development for dengue has been a challenge as the vaccine should be effective against all four serotypes. After so many trials, recently a vaccine has been licensed for human use since 2015 (see the highlight box below). Five more vaccine trials for dengue are going on
- Mosquito control measures.

Dengue vaccine (CYD-TDV):

This vaccine has been licensed for human use since 2015.

- It is a Chimeric Yellow Fever-Dengue, Live-Attenuated, Tetravalent Dengue Vaccine (CYD-TDV); commercially available as dengvaxia (developed by Sanofi Pasteur)
- It uses live attenuated yellow fever 17D virus as vaccine vector in which the target genes of all four dengue serotypes are integrated by recombinant technique
- Age: It is indicated for 9–45 years of age
- Schedule: 3 injections of 0.5 mL administered subcutaneously at 6 month intervals
- It is available as lyophilized form; reconstituted with normal saline
- Contraindications: (i) allergic reactions to vaccine; (ii) immunodeficient individuals (e.g. HIV) (iii) Pregnant and breastfeeding women
- Efficacy against hospitalized dengue illness was found around 80%
- WHO recommends this vaccine to start in high burden countries (seroprevalence >70%)
- Currently, the vaccine is approved in Mexico, Philippines, Brazil, Indonesia, Thailand and Singapore. In India, it is not available yet.

Zika Virus

Zika virus (ZIKV) has recently gained attention due to recent large outbreak occurred in 2015-16 worldwide. It is ssRNA virus, belongs to family Flaviviridae. Monkeys are the reservoirs. It is named after its place of discovery (1947), Zika Forest in Uganda.

Transmission

ZIKV is transmitted by mosquito bite; though other mode of transmission has also been reported.

- **Mosquito borne**—Mainly spread by the *Aedes aegypti* but also by *Aedes albopictus* and other *Aedes* species
- **Mother-to-child** transmission can occur through placenta. It is common in first trimester, occasionally can occur during delivery
- **Sexual transmission:** Transmission has been observed from (i) asymptomatic males to their female partners. Longer shedding of Zika virus in semen has been reported, (ii) Symptomatic females to their male partners.

Epidemiology

Though ZIKV was wide spread among human population, was never a threat to cause disease. Only 14 cases were reported since the discovery (1947) till 2007.

The first outbreak was reported in 2007 in Yap Islands. *Aedes hensilli* was the predominant mosquito. About 49 confirmed and 59 probable cases were reported.

Recent Outbreak (2015–2016)

It began in April 2015 in Brazil and then subsequently spread to other countries in South America, Central America, the Caribbean, Europe USA and Australia.

- During the 2015–16, about 5,28,157 confirmed cases and 18 deaths have been reported worldwide; out of which Brazil alone witnessed nearly 2,00,465 confirmed cases
- Next to Brazil, other countries reported maximum cases were Puerto Rico, Colombia and Mexico
- In February 2016, the WHO declared the Zika virus outbreak a public health emergency of international concern.

Situation in India

Three confirmed cases have been reported from Gujarat in 2017; first report from India. As the vector is prevalent, India has a higher risk of getting affected by ZIKV in near future.

Clinical Manifestations

Incubation period ranges from few days to 1 week. Majority of infections are asymptomatic. The asymptomatic to symptomatic ratio is 5:1.

- **Zika Fever:** Symptomatic people develop minor illness such as fever with rash and conjunctivitis
- **Fetus:** Congenital transmission leads to development of fetal anomaly such as microcephaly
- Few cases of Guillain-Barré syndrome have been reported in patients with ZIKV disease.

Laboratory Diagnosis

The following laboratory tests are performed for confirmation of ZIKV disease.

- **Reverse transcriptase PCR** (RT-PCR) has been the investigation of choice. It can detect ZIKV RNA in blood and urine up to 5 and 7 days of onset of symptoms respectively

  Multiplex real time RT-PCR has been commercially available in India targeting the non-structural 5 (NS5) region of ZIKV, non-structural protein 4 (nsP4) from CHIKV and 3' untranslated region (3'UTR) of DENV 1–4.

- **IgM antibody detection** (ELISA): It appears in blood after 1 week of symptoms and remain positive up to several months. It cross reacts with dengue antibodies

- **Plaque-reduction neutralization test** is more specific serological (antibody detection) test; but it is cumbersome, not widely used.

TREATMENT **Zika virus**

No effective treatment and vaccine is available so far. Only symptomatic treatment is available such as fluid replacement and analgesic such as acetaminophen.

Prevention

ZIKV Vaccine

Though no vaccine has been licensed yet; several vaccine trials are going on for ZIKV.

- **ZIKV DNA vaccine (VRC 705):** The most advanced trial is the one conducted by Vaccine Research Center (VRC) under NIAID, USA. The vaccine candidate is a genetically engineered plasmid encoding Zika virus protein. It has entered phase-2 vaccine trial in 2017 and is expected to get completed by 2019
- **Killed ZIKV vaccine:** Another trial (phase-I) is been evaluated by Bharat Biotech's Hyderabad, India. It uses an African strain of ZIKV.

General Preventive Measures

The following general preventive measures are recommended.

- Mosquito control measures
- Infected patients should prevent mosquito bites for the first week of illness
- **Sex/pregnancy restriction:** CDC has recommended for considering condom use or abstinence for at least 6 months for males and 8 weeks for females after travel to endemic area or developing symptoms or ZIKV diagnosis
- **During outbreak time (2015–16):**
  - Affected countries such as Brazil, and others have advised women to postpone getting pregnant until more was known about the risks
  - Travel of pregnant women from other countries (including India) to ZIKV affected countries had been restricted.

Yellow Fever Virus

Yellow fever is an acute, febrile illness; severe cases are characterized by liver dysfunction which leads to jaundice (hence the name yellow fever), renal dysfunction and hemorrhage, with high mortality.

- **Geographical distribution:** Yellow fever is endemic in West Africa and Central South America. It is not found in the rest of the World including India
- **Typing:** At least **seven genotypes** of yellow fever virus have been identified based on genomic sequence, five in Africa and two in South America. However, antigenically it is homogenous, only one serotype is known to exist.

Transmission

- **Vector:** Humans get the infection by the bite of *Aedes aegypti* or the tiger mosquito
- **Transmission cycle:** Two major cycles of transmission have been recognized:
  1. **Jungle cycle:** This cycle occurs between monkeys and forest mosquitoes. Humans can only get infection occasionally during their forest visits. Monkeys usually suffer from disease.
  2. **Urban cycle:** This cycle occurs between humans and urban mosquitoes (*Aedes aegypti*).

India

Yellow fever has not invaded India yet. However, India has all the potential of developing yellow fever in future because the vector, *A. aegypti*, is widely distributed here, and India has the tropical climatic condition similar to Africa. Various reasons have been hypothesized to explain the absence of yellow fever in India such as:

- **Measures in airport:** Government of India has laid down strict guidelines for vigilance and quarantine of the travelers in the international airports
  - Unprotected (i.e. unvaccinated) travelers coming from endemic zone to India will be kept in quarantine for the longest incubation period; i.e. 6 days
  - Breteau index or the *Aedes aegypti* index should be less than one, surrounding 400 meter of an airport. (**Breteau index:** No. of containers showing breeding of *Aedes aegypti* larvae/No. of houses surveyed × 1000).
- Being endemic for dengue, many individuals in India possess dengue antibodies which are found to be cross reactive and hence provide protection against yellow fever. However, it is observed that yellow fever immunization does not protect from dengue.

Period of Communicability

- **Man:** Patients are infective to mosquito during the first 3–4 days of illness
- ***Aedes:*** After an extrinsic incubation period of 8–10 days, the mosquito becomes infective and once infected, remains infective for life.

Clinical Manifestations

Incubation period is about **3–6 days**. Febrile illness occurs in early stage of the disease and characterized by:

- Presence of fever, chills, headache, dizziness, myalgia, and backache-followed by nausea, vomiting, and relative bradycardia

- Patient is viremic in this stage and may be a source of infection for mosquitoes.

Severe cases are characterized by:

- Hemorrhagic manifestations
- Platelet dysfunction
- Features of liver involvement (hepatitis):
  - Mid-zonal necrosis and presence of councilman bodies
  - Intranuclear inclusions may be seen inside the hepatocytes called **Torres bodies**
  - Appearance of jaundice.
- Renal dysfunction
- Encephalitis occurs very rarely
- Mortality rate is high (>20%), especially among children and elderly.

Laboratory diagnosis

- **Serology:** IgM ELISA can be done after 3 days of onset of symptoms. It gives false positive result in other flavivirus infections such as dengue, West Nile and Zika, and also in people who are recently vaccinated within 30 days. Hence the positive result should be confirmed with a more specific test (e.g. plaque-reduction neutralization test).
- **Molecular method:** RT-PCR detecting specific viral RNA (NS5 region) in blood is more confirmatory than serology. It should be performed within 10 days of onset of symptoms, beyond which RNA becomes undetectable in blood.

Epidemiology

It is estimated that about 200,000 cases of yellow fever with 30,000 deaths occur annually worldwide and majority of outbreaks (~90%) occur in Africa.

- Epidemics usually occur in humid and semi-humid savanna area adjoining a rain forest
- **Infection to case ratio** ranges from 20:1 in non-outbreak season to 2:1 during epidemic
- All age groups are susceptible.

Laboratory diagnosis of yellow fever virus is similar to that of other arboviruses described earlier. Treatment is only by symptomatic care. Preventive measures include vaccination (see the box below) and mosquito control.

Yellow Fever 17D Vaccine

It is a live attenuated vaccine, which is prepared in allantoic cavity of chick embryo (hence, it is contraindicated in people having allergy to egg).

- There is no risk of encephalitis (unlike the previously used mouse brain derived inactivated **Dakar vaccine** which was found to be encephalitogenic)
- **In India:** It is prepared in Central Research Institute (CRI), Kasauli

Contd...

Contd...

- **Strict cold chain** has to be maintained during the transport with a temperature range of -30°C to +5°C
- It is available in **lyophilized form** and has to be reconstituted before use
- Reconstitution is usually done with diluents such as **physiological saline**. Once reconstituted, it should be used within ½ hour
- **Dosage:** Single dose, given subcutaneously
- Vaccine is effective within 7 days of administration, the efficacy lasts for up to 35 years
- **Validity of yellow fever vaccine certificate:** Certificate is issued after 10 days of vaccination and renewed (i.e. reimmunization) every 10 years. This is the recommendation followed for international travel
- Cholera and yellow fever vaccine interact with each other, hence should not be given together (3 weeks gap to be maintained)
- **Contraindications** of yellow fever vaccine include:
  - Children <9 months, (<6 months during epidemic)
  - Pregnancy (except during outbreak)
  - HIV-infected people
  - People with allergy to egg.

TICK-TRANSMITTED FLAVIVIRUSES

ENCEPHALITIS GROUP

Tick-borne Encephalitis (TBE) Viruses

TBE viruses are endemic to most European countries, the Russian Federation and China. They are grouped into three subtypes of viruses:

1. **Central European encephalitis virus** (principal tick vector: *Ixodes ricinus*).
2. **Western Siberian encephalitis virus** (principal tick vector: *Ixodes persulcatus*).
3. **Russian Spring Summer encephalitis** virus (renamed as Far Eastern subtype):
   - **Principal tick vector:** *Ixodes persulcatus.*
   - It is abrupt in onset and more severe than the other types.

Inactivated vaccines: Alum precipitated, formalin-inactivated vaccines are available against all subtypes of viruses.

Powassan Encephalitis Viruses

Powassan virus is another tick-borne encephalitis virus, transmitted by *Ixodes cookei* in eastern Canada and the USA, where it has been linked to 20 recognized human cases of encephalitis.

Louping-ill Virus

It is an acute viral encephalitic disease of sheep, transmitted by tick (*Ixodes ricinus*). Few human cases have been reported from Europe. The name 'louping-ill' is derived from an old Scottish word describing the effect of the disease in sheep whereby they 'loup' or spring into the air.

HEMORRHAGIC FEVER GROUP

Kyasanur Forest Disease (KFD) Virus

Kyasanur Forest disease virus was identified in 1957 from monkeys from the Kyasanur Forest in Shimoga district of Karnataka, India.

Epidemiology

- **Vector:** Hard ticks (*Haemaphysalis spinigera*) are the vectors of KFD virus and once infected, they remain infected for life. At least 15 species of ticks are involved in transmission among animals
- **Hosts:** Monkeys, rodents and squirrels are common hosts which maintain the virus through animal-tick cycles
  - Reservoirs are the rats and squirrels
  - Amplifier hosts are the monkeys, where the virus multiplies exponentially
  - Man is an incidental host and considered as dead end.
- **In monkeys:** KFD virus has been a cause of epizootics with high fatality in primates especially in monkeys, hence known as **Monkey's disease**
- **Seasonality:** KFD is increasingly reported in dry months (January-June) which coincides with human activity in forest
- **Situation in India:** KFD is currently endemic in five districts of Karnataka-Shimoga, North Kannada, South Kannada, Chikkamagaluru and Udupi
  - **Largest outbreak** had occurred in 1983-84, which has witnessed 2,167 cases with 69 deaths. Currently only focal cases occur at a rate of 100-500 cases per year
  - From 2003 to 2012 there were 3,263 suspected cases, with 823 confirmed cases and 28 deaths with a case fatality rate of 3.4%
  - There is a **declining trend** of incidence after the initiation of vaccine in 1999, except for the outbreak that occurred between December 2012 to March 2013 which witnessed 215 suspects with 61 confirmed cases.

Clinical Manifestation in Humans

- Incubation period varies from 3-8 days
- **First stage (hemorrhagic fever):** It starts as acute high fever with malaise and frontal headaches, followed by hemorrhagic symptoms, such as bleeding from the nasal cavity, throat, and gums, as well as gastrointestinal bleeding
- **Second stage** in the form of meningoencephalitis may occur 7-21 days after the first stage.

Laboratory Diagnosis

Diagnosis is made by virus isolation from blood or by IgM antibody detection by ELISA.

- Recently, nested RT-PCR and real time RT-PCR have been developed detecting viral RNA (NS-5 non-coding region) in serum samples and can provide early, rapid and accurate diagnosis of the infection
- Non-specific findings such as leukopenia, thrombocytopenia and decreased hematocrit, albuminuria and abnormal CSF are found in second stage.

Killed KFD Vaccine

A formalin-inactivated chick embryo vaccine has been developed for KFD in the Haffkine institute, Mumbai.

- **Schedule:** Two-doses at interval of 2 months, followed by booster doses at 6-9 months and then every 5 years
- **Target area:** KFD vaccine is recommended in endemic areas of Karnataka (villages within 5 km of endemic foci).

Omsk Hemorrhagic Fever Virus

It is endemic in Western Siberia of Russia, in places including Omsk, Novosibirsk and Tyumen.

- It is transmitted by tick
- Though hemorrhagic fever is the common manifestation, it can occasionally produce neurological involvement.

BUNYAVIRIDAE

The Bunyaviridae family contains about 330 viruses, most of them being arthropod-transmitted with the exception of hantaviruses, which are rodent borne (described in Chapter 49).

Morphology: Bunyaviruses have following morphological features:

- Spherical in shape
- Size: 80-120 nm
- It contains a single-stranded, negative-sense segmented RNA (three segments)
- It is an enveloped virus, which has two glycoproteins.

Several members of this family produce mosquito-borne encephalitis or hemorrhagic fevers in humans and animals.

GENUS BUNYAVIRUS

California Encephalitis Virus Complex

This complex comprises of group of 14 antigenically-related viruses that cause encephalitis in USA. Important ones include:

- **La Crosse virus:** Accounts for majority of cases; transmitted by *Aedes triseriatus*
- California encephalitis virus
- Jamestown Canyon encephalitis virus.

Oropouche Virus

This virus is endemic in Central and South America, characterized by rash and aseptic meningitis. It is transmitted by a midge, *Culicoides paraensis*.

GENUS PHLEBOVIRUS

Sandfly Fever

It is transmitted by sandfly of genus *Phlebotomus papatasi*.

- It is endemic in subtropical zone of the eastern hemisphere particularly in Southern Europe, North Africa, Eastern Mediterranean, Iraq, Iran, Pakistan and India
- It is a febrile illness presenting with chills and malaise
- In India, it has been reported from Aurangabad (Maharashtra).

Rift Valley Fever Virus

- It is endemic in sub-Saharan Africa, Madagascar, and Egypt
- The most common presentation is fever and myalgia. Hemorrhagic manifestations may be seen rarely
- It is transmitted by *Aedes* mosquitoes.

Severe Fever with Thrombocytopenia Syndrome (SFTS) Virus

SFTS is an emerging infectious disease, caused by SFTS virus.

- **Transmission:** It is transmitted by vector Ixodid tick.
- **Clinical feature:** It produces fever, vomiting, diarrhea, multiple organ failure, thrombocytopenia, leukopenia and elevated liver enzyme levels.
- **Epidemiology:** It has caused outbreaks in China's rural areas (2013), Japan and South Korea (2013). Around 2500 cases have been reported till 2014, with a mortality rate ranging from 7% in China and 47% in South Korea. The most recent case was reported from Japan in 2017.

GENUS NAIROVIRUS

Crimean Congo Hemorrhagic Fever Virus

It is a tick-borne virus; transmitted by tick genera *Haemaphysalis* and *Hyalomma*.

- **Zoonosis:** Affects domestic animals and wild animals
- In humans, it causes hemorrhagic fever
- It is endemic in East and West Africa
- **In India:** Focal cases have been reported from Gujarat in 2011 (four cases) and 2013
- Ribavirin has been found to be effective.

Ganjam Virus

In India, it has been isolated from ticks from various places; most recent was from Pune (2004), earliest was from Ganjam (Odisha, 1955).

- One natural case in humans was reported from Vellore (Tamil Nadu)
- It is antigenically related to Nairobi sheep disease virus of Africa.

REOVIRIDAE

Reoviruses are non-enveloped, spherical in shape and possess a double-stranded segmented RNA. Only a few members under the genera *Coltivirus* and *Orbivirus* are arthropod borne. Remaining viruses are discussed in Chapter 49.

GENUS COLTIVIRUS

Colorado Tick Fever Virus

The disease is seasonal (June), found almost exclusively in mountain areas of western USA and Canada such as Colorado and Idaho

- It is characterized by febrile illness, rarely encephalitis
- The infection is acquired through the bite of an infected tick named *Dermacentor andersoni*.

GENUS ORBIVIRUS

Orungo Virus

It is endemic in sub-Saharan Africa. It is either subclinical or causes acute febrile illnesses in man. It is mosquito borne (*Aedes*).

Kemerovo Viruses

This causes a paralytic febrile illness accompanied by meningism following a tick-bite (*Ixodes persulcatus*). Cases have been reported from Russia.

RHABDOVIRIDAE

Vesicular Stomatitis Indiana Virus

This virus causes oral mucosal vesicles and ulcers in cattle, horses and pigs similar to hand foot mouth disease. Human infection is rare.

- It is transmitted by sandfly (*Lutzomyia shannoni*)
- **Medical applications:** It has been used for oncolytic therapy (to reduce tumor size) and anti-HIV therapy (can attack HIV infected T-cells).

Chandipura

Chandipura virus has been associated with a number of outbreaks of encephalitic illness in central India.

- It is transmitted by sandfly
- Children are the most susceptible group for this infection
- It was first isolated from Chandipura village in Maharashtra, India (1965)
- The most recent outbreaks occurred in Andhra Pradesh and Maharashtra in 2003
- Further sporadic cases and deaths in children were observed in Nagpur (2007) and Gujarat (2010).

EXPECTED QUESTIONS

I. Essay:

1. Name the arboviruses prevalent in India along with their vectors and discuss in detail about pathogenesis, clinical features and laboratory diagnosis of Japanese B encephalitis virus.
2. Sunita, a 29-year-old female came to casualty with complaints of high-grade fever, severe joint pain, back pain and myalgia. Gradually, she developed petechial rashes over the body. On examination, she was found to have jaundice, hepatomegaly and a low platelet count (30,000/cmm). A tourniquet test done over the cubital fossa demonstrated 25 petechial spots/square inch area. On enquiry, she told that she has been bitten by the mosquitoes.
   a. What is the clinical diagnosis and how is this disease transmitted?
   b. What are the typical clinical presentation and pathogenesis of this condition?
   c. How will you confirm the diagnosis?

II. Write short notes on:

a. Vaccines for Japanese B encephalitis.
b. Chikungunya.
c. Kyasanur Forest disease.
d. Yellow fever.

III. Multiple Choice Questions (MCQs):

1. False statement regarding Japanese encephalitis:
a. It is caused by flavivirus
b. Transmitted by *Aedes* mosquito
c. Endemic in India
d. Man is dead-end host.

2. Kyasanur Forest disease is transmitted by:
a. Mite b. Louse
c. Tick d. Mosquito

3. Amplifier host in Japanese B encephalitis is:
a. Man b. *Culex* mosquito
c. Pig d. Horse

4. In dengue infection, earliest detectable number of petechial spots per square inch in cubital fossa should be:
a. >5 b. >10
c. >15 d. >20

5. True about Zika virus are all, *except*:
a. Named after Zika forest
b. Highest cases reported from Japan
c. Transmitted by *Aedes aegypti*
d. Sexual transmission is also possible

6. Which is a re-emerging disease?
a. Dengue fever b. Japanese encephalitis
c. Yellow fever d. Chikungunya fever

7. E1-A226V mutation is observed with:
a. Dengue fever b. Japanese encephalitis
c. Yellow fever d. Chikungunya fever

8. Most common cause of acute encephalitis syndrome (AES) in India:
a. Herpes b. Japanese encephalitis
c. Toxoplasma d. Neurocysticercosis

9. How many serotypes dengue virus has?
a. 2 b. 3
c. 4 d. 5

10. Antibody dependent enhancement (ADE) is observed with:
a. Dengue hemorrhagic fever
b. Japanese encephalitis
c. Yellow fever d. Chikungunya fever

11. Which is true about dengue diagnosis?
a. NS1 antigen is positive on day 1
b. Antibody appears in first five days of fever
c. Viral RNA is positive up to 2 weeks of fever
d. Dengue has five genotypes

12. CYD-TDV is a vaccine given for:
a. Yellow fever b. Chikungunya fever
c. Dengue hemorrhagic fever
d. Japanese encephalitis

13. Nakayama strain is used as vaccine for:
a. Yellow fever b. Chikungunya fever
c. Dengue hemorrhagic fever
d. Japanese B encephalitis

14. Contraindications of yellow fever vaccine include all, *except*:
a. During outbreak b. Children <9 months
c. HIV-infected people
d. People with allergy to egg

15. Certificate for yellow fever vaccination is valid for:
a. 1 year b. 5 years
c. 10 years d. Life-long

16. People coming for endemic area not-vaccinated for yellow fever should be quarantine in airport for:
a. 24 hours b. 6 days
c. 10 days d. 2 weeks

17. After travel to endemic area or developing symptoms or Zikavirus diagnosis, the sexual restriction should be followed up to:
a. 6 months for males and 8 weeks for females
b. 6 weeks for males and 8 months for females
c. 6 months for males and females
d. 8 weeks for males and 8 weeks for females

18. Rapid diagnostic tests for dengue virus:
a. Confirmatory
b. Should be confirmed by ELISA
c. Banned in India d. Not available

19. Which of the following disease shows iceberg phenomena:
a. Dengue fever b. Japanese encephalitis
c. Yellow fever d. Chikungunya fever

20. Type of ELISA used for dengue:
a. Direct ELISA b. MAC ELISA
c. Completive ELISA d. ELISPOT

21. State with highest prevalence for Japanese encephalitis in India:
a. Bihar b. Assam
c. Uttar Pradesh d. Karnataka

Answers

1. b **2.** c **3.** c **4.** d **5.** b **6.** d **7.** d **8.** b **9.** d **10.** a **11.** a **12.** c **13.** d **14.** a **15.** c **16.** b
17. a **18.** b **19.** b **20.** b **21.** c

Rhabdoviruses

47
CHAPTER

Chapter Preview

- Rabies virus
- Other rhabdoviruses

The Family Rhabdoviridae comprises of two genera:

1. **Genus *Lyssavirus*:** Contains rabies virus and rabies-related viruses
2. **Genus *Vesiculovirus*:** Contains vesicular stomatitis viruses (described under Arboviruses, Chapter 46).

RABIES VIRUS

Rabies virus causes a rapidly progressive, acute infectious disease of the central nervous system (CNS) in humans and animals, transmitted from another rabid animal. Although the human cases are few in number, rabies is still considered as a major public health problem because it is almost always fatal.

Morphology

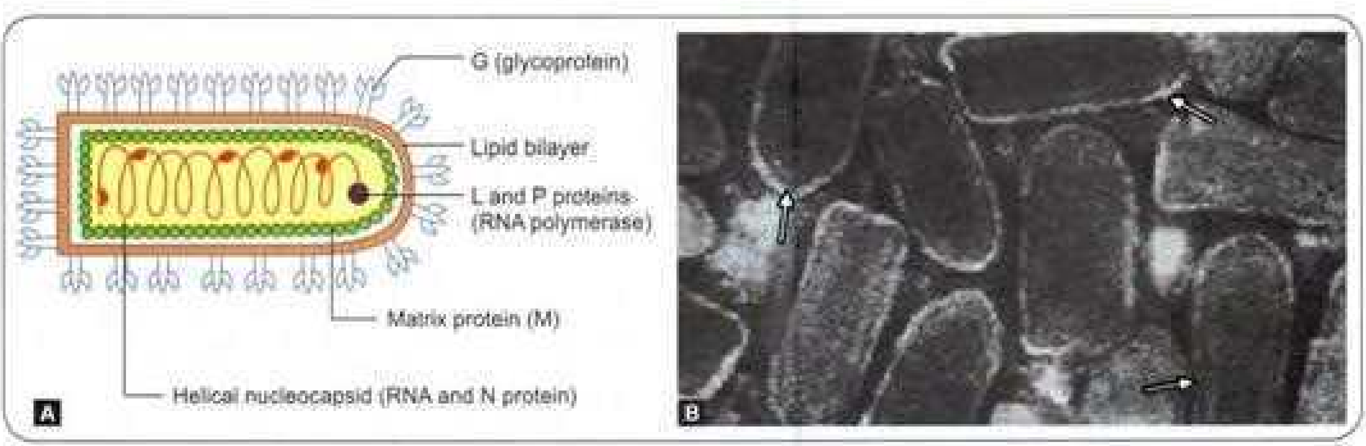
Rhabdoviridae family has a unique morphology.

- **Bullet-shaped** (75 nm in width and 180 nm in length)
- **Enveloped:** They have a lipid envelope in which 10 nm long peplomers or spikes (glycoprotein-G) are embedded. Envelope is lined internally by a layer of matrix protein (Figs 47.1A and B)
- **Nucleocapsid** has a helical symmetry and comprises a single-stranded, negative-sense RNA, nucleoprotein and polymerase proteins.

Antigenic Properties

Rabies virus has only one serotype. However, there are seven antigenic variants that are associated with specific animal reservoirs and found in different geographical areas.

- The variants have minor differences in their antigens (nucleoprotein and glycoprotein G) as well as in their nucleotide sequences
- Glycoprotein-G is the major factor responsible for rabies virus neuroinvasiveness and pathogenicity. It also possesses hemagglutinin activity (Table 47.1).



Figs 47.1A and B: Rabies virus. **A.** Schematic diagram; **B.** Electron micrograph (*arrows showing*)

Source: Public Health Image Library, B. ID#5611/Fred. A. Murphy/Centers for Disease Control and Prevention (CDC), Atlanta (with permission)

Table 47.1: Major antigens of rabies virus

Glycoprotein G	Nucleoprotein
These are peplomers or spikes embedded in envelope	These are capsid proteins associated with viral RNA
Species-specific	Group-specific and it cross reacts with that of rabies-related viruses
Role in pathogenesis: It binds to acetyl choline receptors in neural tissues, which is the first step of pathogenesis (attachment)	Does not have any role in pathogenesis
Diagnostic role: **Antibody detection (HAI):** It induces hemagglutination inhibiting antibodies which can be detected in patient's serum by hemagglutination inhibition (HAI) test	• **Antibody detection (CFT):** It induces complement fixing antibodies which can be detected in patient's serum by complement fixation test (CFT) • **Antigen detection:** Antiserum prepared against the purified nucleocapsid is used in direct IF test
Role in immunity: It induces neutralizing antibodies which are protective in nature and also stimulates cytotoxic T cells	Antibodies are not protective.
Role in vaccination: Being protective in nature, the purified form of the antigen can be used in subunit vaccine	Not used for vaccination

Animal Susceptibility

Rabies virus has a wide host range; infects all warm-blooded animals, including humans.

- **Animal susceptibility to rabies virus:** Susceptibility varies among various animals.
  - Very highly susceptible animals: Foxes, jackals, wolves and cotton rats
  - Highly susceptible animals: Rabbits, cattle, cats, hamsters, raccoons and bats
  - Moderately susceptible animals: Dogs, goats, sheep, horses and non-human primates
  - Low susceptible animals: Opossums.
- Rabies virus also undergoes certain changes when it is serially propagated in animals
- **Street viruses:** These are freshly isolated strains in the laboratory. They mimic the wild viruses; show long and variable incubation periods and produce intracytoplasmic inclusion bodies (Table 47.2).
- **Fixed viruses:** When street viruses are propagated in rabbits by serial brain-to-brain passage; they lose certain properties and become fixed strains (Table 47.2).
  - They do not produce inclusion bodies
  - They do not multiply in extraneural tissues

Table 47.2: Differences between street and fixed rabies viruses

Street viruses	Fixed viruses
Freshly isolated	Isolated after serial intracerebral passage in rabbits/cell lines
Produce Negri body	Do not produce Negri body
Affect salivary glands	Do not affect salivary glands
Pathogenic	Not pathogenic (except- if not inactivated properly)
Incubation Period: 1–3 months (20–90 days)	Incubation Period: 4–6 days
Produce disease	Used for vaccine

  - They do not infect salivary gland
  - They multiply rapidly, and the incubation period is shortened to 4–6 days, hence these strains are best used for vaccination.

Pathogenesis (Fig. 47.2)

Transmission

- **Bite:** Rabies virus is usually transmitted to humans by the bite of an infected animal. However, other modes of transmission have also been rarely reported.
  - **Animal bite:** In India, dogs are responsible for about 97% of human rabies, followed by cats (2%), jackals, mongoose and others (1%) (except rat bite and human bite)
  - **Bat bite** (mostly goes unnoticed): Migrating fruit-eating bats are the most common bats that transmit rabies in America
  - Human-to-human transmission is theoretically possible, but is extremely rare.
- **Non-bite exposures** are rare such as:
  - Lick on abrasion or mucosa
  - Inhalation of virus containing aerosols generated from infected bats
  - Corneal transplantation.

Spread of the Virus

- **Multiply locally:** Virus starts replicating locally at the site of inoculation in muscle or in connective tissue
- **Viral entry to peripheral neurons:** Virus binds to nicotinic acetylcholine receptors present at neuromuscular junctions
- **Neuronal spread:** Rabies virus spreads centripetally along the peripheral motor nerves via retrograde fast axonal transport, at a rate up to 250 mm/day
- It reaches dorsal root ganglia of the spinal cord, and then ascends upward towards CNS
- **Central nervous system (CNS) infection:** It rapidly disseminates to various parts of CNS, most common sites are hippocampus and cerebellum
- **Centrifugal spread:** From CNS, the virus spreads along the sensory and autonomic nerves to various tissues such

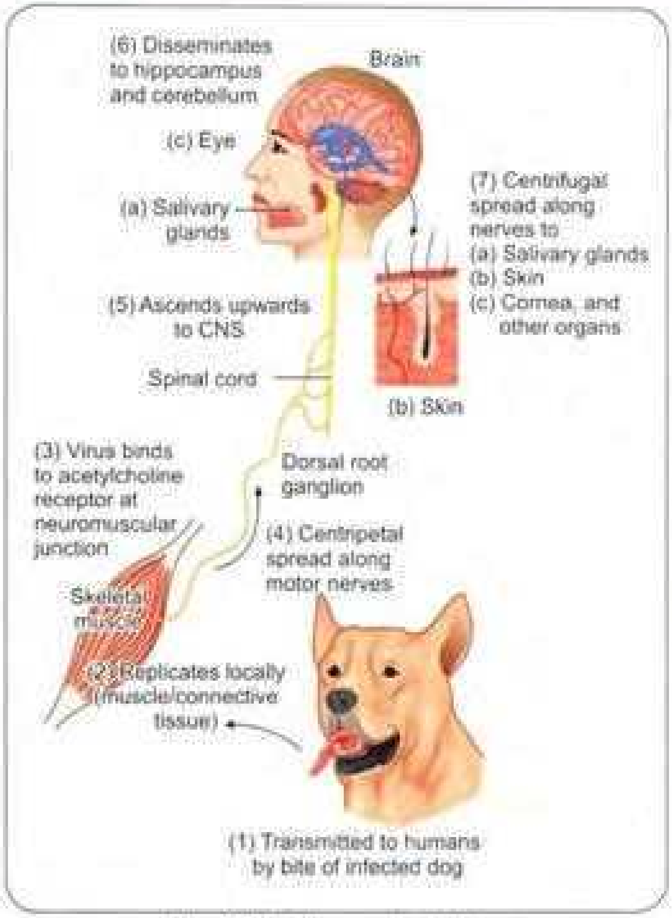


Fig. 47.2: Pathogenesis of rabies

as salivary glands (maximum titer) followed by pancreas, kidney, heart, retina, and cornea. However, viremia does not occur

- **Shed in saliva:** Rabies virus is shed in the saliva of rabid animals which acts as the source of infection to other animals. Viral shedding also occurs in human saliva, but human to human transmission has not been confirmed yet.

Pathological Changes

Histopathological changes produced in brain parenchyma of rabies infected patients include:

- Mononuclear cell infiltration
- Perivascular cuffing of lymphocytes
- Babes nodules consisting of glial cells
- Negri bodies.

Negri Bodies

These are intracytoplasmic eosinophilic inclusions in brain neurons that are composed of rabies virus proteins and viral RNA.

- Detection of Negri bodies confirms post mortem diagnosis of rabies
- However, it may be absent up to 20% of cases; hence absence of Negri body cannot rule out rabies
- Negri bodies are commonly observed in Purkinje cells of the cerebellum and in pyramidal neurons of the hippocampus, and are less frequently seen in cortical and brainstem neurons.

Clinical Manifestations

Incubation Period

Incubation period is prolonged and variable, average being 20–90 days (ranges from 1 week to 19 years).

It is directly related to the distance for the virus to travel from the site of inoculation to CNS. Hence the incubation period is usually shorter in:

- Children than in adults
- Bites on head, neck and upper limbs than legs
- Short people
- Severe lacerations
- Presence of genetic predisposition
- Low host immunity
- **Virus:** High dose of inoculum, ↑ virulence of the strain.

The clinical spectrum is divided into 3 phases as follows.

Prodromal Phase

It lasts for 2–10 days, characterized by non-specific symptoms such as fever, malaise, anorexia, nausea, vomiting, photophobia, sore throat, abnormal sensation (paresthesia, pain, or pruritus) around the wound site.

Acute Neurologic Phase

This may be either encephalitic type (80%) or paralytic type (20%).

- **Encephalitic or furious rabies:** It lasts for 2–7 days, and is characterized by:
  - **Hyperexcitability:** Anxiety, agitation, hyperactivity, bizarre behavior and hallucinations may be seen
  - **Lucid interval:** Period of hyperexcitability is typically followed by complete lucidity that becomes shorter as the disease progresses
  - **Autonomic (sympathetic) dysfunction** features may be seen such as ↑lacrimation, ↑salivation (leads to ***foaming*** at the mouth), ↑ perspiration, gooseflesh, cardiac arrhythmia and priapism
  - **Hydrophobia** (fear of water) or **aerophobia** (fear of air)—The act of swallowing precipitates an involuntary, painful spasm of the respiratory, laryngeal, and pharyngeal muscles. These symptoms are probably due to dysfunction of infected brainstem neurons.
- **Paralytic or dumb rabies:** This occurs in 20% of cases, especially in people who are partially vaccinated or infected with bat rabies virus. It is characterized by flaccid paralysis, often begins in the bitten limb and progressing to quadriparesis with facial paralysis.

However, hydrophobia and other features of encephalitic rabies are typically absent.

Coma and Death

Following acute neurological phase, patient develops coma that eventually leads to death within 14 days. Patients with paralytic rabies may survive longer up to 30 days. However, death is almost certain. Recovery and survival are extremely rare.

LABORATORY DIAGNOSIS — Rabies

- Antigen detection from hair follicles at nape of the neck and from corneal smear—by direct IF test
- Viral Isolation by:
  - Mouse inoculation.
  - Cell lines inoculation—Mouse neuroblastoma and BHK cell lines.
- Antibody detection from serum and CSF—by MNT, RFF-IT, FAVN, IFA, HAI and CFT
- Viral RNA detection—by RT-PCR
- Negri body detection in histopathological staining of brain biopsies (hippocampus)—for postmortem diagnosis of rabies.

Laboratory Diagnosis

Rabies Antigen Detection

Direct immunofluorescence test (direct-IF); also called as direct fluorescent antibody (DFA) test can be performed to detect rabies nucleoprotein antigens in specimens by using specific monoclonal antibodies tagged with fluorescent dye. Because of its high sensitivity and specificity, DFA test is considered as the "gold standard" method for rabies diagnosis.

- The best specimen is hair follicle of nape of neck (most sensitive)
- Corneal impression smear can also be used. It is usually positive in late stage with a sensitivity of 30%.

Immunohistochemistry-based staining can also be performed to detect rabies antigen.

Viral Isolation

- **Mouse inoculation:** Intracerebral inoculation into suckling mice can cause encephalitis and death. The brain biopsies of the inoculated animal are examined for the presence of Negri bodies and rabies antigen
- **Cell lines:** Mouse neuroblastoma cell lines and baby hamster kidney (BHK) cell lines are the preferred cell lines for rabies virus isolation.
  - They can yield virus (2–4 days) much faster than that of mice inoculation
  - Viral growth in the cell lines can be detected by direct-IF test using specific antiserum.

Antibody Detection

Detection of CSF antibodies is more significant than serum antibodies.

- Serum antibodies appear late and can also be present after vaccination
- CSF antibodies appear early and they are produced only in rabies-infected individuals but not in response to vaccination
- Several test formats are available, which detect antibodies by employing antigens such as glycoprotein or nucleoprotein
  - Mouse neutralization test (MNT)
  - Rapid fluorescent focus inhibition test (RFFIT)
  - Fluorescent antibody virus neutralization test (FAVN)
  - Indirect fluorescence assay (IFA)
  - Hemagglutination inhibition test (HAI)
  - Complement fixation test (CFT)
  - Immunoperoxidase inhibition assay.

Viral RNA Detection

Reverse transcription-polymerase chain reaction (RT-PCR) can be used to amplify genes of rabies virus (e.g. genes coding for nucleoprotein or large structural protein) from brain tissue, saliva and skin biopsy samples. It is the most sensitive and specific assay available at present for the diagnosis of rabies.

Negri Body Detection

It is useful for postmortem diagnosis of rabies:

- It is an intracytoplasmic eosinophilic inclusion with characteristic basophilic inner granules
- It is sharply demarcated, spherical to oval, and about 2–10 μm in size; surrounded by a halo
- Most common sites of Negri bodies are neurons of cerebellum and hippocampus; however, they can also be less frequently seen in cortical and brainstem neurons
- Commonly used stains are histological stains such as H and E (Fig. 47.3) and Sellers stains (basic fuchsin and methylene blue in methanol). Basophilic granules are better viewed when stained with Mann's stain
- Negri bodies in animals should be differentiated from other neuronal inclusion bodies of viral infections such as canine distemper virus and some herpesviruses (HSV, pseudorabies virus and B virus); all of which are intranuclear in location
- Immunohistochemistry: Peroxidase labeled specific antibodies are used to detect the viral inclusions in formalin-fixed tissues. It is more sensitive and specific than histological staining methods
- Negri body detection is pathognomonic of rabies. However, it may not be detected in 20% of cases
- Therefore, the absence of Negri bodies does not rule out the diagnosis of rabies.

Fig. 47.3: Negri bodies in brain biopsy by H and E stain (*arrow showing*)

Source: Public Health Image Library, ID# 3377//Dr. Daniel P. Perl/Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*).

TREATMENT **Rabies virus**

There is no specific treatment for rabies. Symptomatic treatment may prolong life, but the outcome is almost always fatal.

- Isolation: Patient should be isolated in a quiet room, protected as far as possible from external stimuli such as bright light, noise, water or cold air which can precipitate spasms.
- Sedatives and anti-anxiety drugs such as morphine can be used.
- Hydration and urination should be properly maintained.

Prognosis

Mortality in rabies is almost 100%; however, it is preventable by administration of post-exposure therapy during the early incubation period. There are seven well-documented cases who survived from rabies—mostly because of taking rabies vaccine in the early incubation period.

Prevention of Human Rabies

Post-exposure prophylaxis (PEP) includes local wound care, and both active and passive immunization.

Local Wound Care

It can greatly decrease the risk of rabies if initiated immediately. Even the patient reports late, would care must be performed as rabies virus is known to persist in skin for long time.

- **Physical cleansing:** All bite wounds and scratches should be washed thoroughly with soap and water for 15 minutes
- **Chemical inactivation:** Antiseptics such as povidone iodine or alcohol can be used to inactivate the residual viruses
- **Biological neutralization** of the virus by giving anti-rabies immunoglobulin
- Devitalized tissues should be debrided
- Tetanus prophylaxis should be given
- Antibiotic treatment is initiated to prevent secondary bacterial infection
- **Suturing is contraindicated:** Bite wounds should not be immediately sutured, as it may help in spreading of the virus into deeper tissues
- Do not touch the wound(s) with bare hand
- Do not apply irritants like soil, oil, lime, herbs, chalk, betel leaves, etc.

Passive Immunization (Rabies Immunoglobulin)

Rabies immunoglobulins (RIG) have the property of binding with the rabies virus, thereby resulting in neutralization and thus loss of infectivity of the virus. Hence, RIGs are usually administered locally at the site of exposure.

- **Two types** of RIGs are available:
  - **Equine rabies immunoglobulin (ERIG):** It is given at dose of 40 IU/kg. Being heterologous in origin (horse), it is associated with serum sickness
  - **Human rabies immunoglobulin (HRIG):** It is given in a dose of 20 IU/kg. It is devoid of side effects
- **Maximum volume** of RIGs should be infiltrated into and around the bite wound(s); remaining volume if any should be administered by deep intramuscular injection at a site distant from the vaccine injection site
- RIG should be administered within 24 hours; maximum up to 7th day.

Active Immunization (Rabies Vaccine)

Rabies vaccine can be categorized into—neural and non-neural vaccine.

Neural Vaccines

These are derived from the nervous tissues of animals infected with the fixed rabies virus. It was developed by Louis Pasteur and modified later.

- Neural vaccines were in use in India for quite a long time, but they are encephalitogenic, poorly immunogenic and are associated with serious risk of neurological complications which occur due to the myelin component of vaccine which is encephalitogenic. They are no longer in use since 2004 and have been replaced by non-neural vaccines
- **Examples include:**
  - **Semple vaccine:** It is derived from infected sheep brain, inactivated with phenol
  - **Beta propiolactone (BPL) vaccine:** It is a modified Semple vaccine which is inactivated with beta propiolactone instead of phenol. It was earlier prepared by Pasteur Institute of India, Coonoor, Tamil Nadu

HIV and Other Retroviruses

48
CHAPTER

Chapter Preview

- Retroviruses
  - Human immunodeficiency virus (HIV)
  - Human T cell lymphotropic virus (HTLV)

RETROVIRUSES

Retroviruses possess a unique enzyme called **reverse transcriptase** that directs the synthesis of DNA from the viral RNA after they infect a host cell.

The family Retroviridae includes three subfamilies and seven genera; out of which two genera contain viruses that are pathogenic to humans (Table 48.1).

- Genus *Lentivirus:* Contains human immunodeficiency virus (HIV)-1 and 2
- Genus *Deltaretrovirus:* Contains human T cell lymphotropic virus-1 (HTLV-1).

Types

Based on the mode of spread from one host cell to other, retroviruses are grouped into two types:

1. **Exogenous retroviruses:** They spread horizontally from one host cell to other. Example include most pathogenic retroviruses (HIV and oncogenic HTLV-I)
2. **Endogenous retroviruses:** They are transmitted vertically from parent host cells to offspring, by integrating the provirus into host cell chromosome.
   - The proviral DNA behaves like a cellular gene and is subjected to regulatory control by the host cell
   - They are usually silent and do not cause any disease or malignancies.

HUMAN IMMUNODEFICIENCY VIRUS (HIV)

Human immunodeficiency virus (HIV) is the etiologic agent of Acquired Immunodeficiency Syndrome (AIDS)- the biggest threat to mankind in last three decades.

History/Origin of AIDS

The first case of AIDS was described from New York (USA) in 1981; which was soon followed by the discovery (isolation) of HIV-1 from Pasteur Institute, Paris in 1983.

- HIV in humans was believed to be acquired from chimpanzee (*Pan troglodytes troglodytes*) by the cross-species infections of simian counterpart of HIV in rural Africa (simian immunodeficiency virus or SIVcpz)
- It has been postulated that though such zoonotic transmission to humans was going on repeatedly over many years in the past, only by the late 20th century the

Table 48.1: Classification of retroviruses

Subfamily	Genus	Example(s)	Feature
Oncovirinae	*Alpharetrovirus*	Rous sarcoma virus	Non-pathogenic to man Contains *src* oncogene
	Betaretrovirus	Mouse mammary tumor virus	Non-pathogenic to man
	Gammaretrovirus	Abelson murine leukemia virus	Non-pathogen to man Contains *abl* oncogene
	Deltaretrovirus	HTLV-I	Causes T cell lymphoma and neurologic disease in man
	Epsilonretrovirus	Walleye dermal sarcoma virus	Non-pathogenic to man
Lentivirinae	*Lentivirus*	HIV-1 and 2	Cause AIDS
Spumavirinae	*Spumavirus*	Simian foamy virus	Non-pathogenic to man

Abbreviations: HTLV-1, human T cell lymphotropic virus-1; HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome.

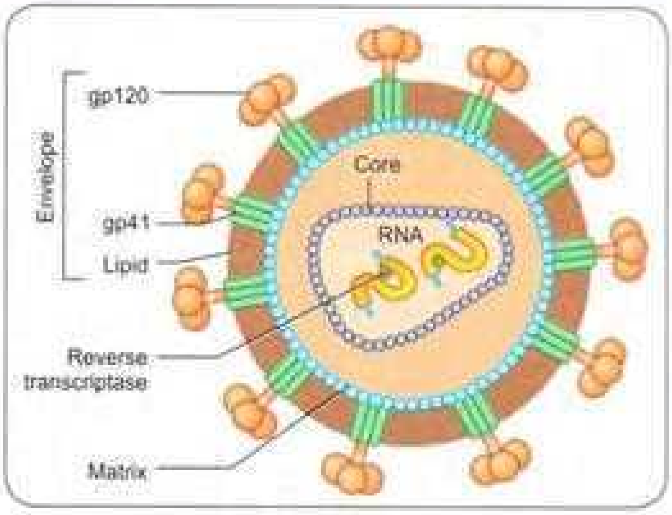


Fig. 48.1: Structure of HIV

virus underwent changes which enabled it to adapt to human environment and to reach the epidemic level.

Morphology

HIV and other lentiviruses have a unique structure (Fig. 48.1): They are spherical and 80–110 nm in size.

- **Envelope:** HIV is an enveloped virus. The envelope is made up of:
  - Lipid part: It is host cell membrane derived
  - Protein part: It has two components:
    1. Glycoprotein: 120 (gp 120)—They are projected as knob like spikes on the surface and
    2. Glycoprotein: 41 (gp 41)—They form anchoring transmembrane pedicles.
- **Nucleocapsid**: Capsid is icosahedral in symmetry, made up of core protein. Inside, there is a dense cylindrical inner core which encloses:
  - **RNA:** Two identical copies of single-stranded positive sense linear RNA
  - Viral enzymes such as reverse transcriptase, integrase and proteases which are closely associated with HIV RNA.

HIV Genes and Antigens

HIV contains three structural genes—*gag*, *pol*, and *env* and six non-structural or regulatory genes.

Structural Genes

Structural genes code for various components of the virus.

- The *gag* gene codes for the core and shell of the virus. It is expressed as a precursor protein, p55* which is cleaved into three proteins:
  - p18—constitutes the matrix or shell antigen
  - p24 and p15—constitute the core antigens.
- The *pol* gene codes for viral enzymes such as reverse transcriptase, protease and integrase. It is expressed as a precursor protein, which is cleaved into proteins p31 (integrase), p51 (reverse transcriptase) and p66
- The *env* gene codes for the envelope glycoprotein (gp 160), which is cleaved into two components:
  1. gp120: It is the main receptor of HIV that binds to CD4 molecules on host cell to initiate the infection
  2. gp41: It is the fusion protein.

Non-structural Genes

Non-structural genes regulate viral replication and are important in disease pathogenesis *in vivo*.

- *Tat* is a transcriptional transactivator gene, i.e. essential for HIV-1 replication
- *Nef* (negative factor gene, p27): It down regulates the CD4 expression on the host cell surface and ↑ viral infectivity
- *Rev* (regulator of virus gene): It enhances expression of structural proteins
- *Vif* (viral infectivity factor gene): It influences the infectivity of viral particles
- *Vpu* gene: It promotes the CD4 degradation and release of progeny viruses from the host cell and are type specific; expressed only by HIV-1
- *Vpr* gene: It increases the transport of viral genome into the nucleus and also arrests host growth
- *Vpx* is found in HIV-2 (and SIV), but not in HIV-1. It is closely related to *Vpr*
- *LTR* (long terminal repeat) sequences are present on both the ends; provide promoter, enhancer and integration signals.

Antigenic Variation and Diversity

HIV shows extensive antigenic diversity because of undergoing high rates of mutation.

- This is believed to be due to the error prone nature of reverse transcriptase enzyme
- Different mutants will be selected under different conditions (host factors, immune responses and tissue types)
- Although mutations may occur in any genes, most notably it is observed in *env* gene
- Unfortunately, envelope proteins happen to be the major target against which antibodies are produced. Hence mutations in *env* gene is the main reason which explains why:
  - HIV evades the host's immune response
  - Vaccination against HIV is extremely difficult

*In HIV, the proteins and glycoproteins are indicated by their mass which is expressed in kilodaltons (e.g. p55- means protein with molecular weight 55kDa.)

HIV Serotyping

Based on sequence differences in *env* gene, HIV comprises of two serotypes HIV-1 and 2.

HIV-1

It is divided into three distinct groups (M, N, and O). Recently, a HIV strain related to gorilla SIV was identified in a Cameroonian woman in 2009 and has been proposed as *group P*.

- 'M' is the dominant group worldwide. It comprises of ten **subtypes** or "clades" (A-J)
- Subtypes are sometimes further split into **sub-subtypes** such as A1 and A2 or F1 and F2
- There are also "**circulating recombinant** forms" or CRFs derived from recombination between different subtypes. For example, CRF01_AE is a recombination between subtypes A and E
- The same infected host may have a group of closely related viral subtypes and/or CRF at a given time which are collectively called as **quasispecies**
- HIV-1 subtypes or clades do not vary in pathogenesis or biology; but they differ in geographical distribution and transmission
- **Geographical distribution**
    - Subtype A is common in West Africa
    - Subtype B is predominant in Europe, America, Japan, and Australia
    - Subtype C is the most common form worldwide (47%). It is also the dominant form in Southern and Eastern Africa, India, and China
    - Greatest diversity: In Cameroon (West Africa), all known HIV groups and subtypes are found. It is probably, considered as the place of origin of the virus.
- **Transmission:** Asian and African subtypes (C and E) are more readily transmitted heterosexually, where as American strains (subtype B) preferentially spread through blood and homosexual contact.

HIV-2

It comprises of eight groups (A-H); they are mainly confined to Africa and few other places including India. Group A is the most common form.

Disinfection and Inactivation

HIV is inactivated completely by treatment with:

- Household bleach (10%) for 10 minutes, used for infected needle or syringe
- Ethanol (50%), isopropanol (35%)
- Lysol (0.5%) for 10 minutes
- Para formaldehyde (0.5%): For 10 minutes
- Hydrogen peroxide (0.3%) for 10 minutes
- Heating: Serum inactivation at 56°C for 10 minutes (dried blood or lyophilized blood products would need—68°C for 72 hours)
- Extremes of pH (pH 1.0, pH 13.0).

Table 48.2: Transmission of HIV

Route of transmission	Risk of transmission (Worldwide, %)	% of total transmission	
		World	India*
Blood transfusion	90–95	5	1**
Parent to child	20–40	10	5.4
Sexual intercourse	**0.1–1**	**75**	**88.9**
Vaginal	0.05–0.1	60	87.4 (heterosexual)
Anal	0.065–0.5	15	1.5 (homosexual)
Oral	0.005–0.1	Rare	Not reported
Injection drug abuse	0.5–1.0	10	1.6%
Needle stick exposure	0.3	0.1	1**
Unknown	–	–	3

*Courtesy: NACO (National AIDS Control Organization, India).
** 1% for Needle stick exposure plus blood transfusion together

Pathogenesis

Mode of Transmission (Table 48.2)

HIV is transmitted through the following modes:

- **Sexual mode** is by far the most common mode of transmission, accounts for 75% of total cases in the world
    - Heterosexual route (male to female via vaginal coitus) is the commonest mode
    - However, the risk of transmission through sexual route is minimal (0.1–1% per coitus)
    - Anal intercourse (among homosexual males or even male to female) has higher risk of transmission than vaginal intercourse.
- **Blood transfusion**, though is the least common mode of transmission (5%) but the risk of transmission is maximum (90–95%)
- **Percutaneous/mucosal** transmission modes such as needle stick injury, injection drug abuse and sharing razors or tattooing or splashes of infected blood on eyes, etc. are among the less effective modes of transmission
- **Perinatal mode:** In the absence of any intervention, the risk of transmission from mother to fetus is about 20–40%
    - Transmission may occur at any time during pregnancy and breastfeeding but the risk is maximum during delivery
    - Risk is maximum if mother is recently infected or has already developed AIDS.
- There is no evidence of HIV transmission by casual contact or kissing or insect bite
- **Viral load** is maximum in blood, genital secretions, and CSF; variable in breast milk and saliva; zero to minimal in other body fluids or urine

- Saliva may contain inhibitory substances like fibronectin and glycoproteins, which prevent transmission of the virus.

Receptor Attachment

The following receptor interaction is essential for HIV entry into the host cell.

- **Main receptor:** HIV enters into the target cells by binding its gp120 to the CD4 receptor on host cell surface. CD4 molecules are mainly expressed on helper T cells; and also on the surface of various other cells like monocytes, macrophages, Langerhans cells, astrocytes, keratinocytes and glial cells
- **A second co-receptor** in addition to CD4 is necessary for fusion of HIV to gain entry into the host cell. Usually, the chemokine receptors act as co-receptors for HIV and act by binding to gp120. Examples include:
  - CXCR4 molecules present on T lymphocytes
  - CCR5 molecules present on cells of macrophage lineage.
- **DC-SIGN**, a dendritic cell-specific lectin receptor present in skin and mucosal surfaces, can also bind to HIV-1 but does not mediate cell entry. Rather, it may facilitate transport of HIV by dendritic cells to lymphoid organs where HIV replicates further in T cells.

Mutation in CCR5 (delta 32 mutation)

This mutation results in blockade of HIV entry into the cells. It is observed principally in some lucky people of Europe and Western Asia who are either:

- **Completely resistant to HIV infection:** If they are homozygous for delta 32 mutation genes (seen in 1% of Northern Europeans, particularly in Sweden) or
- **Susceptible, but progression to AIDS is delayed:** If they are heterozygous for the same gene, seen in 10–15% of Europeans).

Replication (Refer Fig. 41.5)

The replication of HIV occurs through the following steps:

- **Fusion:** Following attachment of receptor and co-receptor to gp120, fusion of HIV to host cell takes place; mediated by the fusion protein gp41
- **Penetration and uncoating:** After fusion, HIV nucleocapsid enters into the host cell cytoplasm, which is followed by uncoating and release of two copies of ssRNA and viral enzymes
- **Reverse transcription:** Viral reverse transcriptase mediates transcription of its ssRNA into ssDNA so that DNA-RNA hybrid is formed. The RNA is degraded by viral endonuclease and ssDNA replicates to form dsDNA.
- Transcription of the DNA occurs to form some of the components of viral proteins
- **Pre-integration complex:** The nucleoprotein complex formed, comprises of linear dsDNA, gag matrix protein, accessory vpr protein and viral integrase. This is called **pre-integration complex**, which is transported into the host cell nucleus
- **Integration:** The viral dsDNA gets integrated into the host cell chromosome; mediated by viral integrase. The integrated virus is called as **provirus**
- **Latency:** In the integrated state, HIV establishes a latent infection for variable period

  However, HIV is different from other latent viruses as it is able to replicate even in latent state and is infectious to other neighboring cells.

Disease Progression

Patients infected with HIV undergo various types of disease progression (Table 48.3); which in-turn depends upon various factors such as host, viral and environmental factors.

Factors Affecting Disease Progression

- ***Viral factors:*** For example, viral fitness, co-receptor usage, generation of escape mutants, latency
- ***Host factors:*** include genetic factors, such as polymorphism of HLA and chemokine receptors.
  - HLA types A24, B35, B8 and C4: Associated with rapid disease progression while
  - HLA B27 and B57: Associated with slow disease progression
  - Mutations in the co-receptor genes CCR5 (see box).
- ***Environmental factors:*** Include nutrition and co-infections. Vitamin A deficiency is linked with HIV susceptibility. Tuberculosis and Hepatitis B can accelerate the disease progression. Hepatitis G co-infection may slow down the progression.

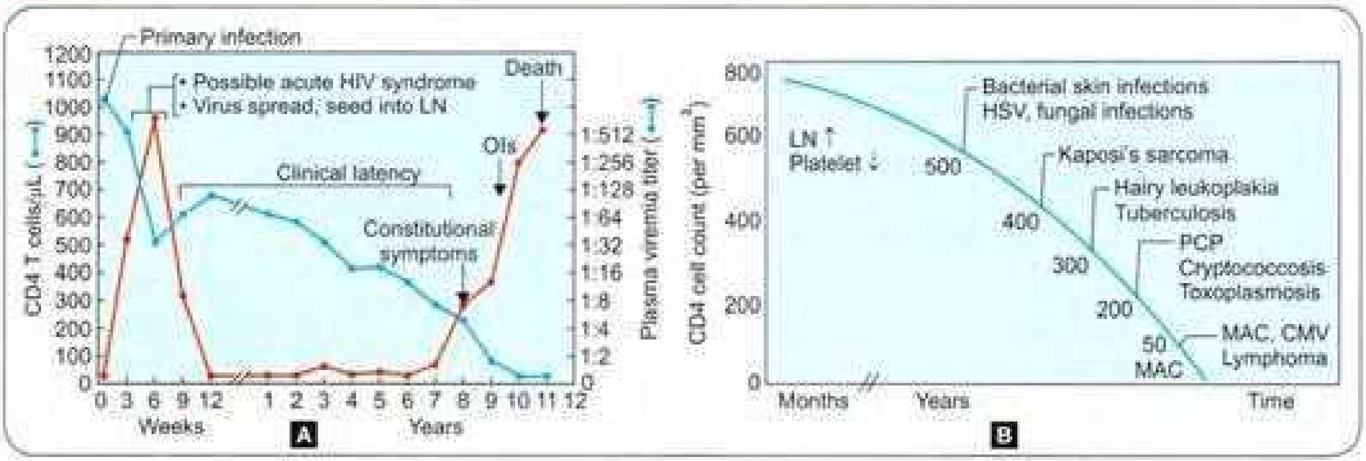
Natural Course (Typical Progressors)

About 80–90% of HIV, infected individuals are "typical progressors," with a median survival time of approximately

Table 48.3: Type of disease progressions in people living with HIV/AIDS (PLHA)

	Develops into AIDS	% of PLHA
Typical progressor	Within 10 years (described in the text)	80–90%
Rapid progressor	Within 2–3 years	5–10%
Long-term Non-progressor (LTNP)	After long time (10–30 years) without ART**. They show <5000 HIV RNA copies/mL. Usually associated with CCR5 mutation	5%
Elite controller (subset of LTNP)	After long time (10–30 years) without ART. They show <50 RNA copies/mL (below detectable level)	<1%

*PLHA: people living with HIV/AIDS; **ART: Antiretroviral therapy.



Figs 48.2A and B: A. Natural course of HIV infection; **B.** Opportunistic infections associated with HIV infection and correlation with CD4 T cell counts

Abbreviations: PCP, *Pneumocystis* pneumonia; MAC, *Mycobacterium avium* complex; CMV, cytomegalovirus; OIs, opportunistic infections.

10 years. The typical course of HIV infection includes the following stages (Fig. 48.2A).

Acute HIV Disease or Acute Retroviral Syndrome

Following infection, HIV is carried to the lymph nodes and other lymphoid tissues where further multiplication occurs inside the T cells.

- ❖ Initially, HIV destroys the infected T cells and spills over into blood stream to cause primary viremia (or acute mononucleosis-like syndrome) which coincides with an initial flu-like illness that occurs in many patients (50–75%) 3–6 weeks after the primary infection
- ❖ There is a significant drop in the numbers of circulating CD4 T cells at this stage.

Asymptomatic Stage (Clinical Latency)

Adequate immune response develops within 1 month in most of the patients.

- ❖ Both effective cell-mediated immune response (HIV specific CD8 T cells) and humoral immune response (HIV specific neutralizing antibodies) come into play
- ❖ As a result, viremia drops down and CD4 T cell count becomes normal
- ❖ It is important to note that this is a state of clinical latency, but **not microbiological latency**
- ❖ The immune response cannot clear the infection completely. HIV-infected cells persist in the lymph nodes, and there is a high level of ongoing viral replication
- ❖ This period of clinical latency is variable, may last for 10 years but ranges from few months to 30 years
- ❖ However, once the latency is broken, the disease progresses rapidly and death usually occurs within 2 years if left untreated.

Persistent Generalized Lymphadenopathy (PGL)

As a result of HIV replication in lymph nodes, 25–30% of infected people who are otherwise asymptomatic, develop lymphadenopathy.

- ❖ PGL is defined as enlarged lymph nodes of more than 1 cm size in two or more non-contiguous sites that persist for at least 3 months
- ❖ PGL must be distinguished from other causes of lymphadenopathies such as lymphoma.

Symptomatic HIV infection (AIDS-related Complex)

After variable period of clinical latency, the CD4 T cell level starts falling. Eventually patients develop constitutional symptoms such as:

- ❖ Unexplained diarrhea, lasting for more than 1 month
- ❖ Weight loss (>10%), fatigue, malaise and night sweat
- ❖ Mild opportunistic infections such as oral thrush.

AIDS

Gradually, the patient moves towards the advanced end stage of HIV infection called AIDS; characterized by:

- ❖ Rapid fall in CD4 T cell count (usually less than 200 cells/µL)
- ❖ High viral load
- ❖ Lymphoid tissue is totally destroyed and replaced by fibrous tissue
- ❖ **Opportunistic infections** set in secondary to profound immune suppression. Depending on the CD4 T cell count, various infections occur (Fig. 48.2B)
- ❖ Development of neoplasia (e.g. CNS lymphoma)
- ❖ Development of direct HIV induced manifestations such as HIV encephalopathy.

Clinical Diagnosis

Classification systems for HIV disease have been developed which are useful for tracking and monitoring the HIV epidemic, for providing clinicians and patients with important information about HIV disease stage and clinical management. Two such systems are currently in use worldwide:

1. **CDC classification system** (Centers for Disease Control and Prevention, revised 1993): This system classifies HIV infection into nine stages based on associated clinical conditions and CD4 T cell count of the patient (refer CDC website)
2. **WHO clinical staging of HIV/AIDS for adults** (World Health Organization, revised 2007) is based only on the clinical conditions associated with the patient. For, resource poor countries like India, where facilities for testing CD4 T cell count are not available widely, WHO clinical staging is more useful. It classifies HIV infection in adults and adolescents (>15 years) into four stages (Table 48.4).

Table 48.4: WHO clinical staging of HIV/AIDS for adults

WHO clinical staging of HIV/AIDS for adults (Revised,2007)

Clinical Stage 1

- Asymptomatic HIV infection
- Persistent generalized lymphadenopathy

Clinical Stage 2

- Unexplained moderate weight loss (<10%)
- Recurrent respiratory tract infections (sinusitis, tonsillitis, otitis media, pharyngitis)
- Herpes zoster
- Angular cheilitis
- Recurrent oral ulcers
- Papular pruritic eruptions
- Seborrheic dermatitis
- Fungal nail infection
- Unexplained persistent parotid enlargement

Clinical Stage 3

- Unexplained severe weight loss (>10%)
- Unexplained chronic diarrhea: >1 month
- Unexplained persistent fever: 1 month
- Oral candidiasis
- Oral hairy leukoplakia
- Pulmonary tuberculosis
- Severe bacterial infection
- Acute necrotizing ulcerative stomatitis, gingivitis, and periodontitis
- Unexplained anemia

Clinical Stage 4

- **HIV wasting syndrome (Slim disease):** Characterized by profound weight loss (>10%), chronic diarrhea (>1 month), prolonged unexplained fever (1 month)

Contd...

Contd...

- **Bacterial opportunistic infections:**
  - Recurrent severe bacterial infections
  - Extrapulmonary tuberculosis
  - Disseminated non-tubercular mycobacterial infection
  - Recurrent septicemia (including non-typhoidal salmonellosis)
- **Viral opportunistic infections:**
  - Chronic HSV infection
  - Progressive multifocal leukoencephalopathy
  - CMV (retinitis, or other organ infection excluding liver, spleen, and lymph node)
- **Fungal opportunistic infections:**
  - *Pneumocystis jirovecii* pneumonia
  - Esophageal candidiasis
  - Extrapulmonary cryptococcosis (meningitis)
  - Disseminated mycoses (histoplasmosis and coccidioidomycoses)
- **Parasitic opportunistic infections:**
  - *Toxoplasma* encephalitis
  - Chronic intestinal cystoisosporiasis (>1 month)
  - Atypical disseminated leishmaniasis
  - Chronic intestinal cryptosporidiosis (>1 month)
- **Neoplasia:**
  - Kaposi's sarcoma
  - Invasive cervical cancer
  - Lymphoma (cerebral, B cell and non-Hodgkin)
- **Other conditions (direct HIV induced):**
  - HIV encephalopathy
  - Symptomatic HIV-associated nephropathy or cardiomyopathy

Epidemiology

Global Situation

Since the discovery of AIDS epidemic, almost 78 million people have been infected and 39 million deaths occurred due to HIV worldwide.

- In 2016, 1.8 million new HIV infections occurred globally with 1 million deaths
- **Prevalence** (number of cases per 100 population): At the end of 2016, about 36.7 million people were living with HIV (including 1.8 million children) with a **global prevalence of 0.8%** [0.7–0.9%] in adults
- **Sub-Saharan Africa** remains the most severely affected region, with nearly one in every 25 adults (4.2% prevalence) living with HIV and accounting for nearly two-thirds of the people living with HIV worldwide. South Africa has the largest HIV epidemic in the world, with 19% of the global PLHA. Swaziland a state in Southern Africa has the world's highest HIV prevalence in adults (26%). In addition, Swaziland's TB incidence rate is the highest in the world, and 80% of TB patients are co-infected with HIV

- **World AIDS Day:** Globally, 1st December is observed as World AIDS Day every year.

HIV/AIDS Situation in India (End of 2015)

By the end of 2015, the adult **HIV prevalence in India** was reported as 0.26% (0.3% in males, 0.22% in females).

- Number of PLHA (people living with HIV/AIDS) were over 2.1 million adults
- **Andhra Pradesh** (undivided) was the worst affected state followed by Maharashtra and Karnataka in terms of PLHA
- However, as far as prevalence is concerned, Northeast states such as Nagaland, Mizoram and Manipur are worst affected
- Manipur has the highest HIV prevalence of 1.15% (only state to have epidemic of HIV), followed by Mizoram (0.80%), Nagaland (0.78%), Andhra Pradesh & Telangana (0.66%), Karnataka (0.45%), Gujarat (0.42%) and Goa (0.40%). Other states to have HIV prevalence greater than the national prevalence (0.26%) are Maharashtra, Chandigarh, Tripura and Tamil Nadu
- **High risk group:** HIV prevalence among female sex workers is 2.2%, men who have sex with men is 4.2%, Hijra/transgender people is 7.5% and IV drug abuser group is 9.9%.

Reservoir

Infected people (both symptomatic as well as asymptomatic) are the only reservoir host. Once infected, they harbor the virus for life.

High-risk Groups

High-risk groups which commonly acquire infection are:

- Homosexuals and people with multiple sex partners, and prostitutes
- Health care workers (via accidental needle pricks or blood splashes on eyes)
- Intravenous drug addicts
- Hemophiliacs and other recipients of blood or blood products
- People with other STIs (sexually-transmitted infections).

Opportunistic Infection

Globally including India, tuberculosis is the most common opportunistic infection that occurs in HIV-infected people (see Fig. 48.2B).

- Common fungal infections are candidiasis (oral thrush) and *Pneumocystis jirovecii*
- Frequent viral infections are herpes simplex mucosal lesions and CMV retinitis
- Common parasitic infections are *Cryptosporidium parvum* diarrhea, *Toxoplasma* encephalitis and *Strongyloides stercoralis* hyperinfection syndrome.

AIDS Control Organization

- **UNAIDS:** The Joint United Nations Program on HIV and AIDS (UNAIDS) is the main advocate for global action on the HIV/AIDS. It has initiated the 'Fast-Track strategy to end the AIDS epidemic by 2030' (Table 48.5)
- **NACO:** National AIDS Control Organization (NACO) has been constituted to implement the HIV/AIDS control program in India. It provides single national plan within one monitoring system
- **SACS:** State AIDS Prevention and Control Societies (SACS) are present in every state/union territory (35 numbers). They implement NACO program at state level
- **National Strategic Plan for HIV/AIDS and STI (2017-2024):** NACO has launched the national strategic plan 'Paving Way for an AIDS Free India'; going in line with UNAIDS for ending the AIDS epidemic by 2030.

Kinetics of Immune Response

An understanding of the kinetics of host immune response following infection is needed before we discuss the laboratory diagnosis; so as to understand the optimal usage of various tests during different stages of HIV disease.

- **Viremia:** Soon following the entry of the virus into the body, there occurs a transient period of high level viremia and p24 antigenemia. However, the levels of these components fall down with concomitant immune response
- **Humoral response** is evidenced by formation of antibodies of different classes (IgM, IgA, IgG) against different **structural proteins** (*gag*: p15, p17, p24, p55; *env*: gp 41, gp 120, gp 160; and *pol*: p31, p51 and p66), **regulatory proteins** (nef, rev, tat) and **accessory proteins** (vif, vpu and vpr)
- All **structural components are strongly immunogenic** and induce formation of antibodies; whereas,

Table 48.5: Fast-Track strategy ending the AIDS epidemic by 2030 by UNAIDS (United Nations Programme on HIV/AIDS)

	Present status (2016)	By 2020	By 2030
% of infected people should know about their disease status	70%	90%	95%
% of infected people receiving antiretroviral treatment (ART)	56%	90%	95%
% of people receiving ART have viral suppression	60–70%	90 %	95 %
New infection among adults	1.8 million	5 Lakh	2 Lakh
To eliminate HIV-related stigma and discrimination	>50%*	Zero	Zero

*Findings from 50 countries indicate that roughly one in every eight people living with HIV is being denied of health services because of stigma and discrimination.

immunogenicity of regulatory and accessory proteins is variable

- **Window period:** Following infection, antibodies appear in serum only after a period of interval, which is called window period. This is about 3 to 12 weeks
- **The antibodies to *gag* protein** (p24 and p55) usually appear first, though antibodies to *env* proteins and *pol* proteins may also be produced simultaneously
- As infection progresses to AIDS, antibody to p24 usually declines as p24 antigen levels rise concomitant with progression of disease to AIDS. However, antibodies to *env* proteins persist throughout the infection
- **Anti-HIV antibodies:** Among the antibodies (IgA, IgM and IgG) appear, only IgG response is consistent and long lasting. Most currently available assays detect IgG antibodies
  - IgM response appears earlier than IgG but sensitivity is low and is detectable for a short period. However, it is valuable for identifying early seroconversion (2-11 days) particularly following needle stick injury and infection in newborn
  - Detection of IgA is useful in specimens such as serum, mucous secretions (saliva, colostrum, genito-urinary secretion, etc.) and in newborn.

LABORATORY DIAGNOSIS — HIV Infection

Specific Tests for HIV Infection

- **Screening tests** (antibody detection):
  - ELISA (takes 2–3 hours)
  - Rapid/Simple test (takes <30 minutes).
- **Supplemental tests** (antibody detection):
  - Western blot assay
  - Immunofluorescence assay
  - Radio-immuno-precipitation assay (RIPA)
  - Line immunoassay (LIA).
- **Confirmatory tests**
  - p24 antigen detection
  - Viral culture—by Co-cultivation technique
  - HIV RNA (best confirmatory method)
    - Reverse transcriptase PCR (RT-PCR)
    - Branched DNA assay
    - NASBA (Nucleic acid sequence-based amplification)
    - Real time RT-PCR- for estimating viral load
  - HIV DNA detection: Useful for diagnosis of pediatric HIV.

Non-specific Immunological Methods

- Low CD4 T cell count
- Hypergammaglobulinemia:
  - Neopterin
  - β2-macroglobulin.
- Altered CD4: CD8 T cell ratio.

Laboratory Diagnosis

Diagnosis of HIV/AIDS is not like other infectious diseases. A number of moral, ethical, legal and psychosocial issues are associated with a positive HIV status. Disease is life long, outcome is invariably fatal, no cure or vaccine is available so far, and in majority, the transmission is through sexual contact. Hence, individuals known to be HIV infected are stigmatized and develop fear of being discriminated and socially out casted. Therefore, the following care should be taken **(3Cs)** while performing the test for HIV.

- **Consent** in written format should be taken before the test is done. Patient should be explained about the nature of the test being performed
- **Confidentiality** of a positive test result is must. Patient name or the word "HIV positive" should not be written on the report form
- **Counseling** should be provided to motivate the individual to tell the spouse/family and induce behavioral change.

Antibody Detection

Detection of anti-HIV antibodies is the mainstay of diagnosis of HIV. Tests to detect specific HIV antibodies can be classified into:

Screening Assays

Screening assays usually take less time (2-3 hours for ELISA, less than 30 minutes for rapid/simple tests):

- **High sensitivity and specificity:** NACO recommends the use of ELISA and rapid kits with a sensitivity of ≥99.5% and the specificity of ≥98%
- **Should be confirmed:** Results of a screening test should never be used as the final interpretation of HIV status as false positive results or technical errors can occur. It is always subjected to confirmatory tests
- Antigens used in most of the screening tests are:
  - HIV-1 specific (p24, gp 120, gp160, gp41)
  - HIV-2 specific gp36.
- They detect HIV-1 and 2 either separately or together.

ELISA (Enzyme-linked Immunosorbant Assay)

ELISA is the most commonly performed screening test at blood banks and tertiary care sites. It is easy to perform, adaptable to large number of samples. It is sensitive, specific, and cost effective.

Types of ELISA kits: Based on type of HIV antigen used, different types of ELISA are available commercially:

- *1st generation ELISA:* Uses crude preparation of HIV antigens. It yields more false positive results, hence not in use now
- *2nd generation ELISA:* Uses recombinant and/or synthetic antigens of HIV. It has improved sensitivity
- *3rd generation ELISA:* Uses recombinant and/or synthetic peptides in an antigen sandwich configuration. Advantages include: (i) very high sensitivity, (ii) able to detect IgM antibody in addition to IgG antibody;

(iii) reduces the window period to 22 days; (iv) detects HIV-1 and HIV-2 simultaneously

- *4th generation ELISA:* Detects both HIV antibodies (IgM and IgG) and antigen (p24) by using combination of recombinant/synthetic peptides as well as monoclonal antibodies respectively. It reduces the window period considerably
- *5th generation ELISA:* It differentiates between HIV-1 and HIV-2 antibodies as well as detects HIV-1 p24 antigen.

Principle of ELISA: Various ELISA formats are in use depending on different principles such as: (i) indirect ELISA, (ii) competitive ELISA, (iii) sandwich ELISA.

Rapid/Simple Test

These assays have been developed for ease of performance and quick results. They generally require less than 30 minutes to perform and do not require special equipment. They are the most commonly used tests in India. They work on various principles such as:

- Dot blot assays (or Immunoconcentration or flow through method, e.g. Tridot test, see Fig. 12.25)
- Immunochromatography (or ICT, lateral flow assay)
- Particle agglutination assays (using latex, gelatin, RBCs)
- Dip stick/Comb tests (Enzyme immune assay-based tests).

Supplemental Tests

These assays are highly specific antibody detection methods; hence used for validation of positive results of screening tests. They are expensive, labor intensive, need expertise to interpret, and may also give equivocal/indeterminate results.

Western Blot

It is the most commonly used supplemental test available and is also recommended by NACO.

- It works on the principle of immunoblot technique (described in Chapter 12)
- It detects individual antibodies in serum separately against various antigenic fragments of HIV such as (Fig. 48.3)

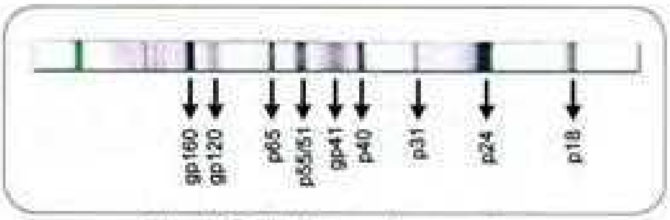


Fig. 48.3: HIV Western blot test strip

Source: Department of Microbiology, JIPMER, Puducherry (with permission).

  - Antibody to *gag* gene products (p55, p40, p24, p18)
  - Antibody to *pol* gene products (p68, p53, p31)
  - Antibody to *env* gene products (gp 120, gp160, gp41).
- The antigen antibody complexes appear as distinct bands on nitrocellulose strip
- Reactive results are interpreted as per:
  - *WHO criteria:* presence of at least two envelope bands (out of gp120, gp160 or gp41) with or without gag or pol bands
  - *CDC criteria:* presence of any two; out of p24, gp120, gp160 and gp41 bands.

Detection of p24 Core Antigen

The p24 antigen becomes detectable after 12-26 days of infection and lasts for 3-4 weeks thereafter. Again, it is elevated during the late advanced stage of AIDS. p24 Ag is detected by 4th generation ELISA (described earlier).

- **It is less sensitive** (~30%) because once the antibody is formed, it binds to the p24 protein and the antigen-antibody complex gets eliminated from blood
- Recently, **antigen dissociation assay** has been developed that involves pretreatment of serum to an agent, that liberates p24 antigen from the immunocomplexes. This has shown better sensitivity
- **Uses of p24 antigen detection test:**
  - For confirmation of diagnosis of HIV/AIDS
  - Diagnosis of HIV during window period
  - To diagnose late stage of HIV/AIDS (immune collapse) or CNS disease
  - Diagnosis of HIV in infants (not reliable)
  - Monitoring the progress of HIV infection
  - To resolve equivocal western blot results.

Viral RNA Detection

Detection of viral RNA is the **"gold standard"** method for confirmation of HIV diagnosis. Various formats are available targeting pol and env genes.

- Reverse transcriptase polymerase chain reaction (RT-PCR)
- Branched DNA assay
- NASBA: Nucleic acid sequence-based amplification
- Real time RT-PCR: For estimating viral load

Apart from the routine diagnosis of HIV, RNA detection has several other uses such as:

- It is the **most sensitive** and **specific** method, detects even few copies of viral RNA and is the best method **for confirmation** of HIV
- It is the best tool for diagnosis of HIV during **window period**, detects HIV earlier than all available methods (10-14 days postexposure)
- **Viral load monitoring:** Real time RT-PCR can quantify the viral load and is the most appropriate tool for monitoring the response to antiretroviral therapy

- **Typing:** RT-PCR can successfully differentiate between HIV-1 and HIV-2 infections and can detect the specific genotype or subtype
- Detection of **drug resistance** genes.

DNA PCR

PCR detecting proviral DNA is extremely useful for diagnosis of pediatric HIV and to differentiate latent HIV infection from active viral transcription. It is also useful during the window period, viral load estimation (real time PCR) and detection of genotypes.

Isolation of the Virus from Blood or Tissues

Isolation is time consuming, expensive, takes longer time (6 weeks or more) and not sensitive. This is due to the lower number of available cells cultured and paucity of infected cells in the sample that are present. Two methods are being used for virus isolation:

1. **In the direct method**, peripheral blood mononuclear cells (PBMCs) from the patient are cultured *in vitro* in presence of mitogen phytohemagglutinin (PHA)
2. **In the co-cultivation method**, PBMCs from heterologous HIV uninfected donor are stimulated with PHA, and after 48-72 hours, the stimulated cells are cultured along with the PBMCs from the patient
   - Viral growth in the culture supernatant is detected either by demonstration of the presence of p24 antigen or reverse transcriptase enzyme or syncytia formation in the infected cells or immunofluorescence assay to detect the viral antigens in the infected cells
   - HIV cultivation is predominantly a research tool and is available only in a few reference laboratories, as it requires at least level-2 containment facility and high degree of expertise.

Non-specific/Immunological Tests

- **CD4 T cell count:** Measurement of CD4 T cell count is carried out by flow cytometry method. It is useful for:
  - Assessing the risk of opportunistic infections (see Fig. 48.2B)
  - Initiation of antiretroviral therapy—previously used. Current guideline says treatment should be started in all patients regardless of CD4 T cell count
  - Monitoring the response to antiretroviral therapy.
- **Abnormal proteins** such as neopterin, beta 2-microglobulin and soluble IL-2 receptor are produced by peripheral blood mononuclear cells; stimulated by interferon-gamma or IL-2 which in turn are produced by HIV activated T_H1 cells.

NACO Strategy for HIV Diagnosis

For the resource poor countries, it is impracticable to confirm the result of HIV screening tests by PCR or western blot as these assays are expensive and available only at limited centers.

NACO (National AIDS Control Organization, India) has formulated a strategic plan for HIV diagnosis. The guidelines are as follows:

- Depending on the situation/condition, for which the test is done, the positive result of the first screening test should be either considered as such or confirmed by another one or two screening tests
- The first screening test should be highly sensitive, whereas the second and third screening tests should have high specificity
- The three screening tests should use different principles or different antigens. The same kit should not be used again
- Supplemental or confirmatory tests should be used only when the screening test(s) results are equivocal/ intermediate.

There are four NACO Strategic Plans/Algorithms (Fig. 48.4):

1. **Strategy I:**
   - **Purpose:** It is done for transfusion and transplantation safety; i.e. for the screening for the blood donors in blood banks
   - Only one test should be done. If found reactive, then the unit of blood is destroyed.
2. **Strategy IIa:**
   - **Purpose:** It is done for sentinel surveillance of HIV infection to estimate the prevalence of infection
   - **UAT:** The method followed here is called as unlinked anonymous testing (UAT), which involves screening of blood specimens taken for purposes other than HIV testing. Then the samples are permanently decoded of personal identifiers. This process occurs without informed consent
   - **Two tests format:** Positive results of the first test should be confirmed by a second test. If the second test is negative, then it is reported as negative.
3. **Strategy IIb:**
   - **Purpose:** It is followed for the diagnosis of HIV/AIDS in symptomatic patients
   - Positive result of first test should be confirmed by a second test. If the second test is negative, then a third test is done for confirmation.
4. **Strategy III:**
   - **Purpose:** It is done for the diagnosis of asymptomatic HIV patients, antenatal screening and screening of patients awaiting surgeries
   - **Three tests format:** All positive results in first test should be confirmed by second and third test. Positive report is sent only if all three test results are found reactive

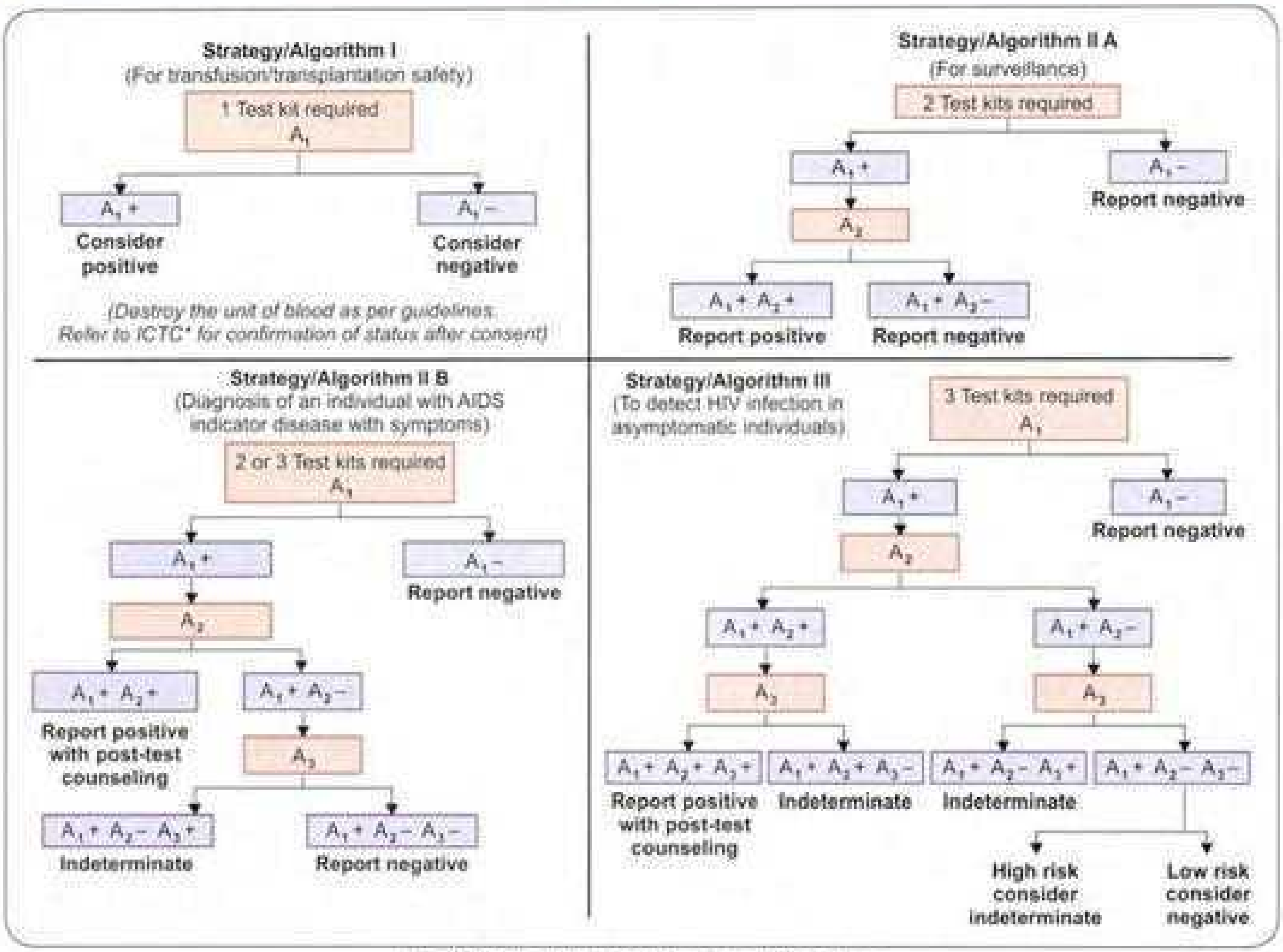


Fig. 48.4: Algorithms for diagnosing HIV infection

* NACO strategies for HIV diagnosis (Adapted from National AIDS Control Organization, NACO)

- For indeterminate results of strategy IIB and III, (i.e. first test positive but second or third test negative), the repeat test is done after 2–4 weeks and the sample should be sent to reference center for confirmation by western blot or RT-PCR.

Prognosis/monitoring of HIV

Various tools available for monitoring the response to antiretroviral therapy include:

- **CD4 T cell count:** Most commonly used
- **HIV RNA load:** Most consistent and best tool at present
- p24 antigen detection
- Neopterin and β2 macroglobulin level.

Note: Viral antibody levels are inconsistent and variable during late stage due to immune collapse; hence not reliable for prognosis.

Diagnosis of Pediatric HIV Infection

The routine screening methods (ELISA or rapid/simple tests) detect IgG antibodies.

- They cannot differentiate between baby's IgG or maternally transferred IgG, hence cannot be used for the diagnosis of pediatric HIV
- As all maternal antibodies would disappear by 18 months; therefore IgG assays can be performed after 18 months of birth.

HIV DNA PCR: This is the most recommended method for diagnosis of pediatric HIV. Baby is tested for HIV DNA PCR at 6 weeks by DBS (Dry Blood Spot) collection. If found positive, then is reconfirmed by a repeat HIV DNA PCR. Then it is reported as positive and the baby is then initiated on lifelong ART.

Other methods for pediatric HIV diagnosis include:

- HIV RNA detection
- p24 antigen detection
- IgG ELISA only after 18 months of age

Diagnosis of HIV in Window Period

Definition: Window period refers to the initial time interval between the exposure and appearance of detectable levels of antibodies in the serum.

- The antibodies appear in blood within 2–8 weeks after infection, but usually become detectable after 3 weeks to 12 weeks with the assays available presently. It can be as low as 22 days; when third generation antibody detection kits with high sensitivity are used
- p24 antigen detection (30% sensitive; by 4th generation ELISA): It can be detected by 12-26 days after infection
- HIV RNA detection (by RT-PCR) is the best method—it detects HIV RNA around 10–14 days after infection.

TREATMENT **HIV**

Antiretroviral Therapy (ART)

Antiretroviral therapy (ART) denotes the approved drugs used for the treatment of HIV/AIDS (Table 48.6). The drugs do not kill all the viruses or cure the disease. However, they have the following goals:

- **Clinical goals:** Prolongation of life; improvement in quality of life and stoppage of the progression of HIV infection to AIDS
- **Virological goals:** Greatest possible reduction in the viral load as long as possible
- **Immunological goals:** Immune reconstitution; both quantitative and qualitative improvement
- **Transmission goals:** Reduction of HIV transmission in individuals.

Previously, NACO guideline recommended the initiation of ART based on (i) CD4 T cell count, (ii) the WHO stage of the disease and (iii) associated opportunistic infections (OIs) such as TB, HBV, HCV or (iv) pregnancy/breastfeeding women.

However according to the revised NACO guideline 2017, ART has to be started in all patients irrespective of CD4 count, clinical stage, age, population or associated OIs.

Highly active antiretroviral therapy (HAART)

HAART is referred to the use of combination of at least three antiretroviral drugs to maximally suppress the HIV and stop the progression of the disease. Monotherapy with single drug is contraindicated due to inefficacy and chance of development of resistance.

NACO recommended HAART Regimen

First line regimen: NACO recommends to include three drugs (2NRTIs/NtRTI + 1NNRTI) as first line regimen.

- **TLE** (tenofovir 300 mg + lamivudine 300 mg+ Efavirenz 600 mg) is the NACO recommended regimen. It should be started for all new patients as single dose daily regimen
- **Older regimens:** If the patients are already on a different ART regimen and responding well—then same regimen can be continued.
  - ZLN (Zidovudine + Lamivudine + Nevirapine)
  - ZLE (Zidovudine + Lamivudine + Efavirenz)
  - TLN (Tenofovir + Lamivudine + Nevirapine).
- **Monitoring:** CD4 count and viral load have to be monitored every 3–6 months to monitor the response to treatment.

Contd...

Contd...

TREATMENT **HIV**

Second line regimen: If the patient is not responding to primary regimen or develops toxicity; then switch over to protease inhibitor-based regimen (1 PI +2NRTIs/NtRTIs). Example includes: tenofovir + lamivudine+ lopinavir or ritonavir.

Opportunistic Infections (OIs)

OIs should be adequately treated with appropriate antimicrobial agents before starting ART.

Cotrimoxazole prophylaxis (for prevention of Pneumocystis pneumonia)

Cotrimoxazole may be initiated in the following scenarios:

- HIV-infected adults with CD4 count <200/cmm
- WHO clinical stage 3 or 4 irrespective of CD4 count.

Problems Pertaining to use of ART

Although early start of ART can reduce the risk of disease progression, there are many other factors that pose deleterious effects on quality of life.

- Toxicity and adverse effects of ARTs, especially lipid abnormalities and drug interactions
- High cost of the regimen
- Risk of development of drug resistance and dissemination of resistant virus
- Limited therapeutic options
- **IRIS (Immune reconstitution inflammatory syndrome):** It can occur in some cases of AIDS during the recovery phase following the start of ART. As the viral load decreases, the immune system begins to recover, which results in an exaggerated immune response to a previously acquired opportunistic infection causing an overwhelming inflammatory response, that paradoxically makes the symptoms of infection worse.

Table 48.6: Antiretroviral drugs

NRTI	NNRTI	PI	Others
Zidovudine	Nevirapine	Saquinavir	**Fusion inhibitor** e.g. enfuvirtide
Stavudine	Efavirenz	Ritonavir	
Lamivudine	Delavirdine	Nelfinavir	**Integrase inhibitor** e.g. raltegravir, dolutegravir
Didanosine	Rilpivirine	Amprenavir	
Zalcitabine	Etravirine	Indinavir	
Abacavir		Lopinavir	**CCR5 receptor inhibitor** e.g. maraviroc
Emtricitabine		Ritonavir	
		Fosamprenavir	**Pharmacokinetic enhancer** e.g. cobicistat
NtRTI		Atazanavir	
Tenofovir		Tipranavir	
		Darunavir	

Abbreviation: NRTI, Nucleoside reverse transcriptase inhibitors; NNRTI, Non-nucleoside reverse transcriptase inhibitors; PI, Protease inhibitors; NtRTI, Nucleotide reverse transcriptase inhibitors.

Post-exposure Prophylaxis

Post-exposure prophylaxis (PEP) is required to reduce the risk of transmission after occupational exposures such as needle stick or sharp injury or mucocutaneous exposure.

- Every hospital should have a nodal center for PEP management and must provide PEP free of cost to the employee
- PEP should be started within 2 hours of exposure if the source is unknown or positive for HIV
- **Regimen (TLE):** Single daily dose of tenofovir (300 mg) plus lamivudine (300 mg) plus efavirenz (600 mg) is given for 28 days.

The revised NACO guideline for post-exposure prophylaxis (PEP), 2015 has been discussed in detail in Chapter 56.

NACO Guidelines to Prevent Neonatal HIV

Pregnant women who are found to be HIV reactive are initiated on lifelong ART (TLE regimen); their newborn (HIV exposed) babies are initiated on 6 weeks of Syrup Nevirapine immediately after birth; which may be extended up to 12 weeks, if the duration of the ART of mother is less than 24 weeks. The baby is also initiated on cotrimoxazole prophylaxis at 6 weeks till 18 months.

HIV Vaccine Strategies

Hurdles to Jump

After 37 years of discovery of HIV/AIDS, still medical research failed to invent an effective approved vaccine. This attributes to various factors:

- High mutability of the virus is the single most important factor
- Concept of live attenuated or even killed vaccine is impracticable due to possible risk of reactivation
- Long latent period between exposure and appearance of symptoms
- Lack of ideal small animal models for studying HIV infection
- Ethical issue: Difficulty to get human volunteers for HIV vaccine trial
- Natural immunity fails to clear HIV as it targets cells of the immune system
- As HIV is a retrovirus: Viral genome soon gets integrated into the host cell genome. Hence, it provides short window of opportunity to control.

Approaches and Trials

The researchers have explored a number of strategies and based on which more than 40 vaccine trials have been conducted in several countries so far.

- **SAV001:** It is a killed whole-virus vaccine trial, going on in Canada (2012–till now). The vaccine candidate is a genetically modified killed virus (by deleting the *nef* and *vpu* genes). Efficacy is good (antibody produced till 52-weeks); without side effect

Contd...

Contd...

- Other previously done unsuccessful trials were:
  - **RV 144 trial:** It used Canarypox virus as vaccine vector inserted with gp 120. It was going on in Thailand (2003–2009). Results showed 31.2% of efficacy at 42 months
  - **Vax Gen trial:** Subunit vaccine using recombinant gp120 antigen (2003)
  - **Step trial:** Adenovirus type 5 was used as vaccine vector (2005–2007)
  - **HVTM 505 trial:** It was based on prime boost strategy (vaccine vector and DNA vaccine). It was stopped in phase IIB stage of trial (2009–2013)
  - **Trials in India:** Used Adeno-associated virus (tgAAC09 trial) and Modified Vaccinia ankara virus (MVA trial) as vaccine vector.

Prime Boost Strategy

Most studies use a combination of the above types of vaccines in the form of '**prime and boost**' vaccines, in which two or more different vaccines are used to broaden or intensify immune responses. Examples include a vector virus is being used to prime a T-cell response; along with a subunit (peptide) booster or DNA vaccine booster is used to produce antibodies.

In spite of intense research, effort and finance involved, none of the trials has been approved for human use till now.

HUMAN T CELL LYMPHOTROPIC VIRUS (HTLV)

Human T cell lymphotropic virus (HTLV) belongs to the family Retroviridae, under the genus *Deltaretrovirus*. Two important members are HTLV-I and HTLV-II.

Human T cell lymphotropic Virus-I (HTLV-I)

Pathogenesis

- **Transmission** of HTLV-I occurs by—(1) from mother to child especially via breast milk (most common); (2) sexual (men to women), (3) infected blood
- **Target cells:** Like HIV, HTLV-I has tropism for CD4 T cells; but occasionally also infects CD8 T cells, dendritic cells and B cells
- **Virus entry** into the host cells is mediated through interaction of the envelope glycoprotein with the host cell receptor **GLUT1** (Human glucose transporter protein-1)
- **Following entry**, it replicates inside the T cells similar to that of HIV. Viral RNA is reverse transcribed to DNA, which integrates into host cell DNA. Once integrated, HTLV-I continues to exist only as a provirus which can spread from cell-to-cell. Unlike HIV, there is no free virions of HTLV-I circulated in blood
- HTLV-I expresses a unique gene called *Tax* gene which acts as a transactivator, causing the transcription of viral proteins in the long terminal repeat that are essential for replication

- *Tax* **gene has oncogenic potential:**
  - It modulates several signaling pathways such as NF-κB and modulate many human growth regulatory genes
  - It promotes the host cell growth cycle by accelerating the transition between G1 and S phase
  - DNA repair pathways (base excision repair and nucleotide excision repair) are affected, leads to DNA mutation.

Epidemiology

- **Distribution:** HTLV-I is endemic in certain parts of Japan (10% prevalence) and the Caribbean basin of Africa but it is also found sporadically elsewhere
- **Genotypes:** It has 7 genotypes; type-A is the most common, others are found only in central Africa except type-C which is endemic only in Asia (Papua New Guinea)

Clinical Manifestations

HTLV-I is a potential human oncogenic virus. It is associated with the following conditions.

- Adult T cell leukemia/lymphoma—it ranges from an indolent and slowly progressive type to a very aggressive type
- Cutaneous T-cell lymphoma
- Tropical spastic paraparesis
- Autoimmune manifestations such as inflammatory disease, uveitis and arthropathies.

Human T cell lymphotropic virus-II (HTLV-II)

HTLV-II is endemic in certain native American tribes and in Africa. Transmission and replication of HTLV-II is similar to that of HTLV-1. However, its pathogenic potential is uncertain.

EXPECTED QUESTIONS

I. Essay:

1. A 25-year-old male with history of multiple sex partners is admitted with complaints of unexplained fever, progressive loss of weight, persistent diarrhea and generalized lymphadenopathy for the past 6 months.
   a. What is the most probable diagnosis?
   b. Draw a labeled diagram of the morphology of the causative agent of this condition.
   c. Discuss the pathogenesis and laboratory diagnosis of the above condition.

II. Write short notes on:

1. Structure of HIV.
2. Epidemiology of HIV/AIDS.
3. NACO strategies.
4. Pediatric HIV.
5. Highly active antiretroviral therapy (HAART).

III. Multiple Choice Questions (MCQs):

1. The gene coding for core of HIV is:
a. *gag* b. *env*
c. *pol* d. *tat*

2. During the window period of patient with AIDS, best diagnostic test is:
a. ELISA b. Western Blot
c. Rapid test d. RT-PCR

3. Best indicator of HIV prognosis:
a. CD4 T cell count b. CD8 T cell count
c. HIV RNA d. ELISA

4. Highest risk of transmission of HIV:
a. Sexual b. Blood product
c. Needle/syringe d. Mother to fetus

5. Most common malignancy in AIDS is:
a. Kaposi sarcoma
b. B-cell lymphoma
c. Leukemia
d. Burkitt's lymphoma

6. According to the revised NACO guideline 2017, ART has to be started in all patients if the CD4 T cell count fall below:
a. <350/μL b. <200/ μL
c. <500/ μL
d. Irrespective of CD4 count

7. Which of the following is a CCR5 receptor inhibitor?
a. Emtricitabine b. Ritonavir
c. Maraviroc d. Fosamprenavir

8. First line regimen of NACO include which combination of ART?
a. Tenofovir + Lamivudine + Efavirenz
b. Zidovudine + Lamivudine + Nevirapine
c. Zidovudine + Lamivudine + Efavirenz
d. Tenofovir + Lamivudine + Nevirapine

9. Which of the following test cannot be used for diagnosis of HIV in infants?
a. HIV RNA detection
b. p24 antigen detection
c. Western blot
d. HIV DNA detection

10. The most common subtype of HIV found form worldwide:
a. HIV-1 subtype A b. HIV-1 subtype B
c. HIV-1 subtype C d. HIV-1 subtype D

11. World AIDS Day is celebrated on:
a. January 1st b. July 1st
c. December 1st d. December 31st

12. Which state in India has the highest HIV prevalence?
a. Manipur b. West Bengal
c. Andhra Pradesh d. Maharashtra

13. The UNAID initiated Fast-Track strategy for ending the AIDS epidemic by the year---
a. 2020 b. 2025
c. 2030 d. 2035

14. **Adult T cell leukemia/lymphoma is caused by:**
 a. HIV-1 b. HIV-2
 c. HTLV-1 d. HTLV-2
15. **According to the revised NACO guideline 2017, which of the following is a determinant to start antiretroviral therapy (ART)?**
 a. CD4 T cell count
 b. WHO stage of the disease
 c. Associated opportunistic infections
 d. None
16. **For screening of HIV in blood bank, which of the following NACO strategy is used:**
 a. I b. IIa
 c. IIb d. III
17. **The Government of India wants to know the prevalence of HIV in a newly formed state 'Telangana'. Which of the following NACO strategy should be used here?**
 a. I b. IIa
 c. IIb d. III
18. **For diagnosis of HIV in symptomatic people, which of the following NACO strategy is used?**
 a. I b. IIa
 c. IIb d. III
19. **A surgeon wants to know HIV status of a patient admitted for hernia surgery. Which of the following NACO strategy is used here?**
 a. I b. IIa
 c. IIb d. III
20. **HIV RNA detection (by RT-PCR) reduces the window period to:**
 a. 10-14 days b. 3 weeks
 c. 4 weeks d. 8 weeks
21. **Which generation of ELISA assay can detect and differentiate HIV-1 antibodies, HIV-2 antibodies and HIV-1 p24 antigen separately?**
 a. Second b. Third
 c. Fourth d. Fifth
22. **The following are advantages of 24 antigen detection test, *except*:**
 a. Used for confirmation of diagnosis of HIV/AIDS
 b. Used for diagnosis of HIV during window period
 c. More sensitive in late stage
 d. Used for monitoring the progress of HIV infection
23. **All the following are *env* gene products, *except*:**
 a. gp 120 b. gp160
 c. gp41 d. p24
24. **The HIV prevalence in world (2016-17) is about:**
 a. 0.26% b. 0.8%
 c. 8% d. 18%
25. **In India, the HIV prevalence in world (2015) is about:**
 a. 0.26% b. 0.5%
 c. 0.8% d. >0.8%
26. **Which of the following region accounts for the highest prevalence of HIV:**
 a. Sub-Saharan Africa b. Asia
 c. America d. Europe
27. **Which is the most common mode of transmission of HIV?**
 a. Sexual b. Blood transfusion
 c. Needle prick d. Mother to child
28. **Which of the following transmission route of HIV carries the maximum risk?**
 a. Sexual b. Blood transfusion
 c. Needle prick d. Mother to child
29. **The transmission risk of HIV following needle prick injury is about______**
 a. 0.3% b. 3%
 c. 10% d. 30%
30. **Delta 32 mutation—all are true, *except*:**
 a. Common in Europe
 b. Increases the chance of acquiring HIV
 c. Mutation in CCR5 molecule
 d. Occurs in long-term non-progressors
31. **Elite controllers have RNA copies:**
 a. <50 /mL b. <500 /mL
 c. <5000 /mL d. <50,000 /mL
32. **According to WHO clinical staging of HIV/AIDS for adults, all the following are associated with clinical stage 3, *except*:**
 a. Oral candidiasis
 b. Herpes zoster
 c. Oral hairy leukoplakia
 d. Pulmonary tuberculosis
33. **Co-cultivation method is used for:**
 a. Hepatitis B b. Hepatitis C
 c. Herpes d. HIV
34. **For monitoring the response to antiretroviral therapy- all the following are useful, *except*:**
 a. CD4 T cell count b. HIV RNA load
 c. p24 antigen d. Western blot
35. **For monitoring the response to antiretroviral therapy, the best method is:**
 a. CD4 T cell count
 b. HIV RNA load
 c. p24 antigen detection
 d. Neopterin and β2 macroglobulin level
36. **Which of the following is the best method for diagnosis of HIV in infants?**
 a. HIV RNA b. p24 antigen
 c. Western blot d. HIV DNA
37. **After what age, HIV antibody detection (ELISA) can be performed reliably in children?**
 a. 6 months b. 12 months
 c. 18 months d. 24 months
38. **The risk for Pneumocystis pneumonia in HIV is maximum when the CD4 T cell count falls below-**
 a. 200 b. 350
 c. 500 d. 700

Answers

1. a **2.** d **3.** c **4.** b **5.** a **6.** d **7.** c **8.** a **9.** c **10.** c **11.** c **12.** a **13.** c **14.** c **15.** d **16.** a
17. b **18.** c **19.** d **20.** a **21.** d **22.** c **23.** d **24.** b **25.** a **26.** a **27.** a **28.** b **29.** a **30.** b **31.** a **32.** b
33. d **34.** d **35.** b **36.** d **37.** c **38.** a

Miscellaneous RNA Viruses

49
CHAPTER

Chapter Preview

- Rodent-borne viruses
  - Hantaviruses
  - Arenaviruses
- Filoviruses
  - Ebola virus
  - Marburg virus
- Coronaviruses
- Slow viruses and prions
- Rotavirus and other agents of viral gastroenteritis
- Bornavirus

RODENT-BORNE VIRUSES

Rodent-borne viruses or roboviruses are transmitted from rodents to man by contact with infected body fluids or excretions. They are maintained in nature by transmission from rodent to rodent without participation of arthropod vectors.

Major rodent-borne viruses include:

- **Hantaviruses:** They cause two categories of manifestations:
  - Hemorrhagic fever with renal syndrome is caused by several members of hantaviruses such as Hantaan virus, Dobrava virus, Puumala virus and Seoul virus
  - Hantavirus pulmonary syndrome is caused by another member, Sin Nombre virus.
- **Arenaviruses:**
  - New world viruses: Examples include Junin, Machupo, Guanarito and Sabia viruses. They cause South American hemorrhagic fever
  - Old world viruses: Examples include Lassa viruses and lymphocytic choriomeningitis viruses.

Hantaviruses

Genus *Hantavirus* belongs to the family Bunyaviridae.

- They are spherical, enveloped viruses; contain triple-segmented, negative-sense ssRNA
- Worldwide, about 1–2 Lakh cases of hantavirus infections occur annually
- **Reservoir:** Rodents are the reservoir of infection, but they do not suffer from the disease. They harbor the virus lifelong and transmit to other rodents horizontally
- **Transmission** to humans occurs by inhaling aerosols generated from rodent excreta (urine, feces and saliva).

Clinical Manifestations

Hantaviruses cause two fatal human diseases; hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome.

Hemorrhagic Fever with Renal Syndrome (HFRS)

It is an acute viral infection, characterized by:

- **Interstitial nephritis** that leads to renal failure in severe forms of the disease
- **Hemorrhagic fever and shock.** Several hantaviruses can cause this condition:
- Hantaan and Dobrava viruses cause the severe form of HFRS in Asia, particularly in China, Russia, and Korea, and in Europe (Balkans)
- Seoul virus causes a less severe form of HFRS throughout Eurasia.
- Puumala virus causes a mild form of nephritis called nephropathia epidemica, prevalent in Scandinavia.

Hantavirus Pulmonary Syndrome

Sin Nombre virus is the agent of hantavirus pulmonary syndrome which is prevalent in America.

- It has caused an outbreak of severe respiratory illness which occurred in the United States in 1993
- The deer mouse (*Peromyscus maniculatus*) is the primary rodent reservoir.

Laboratory Diagnosis

The laboratory diagnosis of hantavirus infections include:

- Viral RNA detection by RT-PCR
- Viral antigen detection in fixed tissues by immunohistochemistry

- Specific antibodies can be detected by ELISA using recombinant proteins. Detection of IgM or four-fold rise in IgG is considered significant
- Isolation of hantaviruses is difficult and requires specific biosafety cabinets.

TREATMENT **Hantaviruses**

There is no specific antiviral therapy for hantaviral diseases. Only supportive symptomatic treatment is given such as maintenance of airways and renal function.

Preventive Measures

Preventive measures include rodent control and avoidance of contact with rodents and rodent droppings.

Arenaviruses

Arenaviruses are pleomorphic, 50-300 nm in size, enveloped with large, **club-shaped peplomers** and contain a segmented ssRNA **(two segments)**.

- **Reservoir:** Rodents are the reservoir and each virus is associated with a single rodent species
- **Transmission:** Humans are infected by direct contact or inhalation of aerosols generated from rodent excreta
- **Classification:** Based on RNA sequence differences and geographical distribution, arenaviruses are classified into old world and new world viruses (Table 49.1).

Old World Viruses

- **Lassa fever virus** is endemic in western African countries

Table 49.1: Rodent-borne Arenavirus infections

Virus	Disease	Vector	Distribution
Old world complex			
Lassa virus	Lassa fever	Mouse (*Mastomys natalensis*)	West Africa
LCM virus	Lymphocytic choriomeningitis	House mouse (*Mus musculus*)	Worldwide
New world complex			
Junin virus	Argentine hemorrhagic fever	Drylands vesper mouse (*Calomys musculinus*)	Argentina
Machupo virus	Bolivian hemorrhagic fever	Large vesper mouse (*Calomys callosus*)	Bolivia
Guanarito virus	Venezuelan hemorrhagic fever	Short-tailed Cane mouse (*Zygodontomys brevicauda*)	Venezuela
Sabia virus	Brazilian hemorrhagic fever	Unknown	Brazil
Whitewater Arroyo virus	Hemorrhagic fever	Woodrat (*Neotoma*)	Southwestern USA

  - Reservoir is house rat (*Mastomys natalensis*)
  - Human infection is manifested as hemorrhagic fever, pneumonia, cardiac and renal damage. Permanent deafness is a common complication, occurs in 25% of cases following recovery
  - Pregnancy: Both fetal death (90%) and maternal deaths (30%) can occur especially in third trimester
  - Treatment: Ribavirin is the drug of choice for Lassa fever.
- **Lymphocytic choriomeningitis virus** (LCM) is widespread in Europe and the Americas
  - The primary host is the common house mouse, *Mus musculus*
  - Human infection (rare): LCM virus causes aseptic meningitis or a mild systemic influenza-like illness in humans. Rarely, encephalomyelitis or fatal systemic disease may develop. Infection is severe in immunocompromized people
  - *Fetus:* Vertical transmission occurs early in pregnancy following which it can cause hydrocephalus, blindness or even fetal death.

Laboratory diagnosis of both Lassa and LCM virus includes:

- ELISA detecting serum IgM and IgG antibodies
- Immunohistochemical staining of tissues for viral antigen detection
- RT-PCR for detecting viral RNA
- Viral culture using Vero cells.

New World Viruses

They cause South American hemorrhagic fever. Various examples include:

- **Junin virus** causes Argentine hemorrhagic fever affecting agricultural workers. In Argentina, more than 18,000 cases were reported during 1958-1980 and many cases still occur thereafter with a mortality rate of 15-20%. Rodent, *Calomys musculinus* is the reservoir
- **Machupo virus** causes Bolivian hemorrhagic fever. About 2000-3000 persons were affected, with a case-fatality rate of 20%. Rodent, *Calomys callosus* is the reservoir
- **Guanarito virus** is the agent of Venezuelan hemorrhagic fever
- **Sabia virus** causes hemorrhagic fever in Brazil
- **Whitewater Arroyo viruses** cause hemorrhagic fever in Southwestern USA.

FILOVIRUSES

Family Filoviridae contains two antigenically distinct genera—*Ebolavirus* and *Marburg virus*; both cause African hemorrhagic fever.

- **Morphology:** They are pleomorphic, mostly appear as long **filamentous threads**, ranging from 80-1000 nm, the average size being 665 nm (Marburg) to 805 nm (Ebola)

❖ **Highly fatal:** A great matter of concern is, of all the viral hemorrhagic fevers, Marburg and Ebola viruses have the highest mortality rates (25-90%).

Ebola Virus

Ebola virus has become a global threat, because of its recent explosive outbreak in 2014; which was declared by WHO, as a public health emergency of international concern.

History

Ebola virus disease in humans appeared first in 1976 in two simultaneous African outbreaks occurring in Sudan, and Democratic Republic of Congo. The latter outbreak occurred in a village near the **Ebola River**, from which the virus takes its name.

Species

Ebola virus has five stable subtypes or species (*Zaire, Sudan, Reston, Taï Forest* and *Bundibugyo*); all differ from each other by up to 40% of their nucleotide sequences.

❖ Species are of epidemiological importance
❖ The virus that had caused the 2014 West African outbreak belongs to the *Zaire* species.

Geographical Distribution

Since its discovery, Ebola virus has caused several outbreaks in various African countries affecting more than 31,047 documented cases with nearly 12,889 (41%) deaths.

❖ The most recent outbreak in the world was from Democratic Republic of the Congo (May to July 2017): reported 8 cases with 4 deaths
❖ **India:** There is no confirmed case documented yet.

West African Epidemic (2014–16)

The largest outbreak occurred in 2014–16; reported 28,616 cases with 11,310 deaths (40% mortality).

❑ Three primary countries affected were—Guinea, Liberia and Sierra Leone
❑ However few cases have also been reported from several other countries.

Reservoir

The reservoir hosts for Ebola viruses are unknown, but are suspected to be infected animals, such as a **fruit bat** or **primates** (apes and monkeys).

Transmission

In every outbreak, Ebola virus is introduced to human population through **close contact** with the blood, secretions, organs or other body fluids of infected animals such as chimpanzees, gorillas, fruit bats or monkeys.

❖ **Human-to-human transmission:** Once introduced to humans, Ebola virus spreads among people via **direct contact** (through broken skin or mucous membranes of eyes, nose, or mouth) with:
  ▪ Blood, secretions, organs or other bodily fluids of infected people
  ▪ Infected surfaces and materials (e.g. bedding, clothing, syringes, etc.).
❖ Health-care workers and close contacts/family members of infected individuals are at greater risk of contracting the infection
❖ Ebola virus can stay in semen for up to 3 months, although sexual transmission has not been reported yet.

Clinical Manifestations

❖ Incubation period is about 2–12 days (average being 8–10 days)
❖ Common symptoms include fever, headache, muscle pain and sore throat, followed by:
  ▪ Abdominal pain, vomiting and diarrhea
  ▪ Rash with hemorrhages (bleeding or bruise), often leading to shock and death.

Laboratory Diagnosis

❖ **Serum antibody** detection:
  ▪ ELISA detects both IgM and IgG separately by using recombinant nucleoprotein (NP) and glycoprotein (GP) antigens
  ▪ IgM appears after seven days of symptoms and lasts for 3–6 months. IgG appears after 2 weeks and persists for 3–5 years or more
  ▪ Other antibody detection assays include immunofluorescence test and antibody-phage indicator assay.
❖ **Serum antigen** is detected by capture ELISA. The target proteins are NP, VP40, and GP. Immunohistochemical staining and histopathology can also be used to localize Ebola viral antigen in tissue
❖ **Molecular methods** such as RT-PCR and real time RT-PCR assays are useful to detect specific RNA such as NP and GP gene. Virus is detectable after 3 days of onset of fever and remains positive for 2–3 weeks
❖ **Electron microscopy** of the specimen shows typical filamentous viruses (Fig. 49.1)
❖ **Virus isolation in Vero cell line:** Processing the specimen should be carried out in biosafety level-4 cabinets as there is a great risk of laboratory spread of the virus.

TREATMENT **Ebola virus**

Supportive care such as rehydration and symptomatic treatment improves survival. No proven treatment or vaccine is available yet.

Prevention (General Measures)

People who may be exposed to patients suspected with Ebola should follow the following steps:

❖ Practice proper infection control and sterilization measures such as strict hand hygiene and personal protective equipment (PPEs)
❖ Isolate patients with Ebola from other patients

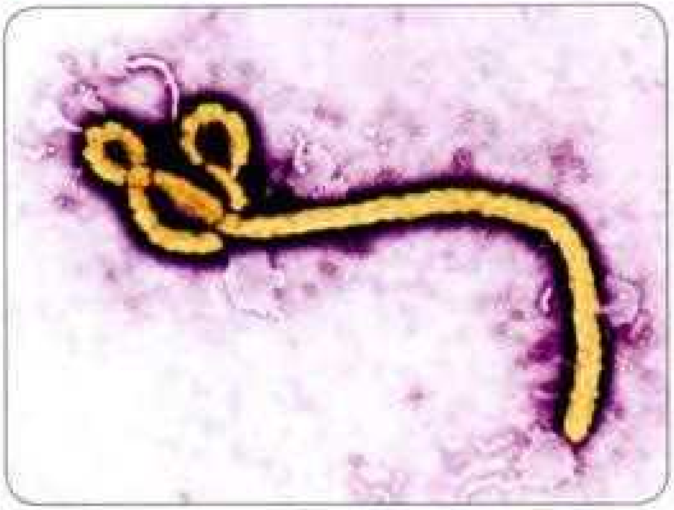


Fig. 49.1: Ebola virus, filamentous shaped (Electron micrograph)

Source: ID# 10815, Public Health Image Library, /Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*).

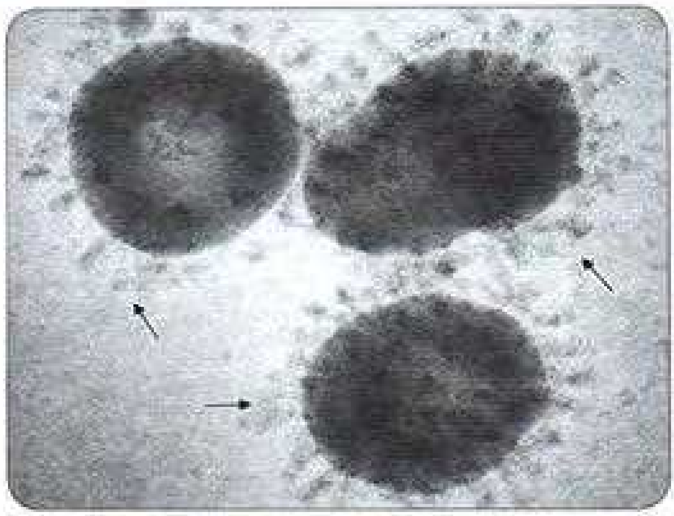


Fig. 49.2: Coronavirus (Petal or club-shaped peplomers). Electron micrograph (arrows showing)

Source: ID# 10270, Public Health Image Library, /Centers for Disease Control and Prevention (CDC), Atlanta/Dr. Fred Murphy; Sylvia Whitfield (*with permission*).

- Avoid direct or indirect contact (clothes, bedding, needles) with blood or body fluids or other secretions of suspected Ebola cases
- Avoid attending funeral or wear PPE while attending funeral
- If traveling to Ebola outbreak area, should be monitored for 21 days after returning.

Vaccine

- No Ebola vaccine is approved for human use yet. However, several vaccine trials are going on
- **STRIVE trial** (Sierra Leone Trial to Introduce a Vaccine against Ebola): The vaccine candidate used here is 'rVSV-ZEBOV,' which is recombinant vesicular stomatitis virus—*Zaire* Ebolavirus vaccine.

Marburg Virus

Marburg virus disease was first reported in Germany and Yugoslavia (1967) among laboratory workers exposed to tissues of African green monkeys imported from Africa.

- Since then, over 450 cases have been reported in various African countries such as Kenya, South Africa, Democratic Republic of Congo, Uganda and Angola
- The most recent outbreak was in Angola (2005), affecting 252 people with 227 deaths (with mortality rate of 90%).

CORONAVIRUSES

Morphology

Coronaviruses are enveloped; carrying petal or club-shaped or crown-like peplomer spikes giving appearance of solar corona (Fig. 49.2).

- They are large (120–160 nm) spherical viruses having a helical symmetry
- They possess linear, positive-sense ssRNA of 26 to 32 kbp size, largest among the non-segmented RNA viruses.

Classification

Coronaviridae family contains two subfamilies: Coronavirinae and Torovirinae. The former has been grouped into four genera—*Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Deltacoronavirus*. Most of them infect animals except *Gammacoronavirus* species, which are the pathogens of birds. Human infection is uncommon except few who have adapted to human conditions.

Human Coronavirues

There are six recognized coronaviruses that are known to cause human infections; most of them belong to *Betacoronavirus* except the first two which belong to *Alphacoronavirus*.

1. Human coronavirus 229E.
2. Human coronavirus NL63 (New Haven Coronavirus).
3. Human coronavirus OC43.
4. Human coronavirus HKU1.
5. SARS-CoV (Severe Acute respiratory syndrome coronavirus).
6. MERS-CoV (Middle East respiratory syndrome coronavirus).

Most human coronaviruses are widespread affecting people of most part of the world and produce mild upper respiratory tract infection and occasional diarrhea.

Two exceptions are SARS-CoV and MERS-CoV which are geographically restricted, transmitted from man to man and have produced outbreaks of severe respiratory disease with higher mortality.

Transmission

Human coronaviruses spread by coughing, sneezing, and close personal contact, such as touching mouth, nose, or eyes or shaking hands. SARS-CoV can also spread via droplets and rarely through airborne.

Sars-CoV (severe acute respiratory syndrome coronavirus)

- **History:** SARS was first recognized in China in 2003 by WHO physician Dr Carlo Urbani. He diagnosed it in a businessman who had traveled from China, through Hong Kong, to Hanoi, Vietnam. The businessman and the doctor who first diagnosed SARS both died from the illness
- **Epidemiology:** During 2003 outbreak, the SARS virus, spread from Asia to various regions of the world causing nearly 8098 cases in 29 countries, with over 774 deaths. However, India remained free from the infection. Since 2004, no case has been reported from anywhere in the world
- **Source:** SARS-CoV infection in humans is believed to be contracted from animals, including monkeys, Himalayan palm civets, raccoon dogs, cats, dogs, and rodents
- **Clinical manifestation** includes severe lower respiratory tract infection; characterized by muscle pain, headache, sore throat and fever, followed by the onset of respiratory symptoms mainly cough, dyspnea and pneumonia.

MERS-CoV (middle east respiratory syndrome coronavirus)

MERS-CoV has recently caused a severe form of lower respiratory illness with a mortality of 30%.

Epidemiology: It was first reported in Saudi Arabia in 2012.

- Between 2012-18, about 2,143 laboratory-confirmed cases MERS-CoV with 750 deaths have been reported to WHO from 27 different countries
- Soudi Arabia accounted for 82% of the cases followed by other middle east countries
- It is not reported from india yet.

Source though unknown, it is believed to have been acquired from camels and bats.

People at increased risk for MERS-CoV infection include:

- Recent history of travel from the Arabian Peninsula within 14 days
- Close contacts of a confirmed case of MERS
- Healthcare personnel not using recommended infection control precautions
- People with exposure to infected camels.

Clinical manifestation

- Incubation period is about 2–14 days
- Severe acute respiratory symptoms such as fever, cough and shortness of breath may appear
- Some people may develop gastrointestinal symptoms including diarrhea and nausea/vomiting
- Complications such as pneumonia and kidney failure occur, especially in people with underlying comorbid conditions.

Laboratory diagnosis

- **Detection of antibodies** in serum indicates past-exposure. ELISA is used for screening of antibodies; which should be confirmed by IFA and microneutralization assays.
- **Molecular method:** Detection of specific MERS-CoV RNA by real-time RT-PCR in blood indicates active infection. However, laboratory confirmation requires detection of at least two MERS CoV specific genes such as *upE* and *ORF1b* present in in upstream of the E gene.
- **Antigen detection:** Capture ELISA detecting nucleocapsid protein in nasopharyngeal aspirate is under evaluation.

Vaccine for MERS-CoV

No vaccine has been licensed for human use yet. However there are 14 vaccine trials going on. The most advanced trial is a DNA vaccine (in phase 1) in South Korea; based on spike S protein.

Laboratory Diagnosis (Coronaviruses)

- **Antigen detection:** Coronavirus antigens in the respiratory epithelial cells may be detected by ELISA using specific monoclonal antibody
- **Electron microscopy** can be used to detect enteric coronaviruses from stool
- **RNA detection:** RT-PCR assays are useful to detect coronavirus RNA in respiratory secretions (targeting pan-coronavirus polymersae gene) and in stool samples and SARS-CoV RNA from blood
- **Isolation** of human coronaviruses in cell culture has been extremely difficult. Traditional **tracheal ring culture** is no longer in use. SARS-CoV was isolated from respiratory specimens using Vero cell line
- **Serum antibody detection:** ELISA and hemagglutination inhibition test are available. Rising titer of antibody between acute and convalescent sera can be used to establish the diagnosis.

Treatment and Prevention (Coronaviruses)

There are no specific drugs or vaccine available for coronavirus infections.

- **Control measures** include:
  - Isolation of patients
  - Quarantine of exposed people
  - Travel restrictions if needed
  - Use of gloves, gowns, goggles and respirators by healthcare workers
  - Thorough and frequent hand washing
  - Avoiding personal contact, such as kissing, or sharing cups or eating utensils, with sick people
  - Avoiding contact with camels, uncooked camel milk or meat.
- **Taking effective control measures** was the main reason behind preventing SARS transmission in 2003.

SLOW VIRUSES AND PRIONS

Slow virus diseases including prion diseases are a group of neurodegenerative conditions affecting both humans and animals, characterized by:

- **Long incubation period,** ranging from months to years because of the long doubling time of slow viruses of 5.2 days or more
- **Predilection for CNS:** Slow viruses usually affect the central nervous system (CNS):
    - This cause vacuolation of neurons (spongiform changes), with deposition of amyloid like plaques and gliosis
    - Common symptoms include loss of muscle control shivering, tremors and dementia
    - Invariably fatal
- Strong **genetic** predisposition
- Slow viruses and prions **lack in antigenicity**; hence, there is:
    - Lack of immune response and interferon production against the viral proteins
    - Lack of associated inflammation.
- Does not produce cytopathologic effect *in vitro.*

Slow virus diseases are either caused by (Table 49.2):

- **Conventional viruses:** Examples include:
    - Subacute sclerosing panencephalitis
    - Progressive multifocal leukoencephalopathy
    - Visna and Maedi.
- **Unconventional** transmissible slow viruses-termed as "prions".

Slow Virus Diseases Due to Conventional Viruses

Subacute Sclerosing Panencephalitis (SSPE)

SSPE is a rare disease of young adults caused by a defective measles virus, characterized by slow progressive demyelination of the CNS ending in death.

- Such measles viruses have a defective transcription and are unable to synthesize envelope proteins; hence, for survival, they establish a chronic persistent infection in the neural cells. Large numbers of viral nucleocapsid structures are produced in the neurons and glial cells
- **Diagnosis:** Patients with SSPE have high titers of anti-measles antibody in cerebrospinal fluid (CSF) except that antibody to the M protein is frequently lacking. However, virus isolation is very difficult.

Progressive Multifocal Leukoencephalopathy (PML)

PML is caused by JC virus a member of the family Polyomaviridae.

- It infects the oligodendrocytes of brain and causes demyelination of CNS
- PML occurs in about 5% of patients with AIDS or other immunosuppressed conditions.

Visna and Maedi

Visna and Maedi viruses are closely related retroviruses that cause slowly developing infections in sheep.

- Visna virus causes demyelination of CNS
- Maedi virus causes a slow progressive fatal hemorrhagic pneumonia of sheep

Table 49.2: Slow viral diseases

Group	Disease	Agent	Hosts	Nature of disease
	Due to Conventional viruses			
	Subacute sclerosing panencephalitis	Measles virus variant	Humans	Chronic sclerosing panencephalitis
C	Progressive multifocal leukoencephalopathy	• Polyomavirus • JC virus	Humans	CNS demyelination
A	Visna virus	Retrovirus	Sheep	CNS demyelination
	Maedi virus	Retrovirus	Sheep	Progressive pneumonia
	Due to Unconventional viruses: Prions			
	Kuru	Prion	Humans, monkeys chimpanzees	
	Creutzfeldt-Jakob disease	Prion	Humans, monkeys chimpanzees	
	Gerstmann-Sträussler-Scheinker disease	Prion	Humans	
B	Fatal familial insomnia	Prion	Humans	Spongiform encephalopathy
	Scrapie	Prion	Sheep, goats, mice	
	Bovine spongiform encephalopathy	Prion	Cattle	
	Transmissible mink encephalopathy	Prion	Mink	
	Chronic wasting disease	Prion	Mule deer, elk	

Abbreviations: JC, John Cunningham; CNS, central nervous system.

Table 49.3: Differences between PrP^C and PrP^{Sc}

Features	PrP^C	PrP^{Sc}
Full form	Prion protein cellular	Prion protein scrapie
	Normal isoform of prion protein present in man/animals	Prion protein that causes prion disease in man/animals
Structure	Elongated polypeptide, rich in α-helix and has little β-structure	• Globular polypeptide • Contains less α-helix but more β-structure
Location	Anchored to the cell membrane	Cytoplasmic vesicles
Protease	Sensitive	Resistant
Turnover	Hours	Days

- ❖ Symptoms appear after a long incubation period of months to years
- ❖ Disease progresses either rapidly (weeks) or slowly (years).

Slow Virus Diseases due to Unconventional Viruses/Prion Diseases

Prions are infectious protein particles that lack any nucleic acid. They are filterable like viruses; but are resistant to wide range of chemical and physical agents of sterilization. There are several prion diseases of humans and animals; *Scrapie* being the prototype.

Prion proteins have two isoforms

- ❖ PrP^{Sc} is the prion protein that causes disease. It is so named because, it is identified in the purified preparations of scrapie prions
- ❖ PrP^C is the normal cellular isoform of the prion protein present on the cell membrane of mammals. It is encoded in chromosome 20. It is the precursor of PrP^{Sc}, they differ from each other in many respects (Table 49.3).

Mechanism of Prion Diseases

Though many theories have been proposed but that of **Stanley B Prusiner** (Nobel prize winner, 1997) had clearly explained the detailed mechanism, how an aberrant protein could cause disease.

- ❖ Once infected, the prion proteins (PrP^{Sc}) are carried to the neurons. They bind to the normal PrP^C on the cell surface
- ❖ This causes the release of PrP^C from cell surface followed by their conversion into the disease-causing isoform (PrP^{Sc}). This is a **post-translational modification** by which the elongated polypeptide PrP^C become globular polypeptide PrP^{Sc}
- ❖ The cell synthesizes new PrP^C and the cycle is repeated; as a result, large amount of PrP^{Sc} is formed
- ❖ PrP^{Sc} are aggregated as **amyloid-like plaques** in the brain. As these plaques consist of host proteins, there is lack of an immune response or inflammation
- ❖ PrP^{Sc} are internalized by neurons and get accumulated inside the **cytoplasmic vacuoles** giving the cell a spongiform appearance
- ❖ **Spongiform encephalopathy** is the main pathology seen in CNS, characterized by:
  - Vacuolation of the neurons
  - Formation of amyloid-containing plaques and fibrils
  - Proliferation and hypertrophy of astrocytes
  - Fusion of neurons and adjacent glial cells.

Clinical Manifestations of Prion Diseases

Incubation period of prion diseases varies from months to years (longest being 30 years). But once the disease sets in, progression is fast.

- ❖ Prodromal phase lasts for 3–5 months, followed by appearance of manifestations such as loss of muscle control, shivering, myoclonic jerks, tremors, loss of coordination and rapidly progressive dementia
- ❖ Death occurs within 1 year of onset of disease.

Prion Diseases of Animals

- ❖ **Scrapie:** It is the prototype of prion diseases that has been extensively studied

  Natural scrapie is a prion disease of sheep.
  - Transmission occurs vertically in sheep from parent to offspring and less often by direct contact
  - After an incubation period of 2 years, the affected sheep become irritable and develop intense pruritus, scraping themselves against trees and rocks; hence, the name scrapie is given
  - Gradually, emaciation and paralysis occurs leading to death.

  Experimental scrapie: The disease can be experimentally transmitted to various animals (several breeds of sheep as well as other animals) by injection of neural tissues of infected sheep.
  - In hamsters and mice, the incubation period is less, which has facilitated the study of the disease
  - Susceptibility: Different breeds of sheep exhibit marked genetic differences in susceptibility to infection (ranging 0–80%); whereas goats are almost 100% susceptible.
- ❖ **Mink encephalopathy:** It is a scrapie-like disease of mink transmitted by feeding the minks on scrapie infected sheep meat
- ❖ **Bovine spongiform encephalopathy (BSE, "mad cow disease"):** It has been enzootic in cattle in Great Britain since 1986
  - The epidemic peaked in 1993 infecting over 1 million of cattle with infection spreading to European countries
  - BSE is transmitted due to the practice of feeding the cattle with meat and bone meal contaminated with scrapie or BSE prions.

Human Prion Diseases

The various human prion diseases are as follows:

- **Kuru:** It was seen only in the Eastern Highlands of New Guinea and was spread by customs surrounding ritual cannibalism of dead relatives infected with the disease. Since this practice has ceased, the disease has disappeared now
- **Creutzfeldt-Jakob disease (CJD):** It is the most common form of prion disease in humans. It typically presents with dementia and myoclonus, is relentlessly progressive, and generally causes death within a year of onset. Types of CJD include:
  - **Classical or sporadic (sCJD):** It is caused by the spontaneous misfolding of prion-protein in an individual. This accounts for 85% of cases of CJD.
  - **Familial (fCJD):** It accounts for the majority of the other 15% of cases of CJD. fCJD and its variants Gerstmann Sträussler-Scheinker syndrome and fatal familial insomnia are hereditary, run in families; caused by germ line mutations in PrP gene.
  - **Iatrogenic (iCJD):** It affects people of 50–75 years age; caused by contamination with tissue from an infected person, usually as the result of a medical or surgical procedures such as blood transfusion, use of human-derived pituitary growth hormones, gonadotropin hormone therapy, and corneal and meningeal transplants.
  - **Variant (vCJD):** In contrast to the classical CJD, vCJD occurs below 30 years and is believed to be transmitted through the consumption of contaminated beef with BSE prions. More than 190 cases of vCJD have occurred, mainly from Britain (where BSE is prevalent).

Laboratory Diagnosis

- **Measurement of PrP^{Sc}** by conformation dependent immunoassay is the most definitive diagnostic tool for prion diseases
- **Brain MRI:** More than 90% of patients show increased intensity in the basal ganglia and cortical ribboning
- **Neuropathological diagnosis** in brain biopsies: The pathologic hallmarks of prion diseases seen under light microscopy, are spongiform degeneration and astrocytic gliosis with lack of inflammatory response
- **Sequencing the PRNP gene** to identify the mutation: This is important in familial forms of prion diseases
- **Stress protein 14-3-3** is elevated in the CSF
- **Abnormal EEG** (electroencephalogram): In late stage of the disease, high-voltage, triphasic sharp discharges are observed.

TREATMENT **Prion diseases**

There is no known effective therapy for preventing or treating prion diseases. Several trials using drugs such as quinacrine and anti-PrP antibodies have shown to eliminate PrP^{Sc} from the cultured cells, but they failed to do so *in vivo*.

Decontamination

Prions are extremely resistant to most of the common sterilization procedures. Recommended methods for sterilization of material contaminated with prion proteins are:

- Autoclaving at 134°C for 1–1.5 hour
- Treatment with 1 N NaOH for 1 hour
- Treatment with 0.5% sodium hypochlorite for 2 hours.

Prions if bound to the stainless steel should be treated with an acidic detergent solution prior to autoclaving; rendering them susceptible to inactivation.

VIRAL GASTROENTERITIS

Viral etiology accounts for the most of the acute infectious gastroenteritis worldwide (Table 49.4). Viral gastroenteritis most commonly occurs among children. However persons of all ages can be affected. Several enteric viruses can cause acute gastroenteritis in humans, most common being rotavirus.

Rotavirus

Rotaviruses are the most common cause of diarrheal illness in children.

Morphology

Rotaviruses are about 60–80 nm in size and possess icosahedral symmetry.

Table 49.4: Viruses causing gastroenteritis

Virus	Genome	Gastroenteritis features
Rotavirus*	Segmented dsRNA	**Group A:** Most common cause of severe endemic diarrheal illness in children worldwide **Group B:** Causes outbreaks of diarrhea in adults in China
Caliciviruses		
Norovirus	ssRNA	Causes outbreaks of vomiting and diarrheal illness in all ages (especially in older children and adults)
Sapovirus	ssRNA	Causes sporadic cases and occasional outbreaks of diarrheal illness in infants, young children, and in elderly
Astrovirus	ssRNA	
Adenovirus* (type 40 and 41)	dsDNA	Second most common viral agent of endemic diarrheal illness of infants and young children worldwide

*Clinical severity is maximum.

- Surrounded by a triple layered, wheel-shaped capsid (Rota in latin meaning 'wheel')
- Possess segmented dsRNA (11 segments)
- **Proteins:** There are six structural viral proteins (VP1 to VP7 except VP5) and six non-structural proteins (NSP1-6)
- Viral protein (VP6) is group-specific
- **VP7** (forms the outer capsid layer) and **VP4** (forms spikes that emanate through the outer capsid layer) are strong inducers of neutralizing antibodies and are type specific.

Classification of Rotaviruses

Rotaviruses belong to the family Reoviridae; the only virus family to have dsRNA.

Traditional groups: Rotaviruses are further classified into seven major groups (A–G) based on the antigenic composition of the group specific VP6. Most human diarrhea is caused by group A and, to a much lesser extent, by groups B and C

Binary system of typing: This is the recent classification of rotaviruses; based on a binary system; i.e. combination of both serotyping and genotyping methods

- Genotyping method is based on gene coding for VP4 antigen (a protease sensitive protein or P-type antigen). Genotype numbers are expressed asP[1], P[2], etc.
- Serotyping method is based on VP7 antigen (a glycoprotein or G-type antigen). Serotype numbers are expressed as G1, G2, etc.
- Currently, 19G and 28[P] types are known. The most common type seen in the world as well as in India is **G1P[8]** type, which accounts for nearly 70% of total isolates
- The diversity among the rotavirus types is more commonly encountered in areas with poor hygiene.

Pathogenesis

Rotaviruses are transmitted by fecal-oral route, then they progress further ultimately destroying the mature enterocytes in the villi of the proximal small intestine; however, the gastric and colonic mucosa are spared.

- They multiply in the cytoplasm of enterocytes and damage their transport mechanisms resulting in secretory diarrhea
- The non-structural protein-NSP4, acts as enterotoxin and induces secretion by altering epithelial cell function and permeability
- Damaged cells may slough into the intestinal lumen and release viruses in the feces; the count may exceed up to 10^{10} viral particles per gram of feces
- Viral excretion usually lasts 2-12 days, but may be prolonged in patients with poor nutrition or HIV infection.

Clinical Manifestation

The incubation period is about 1-3 days. It has an abrupt onset, characterized by vomiting followed by watery diarrhea, fever and abdominal pain.

- Recovery usually occurs in majority, but a few children may suffer from severe loss of electrolytes and fluids leading to dehydration
- Infected adults are usually asymptomatic but show seroconversion. However, epidemics or large outbreaks have occurred in adults, especially in closed populations (e.g. geriatric ward)
- Group B rotaviruses have been implicated in large outbreaks of severe gastroenteritis in adults in China.

Epidemiology

Rotaviruses are the single most important cause of gastroenteritis in young children.

- Worldwide, about 3-5 billion diarrheal episodes in children occur annually resulting in nearly 1 million deaths especially from sub-Saharan Africa and Southern Asia
- **In developing countries like India:** Rotavirus illness occurs at a younger age, is less seasonal and more frequently caused by diverse and uncommon serotypes
- Whereas in temperate climate, Group A rotavirus causes outbreaks in cooler months.

Laboratory Diagnosis

- **Direct detection of virus:** Feces collected early in the illness is the most ideal specimen. Rotaviruses can be demonstrated in stool by:
  - **Immunoelectron microscopy (IEM):** Rotaviruses have a sharp edged triple shelled capsid; look like the spokes grouped around the hub of a wheel (Fig. 49.3)
  - **Isolation** of rotavirus is difficult. Rolling of tissue cultures may be attempted to enhance replication.
- **Detection of viral antigen** in stool by ELISA and latex agglutination-based methods

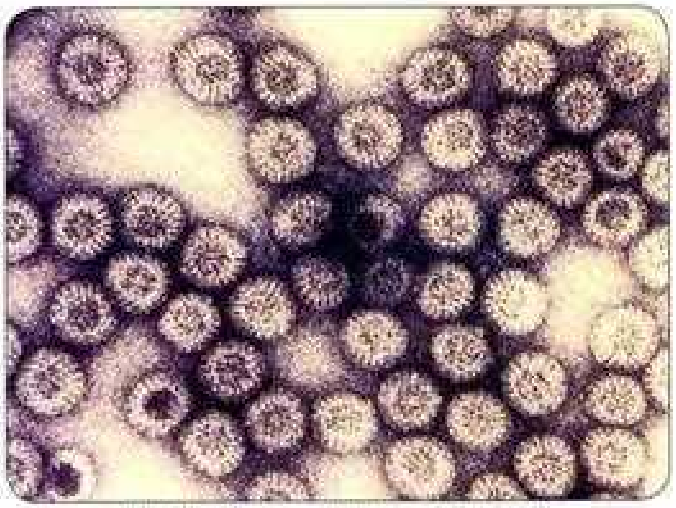


Fig. 49.3: Rotavirus (electron micrograph)

Source: Public Health Image Library, /ID# 15194/Dr Erskine L Palmer/Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

- **RT-PCR** is the most sensitive detection method for detection of rotavirus from stool
- **Typing methods:** G serotypes and P genotypes of rotaviruses can be detected by RNA sequence typing and neutralization test respectively
- **Serologic tests** (ELISA) can be used to detect the rise of antibody titer. This may be useful for seroprevalence purpose.

TREATMENT **Viral gastroenteritis**

Treatment is mainly supportive, to correct the loss of water and electrolytes such as oral or parenteral fluid replacement.

Prevention

Vaccine

Two brands of rotavirus vaccine are available:

Rotavac

It contains live attenuated Rotavirus 116E (G9P[10] strain). It provides cross protection against many types including G1P[8] type.

- It is manufactured by Bharat biotech, India
- It is introduced under national immunization schedule of India in selected states
- Three doses (0.5 mL, i.e. 5 drops/dose): administered at 6, 10 and 14 weeks along with DPT and OPV
- Overall efficacy in first 2 years of life is about 55%
- Side effects (≥5%): crying, irritability, fever and diarrhoea. No vaccine induced intussusception has been reported.

Rotarix

Rotarix contains live attenuated G1P[8]strain; also provides cross protection against G3, G4 and G9. It has to be reconstituted before use. Given as two doses: 1st at 6 week and 2nd dose is given 4 weeks later.

General preventive measures to improve hygiene and sanitation are necessary for prevention of infection.

Other Agents of Gastroenteritis

- **Family Caliciviridae** comprises of four genera; out of which two consists of important agents of human diarrhea—(1) genus *Norovirus*, which includes the Norwalk viruses and (2) genus *Sapovirus*, which includes the Sapporo-like viruses:
  - They are icosahedral, 27–40 nm in size; possess **cup-like depressions** (calyx meaning cup), on the capsid surface typically observed under electron microscope. They are single stranded (+)sense RNA and a single major structural protein
  - Norwalk virus is the most important cause of epidemic viral gastroenteritis in adults
  - Sapoviruses cause sporadic cases and occasional outbreaks of diarrheal illness in infants, young children, and the elderly
  - Laboratory diagnosis and treatment are similar to that of rotavirus. They are not cultivable.
- **Adenoviruses (types 40 and 41)** are the second most common viral agents of endemic diarrheal illness of infants and young children worldwide, responsible for 2-12% of all diarrhea episodes in young children
- **Astroviruses** exhibit a distinctive **star-like** morphology under the electron microscope
  - They are of 28–30 nm size with an icosahedral symmetry and contain a positive sense, ssRNA
  - At least seven serotypes have been identified, of which serotype-1 is the most common
  - Astroviruses cause sporadic cases and occasional outbreaks of diarrhea in infants, young children and in elderly.
- **Respiratory viruses:** Diarrhea has also been reported as a part of manifestations of certain respiratory viruses such as:
  - SARS coronavirus
  - Influenza A/H5N1 virus
  - Influenza A/H1N1 virus (the 2009 pandemic strain).
- **Toroviruses** and **Picobirnaviruses** cause gastroenteritis in a variety of animals, but their role as primary cause of gastroenteritis in humans remains unclear.

BORNA DISEASE VIRUS

Borna disease virus (BDV) is a highly neurotropic virus which causes neuropsychiatric disorders in horses and sheep, manifested by behavioral abnormalities usually ending in death.

- It is seen in certain areas of Germany
- The disorder is immune-mediated; characterized by depostion of inflammatory cells in the brain
- It belongs to the family Bornaviridae. It is enveloped, contains negative sense ssRNA, which replicates in the nucleus
- Human infection has not been established yet, though serologic data suggest that BDV may be associated with neuropsychiatric disorders in humans.

EXPECTED QUESTIONS

I. Write short notes on:
1. Ebola virus.
2. SARS CoV.
3. Prion diseases.
4. Viral gastroenteritis.

II. Multiple Choice Questions (MCQs):

1. **About Ebola virus true statement is:**
   a. Incubation period is less than 48 hours
   b. Transmission is by oral route
   c. Specific treatment is available
   d. Restricted to Guinea, Liberia and Sierra Leone
2. **The most common viral cause of gastroenteritis:**
   a. Rotavirus b. Norwalk virus
   c. Adenovirus d. Hepadnavirus
3. **All are true about SARS, *except*:**
   a. Seen in India b. Spreads by droplet
   c. Diagnosed by PCR d. Type of coronavirus
4. **Which of following is correct about prions?**
   a. Destroyed by autoclaving at 121°C
   b. Long incubation period
   c. Immunogenic d. Nucleic acid present

Answers

1. d **2.** a **3.** a **4.** b **5.** c

Hepatitis Viruses

50 CHAPTER

Chapter Preview

- Hepatitis A virus (HAV)
- Hepatitis B virus (HBV)
- Hepatitis C virus (HCV)
- Hepatitis D virus (HDV)
- Hepatitis E virus (HEV)
- Hepatitis G virus (HGV)

INTRODUCTION

Hepatitis viruses are heterogeneous group of viruses that are taxonomically diverse (belong to different families) but all are hepatotropic; cause acute inflammation of the liver producing identical histopathologic lesions and similar clinical illness such as fever, nausea, vomiting, and jaundice.

Hepatitis viruses are classified into six types (Table 50.1):

1. Hepatitis A virus (HAV): It causes infectious hepatitis.
2. Hepatitis B virus (HBV): It causes serum hepatitis.
3. Hepatitis C virus (HCV): It is common cause of post-transfusion hepatitis.
4. Hepatitis D virus (HDV): It is a defective virus, needs HBV for its replication.
5. Hepatitis E virus (HEV): It is the agent of enterically transmitted non-A, non-B hepatitis.
6. Hepatitis G virus (HGV).

Table 50.1: Features of hepatitis viruses

Properties	HAV	HBV	HCV	HDV	HEV
Common name	Infectious hepatitis	Serum hepatitis	Non-A non-B or post-transfusion hepatitis	Delta agent	Non-A non-B enteric transmitted hepatitis
Family	Enterovirus-72 (Picornaviridae)	Hepadnaviridae	Flaviviridae	Unclassified viroid-like	Unclassified Caliciviridae-like
Genus	*Hepatovirus*	*Orthohepadnavirus*	*Hepacivirus*	*Deltavirus*	*Hepevirus*
Virion	27 nm, icosahedral	42 nm, spherical	60 nm, spherical	35 nm, spherical	30–32 nm, icosahedral
Envelope	No	Yes (HBsAg)	Yes	Yes (HBsAg)	No
Genome	ssRNA	dsDNA	ssRNA	ssRNA	ssRNA
Stability	Heat and acid-stable	Acid-sensitive	Ether-sensitive, acid-sensitive	Acid-sensitive	Heat-stable
Onset	Abrupt	Insidious	Insidious	Insidious	Abrupt
Age	• Children • Young adults	• Young adults • Toddlers and babies	Any age, but more common in adults	Any age (similar to HBV)	Young adults (20–40 years)
Route	Fecal-oral	• Percutaneous (MC) • Sexual • Vertical	• Percutaneous (MC) • Sexual (+/–) • Vertical (+/–)	• Percutaneous (MC) • Sexual (++) • Vertical (+)	Fecal-oral
IP (days)	15–45 (Average 30)	30–180 (Average 60–90)	15–160 (Average 50)	30–180 (Average 60–90)	14–60 (Average 40)
Fulminant disease	Rare (0.1%)	Rare (0.1–1%)	Rare (0.1%)	Frequent (5–20%)	• Usually rare (1–2%) • Pregnancy: 20–40%

Contd...

Contd...

Properties	HAV	HBV	HCV	HDV	HEV
Carrier	None	Yes (0.1–30%)	Yes (1.5–3.2%)	Variable	None
Chronicity	None	Occasional (1–10%)	Common (85%)	Common	None
Oncogenic	No	Yes (neonate)	Yes	+/-	No
Prevalence	High	High	Moderate	Low, regional	Regional
Associated other features	Secondary attack rate 10–20%	HCC, cirrhosis, Autoimmune disorder like AGN, arthritis, PAN	HCC, cirrhosis, Autoimmune disorders like AGN, arthritis, cryoglobulinemia	HCC, cirrhosis, fulminant hepatitis	• Secondary attack rate (1–2%) • Rarely seen in western countries
Prognosis	Excellent	Worse with age	Moderate	• Acute-good • Chronic-poor	Good
Prophylaxis	• Immunoglobulin • Inactivated vaccine	• HBIG • Recombinant vaccine	None	HBV vaccine (no vaccine for HBV carriers)	Vaccine (HEV239) (only in China)
Therapy	None	• Tenofovir • Telbivudine	Directly-acting antiviral agents	Interferon ±	None

Abbreviations: MC, most common; HCC, hepatocellular carcinoma; HBsAg, hepatitis B surface antigen; AGN, acute glomerulonephritis; HBIG, hepatitis B immunoglobulin; IP, incubation period; PAN, polyarteritis nodosa.

"Hepatitis F" (1994) was proposed for its association with transfusion-associated hepatitis, but further investigations failed to confirm the existence of the virus, therefore, it was delisted as a cause for infectious hepatitis.

However, there are many viruses other than hepatitis viruses that can cause sporadic hepatitis, such as yellow fever virus, cytomegalovirus, Epstein-Barr virus, herpes simplex virus, rubella virus, and enteroviruses.

HEPATITIS A VIRUS

Hepatitis A virus belongs to the family Picornaviridae. It was originally designated as "enterovirus 72", but based on the nucleotide and amino acid sequences, it was later assigned to a new genus, *Hepatovirus* under Picornaviridae family.

Morphology

- HAV is 27–32 nm in size, spherical particle with icosahedral symmetry, containing a linear ssRNA
- It has only one serotype, doesnot cross-react with other hepatitis viruses; however it can be typed into seven genotypes based on gene sequences.

Resistance

HAV is relatively resistant to disinfectants.

- It is stable to acid, heat (60°C for 1 hour), and ether (20%) and can be preserved in dried state for 1 month and stored for years at -20°C
- HAV is destroyed by autoclaving, by boiling in water for 5 minutes, by hot air oven, by ultraviolet (UV) rays and by treatment with formalin or chlorine (10–15 ppm for 30 minutes).

Mode of Transmission

- HAV is transmitted principally by fecal-oral route
- Rarely, HAV can also be transmitted by sexual (homosexuals through orogenital contact) and parenteral routes (infected blood products or needle pricks).

Epidemiology

Hepatitis A is the most common cause of acute viral hepatitis in children.

- **Hosts:** Humans are the only host for HAV. However, experimental infection may be induced in chimpanzees
- **Age:** Children and adolescents (5–14 years of age) are most commonly affected, majority remain **subclinical** (80–95%), but excrete virus in feces for longer time. Adults are more icteric (75–90%) than children with higher mortality rate. Anicteric to icteric cases ratio is about:
  - In children: 12:1
  - In adults: 1:3.
- **Risk factors:** Poor personal hygiene and overcrowding are the most important risk factors
  - In **developing countries** including India with poor personal hygiene and overcrowding, most of the children (90%) are infected with HAV by the age of 10 years. Adults have protective antibodies and are mostly immune to HAV
  - However, in **developed countries** with improved hygiene, the incidence is decreasing and there is trend of shift of infection towards the older age.
- **Outbreaks** are common in summer camps, day care centers, families and institutions, neonatal ICUs, and among military troops

- **Recurrent epidemics** and sudden, explosive epidemics are common and usually result from fecal contamination of a single source (e.g. drinking water, milk or food such as raw vegetables, salad, frozen strawberries, green onions and shellfish). The largest epidemic was reported from Shanghai (1988, >3 lakh cases)
- **Seasonal incidence:** Though HAV infection is widespread throughout the year, it tends to peak in late rainfall and in early winter
- **Virus excretion:** Viral excretion in feces may be 2 weeks before to 2 weeks after the appearance of jaundice (however, viremia occurs from -2 weeks to +1 week of jaundice).

Clinical Manifestation

- Incubation period is about 15-45 days (mean 30 days)
- Onset is relatively abrupt (subacute)
- **Clinically,** HAV infection is indistinguishable from other hepatitis viruses; characterized by:
    - Pre-icteric phase (mainly gastrointestinal symptoms such as nausea and vomiting) followed by:
    - Icteric phase or jaundice (dark urine, yellowish sclera and mucus membrane).
- Complete recovery occurs in most (98%) cases
- There is no chronic or carriers state
- **Complications may occur rarey such as:**
    - Fulminant hepatitis; characterized by severe necrosis of hepatocytes, may occur rarely
    - Relapsing hepatitis; may develop weeks to months after apparent recovery from acute hepatitis
    - Cholestatic hepatitis; characterized by protracted cholestatic jaundice and pruritus.

Laboratory Diagnosis

- **Anti-HAV antibody detection:**
    - IgM antibodies appear during the acute phase, peak about 2 weeks after the elevation of liver enzymes and disappear within 3-6 months (Fig. 50.1)
    - IgG antibodies appear a week after the appearance of IgM and persist for decades
    - **Interpretation:**
        - Anti-HAV IgM positive-indicates acute infection with HAV
        - Anti-HAV IgG antibody detection in the absence of IgM indicates past infection or recovery
        - ELISA is the method of choice; however many rapid test formats are also available.
- **Detection of HAV particles:** HAV appears in stool from -2 to +2 weeks of jaundice. It can also be detected from liver, bile, and blood by immunoelectron microscopy
- **HAV antigen detection:** ELISA format is available to detect HAV antigen from stool sample from -2 to +2 weeks of jaundice

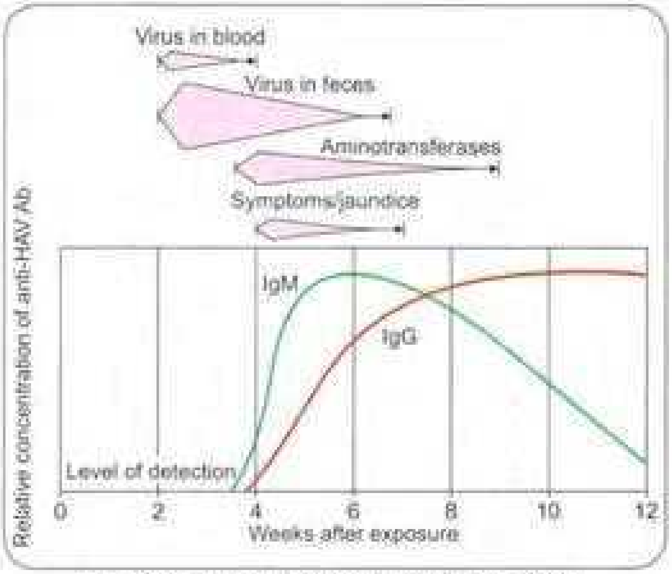


Fig. 50.1: Course of HAV markers with symptoms

- **Isolation:** HAV is very difficult to grow in cell line; though various primate cell lines are susceptible. HAV is the only hepatitis virus where isolation has been attempted
- **Non-specific findings:** Such as elevated liver enzymes and serum bilirubin level.

TREATMENT **Hepatitis A virus**

There is no specific antiviral drug available against HAV.

Prevention and Containment of Infection

General preventive measures should be attempted to improve hygiene such as:

- Hand washing before and after use of toilet
- Sanitary disposal of infected fecal material by disinfection with 0.5% hypochlorite
- Purification of drinking water by effective filtration and adequate chlorination (with at least 1 mg/L of residual chlorine)
- Use of boiled water (boiling for at least 5 minutes) during outbreaks.

Vaccines

- **Formaldehyde inactivated vaccine:** It is prepared from human fetal lung fibroblast cell lines such as MRC-5 and WI 38. It is given to children after 12 months of age. Single dose is administered by intramuscular route (deltoid) followed by booster at 6-12 months gap. Its protective efficacy is about 94%
- **Live attenuated vaccine:** It is given as single dose, subcutaneously. It uses H2 and L-A-1 strains of HAV, prepared in human diploid cell line (China)

- Both vaccines are highly immunogenic, produce long lasting immunity, possibly life-long.

Human Immunoglobulin (HAV-Ig)

It is extremely useful for post-exposure prophylaxis of **intimate contacts** (household, day care centers) of persons with hepatitis A or to the **travelers**.

- Dosage of 0.02 mL/kg is recommended which gives protection for about 1–2 months
- It should be administered as early as possible after exposure; (within 2 weeks)
- However, HAV-Ig is not necessary for those who have already vaccinated, casual contacts (office, factory, school, or hospital), and elderly persons (likely to be immune).

HEPATITIS B VIRUS

Hepatitis B virus (HBV) is the most widespread and the most important type among hepatitis viruses. Though it commonly produces an acute self-limiting hepatitis which may be subclinical or symptomatic, it is also capable of causing a range of hepatic complications including chronic hepatitis, fulminant hepatitis, cirrhosis of liver and liver cancer.

- HBV is the only DNA virus among hepatitis viruses. It was discovered by Blumberg in 1963
- It belongs to the family Hepadnaviridae, under the genus *Orthohepadnavirus*. This family also includes hepatitis viruses of lower animals (e.g, woodchucks, squirrels and ducks).

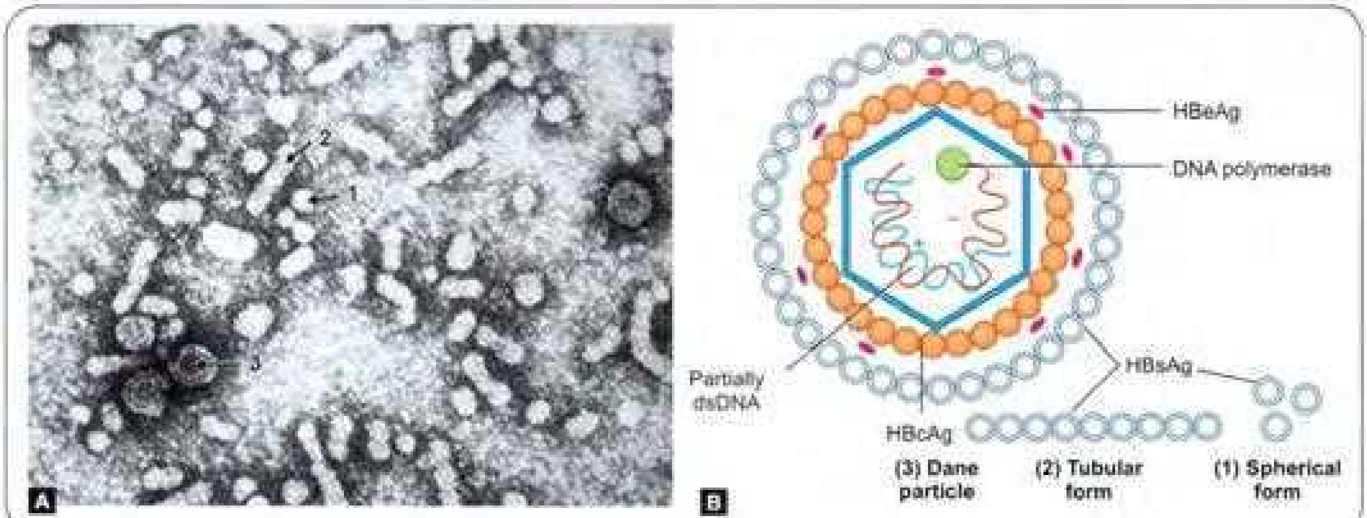
Morphology

Electron microscopy of serum of the patients infected with HBV reveals three morphologic forms (Figs 50.2A and B):

1. **Spherical forms:** Most numerous, small forms measuring 22 nm in diameter. These particles are exclusively made up of HBsAg.
2. **Tubular or filamentous forms:** They also have the same diameter, but 200 nm long. They are also exclusively made up of HBsAg.
3. **Complete form or Dane particles:** They are less frequently observed. They are larger, 42 nm size spherical virions; made up of:
   - **Outer surface envelope:** HBsAg (Hepatitis B surface antigen)
   - **Inner 27 nm size nucleocapsid:** It consists of core antigen (HBcAg) and pre-core antigen (HBeAg) and partially double stranded DNA.

Viral Antigens

- **Hepatitis B surface antigen (HBsAg):** HBsAg was previously called **Australia antigen** as it was first observed in the serum of an Australian Aboriginal person (1965)
- HBsAg is antigenically complex and contains two components—(1) common group reactive antigen 'a' epitope and (2) two pairs of type specific antigens d/y and w/r; only one member of each pair being present at a time
- Thus, four subtypes of HBsAg have been observed: adw, ayw, adr, and ayr



Figs 50.2A and B: A. Electron microscopic appearance of hepatitis B virus, showing 1-spherical form, 2-tubular form and 3-Dane particle; **B.** Schematic diagram

Source: A. Public Health Image Library, /Centers for Disease Control and Prevention (CDC); B. Atlanta/ ID# 5631 (*with permission*).

- **Hepatitis B core antigen (HBcAg):** HBcAg forms the intracellular core protein. It is not secreted and does not circulate in blood, but can be demonstrated in hepatocytes by immunofluorescence
- **Hepatitis B precore antigen (HBeAg):** HBeAg is a non-particulate soluble antigen possessing a signal protein which enables it to be secreted. It is therefore present in circulation.

Typing of HBV

Serotypes

HBV is divided into four major serotypes (adr, adw, ayr, ayw) based on antigenic epitopes present on its envelope protein HBsAg.

- The immunity is not serotype specific as the dominant 'a' antigen is shared by all. But they are useful for epidemiologic investigations, as all the cases during an epidemic would likely to have the same subtype
- Serotypes exhibit distinct geographical distribution
  - adw is the predominant subtype in Europe, Australia and America
  - In India, adr is the prevalent subtype in South and East India; whereas ayw is prevalent in Western and Northern India.

Genotypes

HBV can also be divided into eight genotypes (A-H) according to overall nucleotide sequence variation of the genome. Genotypes A and D are prevalent in India.

Viral Genome

The HBV genome (Fig. 50.3) consists of partially circular dsDNA of 3200 bp in length.

- The minus strand of DNA (L or long strand) is complete and full-length and is identical in all HBV isolates

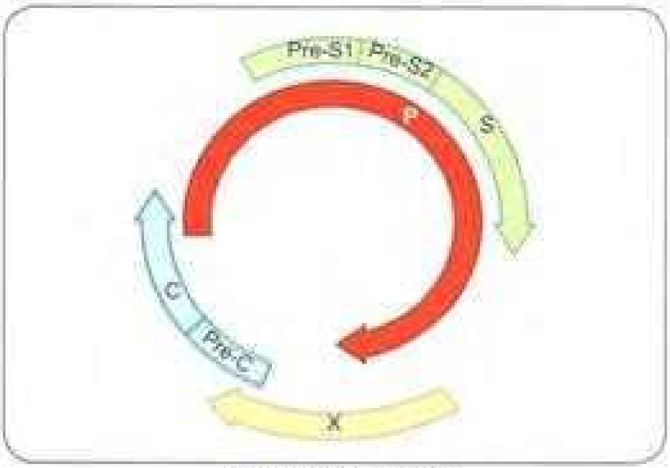


Fig. 50.3: HBV genome

- The positive strand (S or short strand) is incomplete and of variable length (50-80%).

Hepatitis B virus genome is compact and consists of four overlapping genes:

1. **S gene:** It has three regions—(1) S gene (2) pre-S1 and (3) pre-S2. They code for surface antigen (HBsAg).
   - S region codes for the major protein (S)
   - Product of S region combines with that of adjacent Pre-S2 region to form the middle (M) protein
   - Pre-S1, Pre-S2 combines with S to code for Large (L) protein
   - The L protein is present only in the virion, while the M and S proteins are found in the circulating HBsAg particles also.
2. **C gene:** It consists of pre-C and C-regions, which code for two nucleocapsid proteins
   - Pre-C region codes for HBeAg
   - C-region codes for HBcAg.
3. **X gene:** It codes for HBxAg, which can activate the transcription of cellular and viral genes.
   - It may contribute to carcinogenesis by binding to p 53
   - HBxAg and its antibody are elevated in patients with severe chronic hepatitis and hepatocellular carcinoma.
4. **P gene:** It is the largest gene and codes for polymerase (P) protein which has three enzymatic activities:
   1. DNA polymerase activity.
   2. Reverse transcriptase activity.
   3. RNase H activity.

Hepatitis B Virus Mutants

Mutations in various genes of HBV can lead to emergence of mutant strains. Three types of such mutations have been identified, which are as follows:

1. Precore Mutants

Precore mutants have mutations in precore region (which abolishes HBeAg production) or basal core promoter region (down regulates HBeAg production).

- **Viral mutation:** Though several mutations are identified; most commonly encountered is a **nonsense mutation** in the pre-C gene leading to formation of premature stop codon
- **Geographical distribution:** Precore mutants have been identified in Mediterranean countries and in Europe
- Patients infected with precore mutants may be diagnosed late and they tend to have **severe chronic hepatitis** that progresses more rapidly to cirrhosis
- Such patients do not produce HBeAg, although other viral markers are present as such
- **BCP mutation:** Mutation in basal core promoter region is common with HBV genotype C and is associated with increase the risk for hepatocellular carcinoma.

2. Escape Mutants

The escape mutants of HBV have mutations in the S gene which leads to alteration of HBsAg.

- ❖ Mutation occurs in the immunodominant *a* antigen of HBsAg (single amino acid substitution from glycine to arginine, occurs at position 145)
- ❖ Escape mutants are observed in three situations:
  1. Infants born to HBeAg positive mothers.
  2. Liver transplant recipients who underwent the procedure for hepatitis B and who were treated with a high-potency human monoclonal anti-HBs preparation.
  3. A small proportion of recipients of active and passive immunization, in whom antibody pressure may favor evolutionary change in gene coding *a* antigen.
- ❖ **Underdiagnosed:** Patients infected with escape mutants lack HBsAg and therefore are often underdiagnosed by routine serological test for HBsAg
- ❖ **Vaccine failure:** Escape mutants are capable of causing infection in vaccinated individuals as anti-HBs present in them cannot neutralize these HBsAg negative mutants.

3. YMDD Mutation

Hepatitis B virus infected patients on lamivudine therapy may develop resistance to the drug due to mutation in the YMDD locus present in the HBV reverse transcriptase region of polymerase gene.

Hepatitis B Replication (Fig. 50.4)

Replication of HBV is quite different from other DNA viruses.

- ❑ The HBV attaches to host cells by Pre-S region of HBsAg, penetrates into cytoplasm and gets uncoated to release viral DNA and polymerase
- ❑ In the host cell nucleus, the partially dsDNA of HBV gets converted into covalently closed circular dsDNA (cccDNA) mediated by host enzymes
- ❑ The cccDNA serves as the template for the production of HBV mRNAs and pregenomic RNA by undergoing transciption
- ❑ HBV mRNAs translate to form various components of viral proteins (e.g. the core protein, HBcAg)
- ❑ The pregenomic RNA comes to cytoplasm and gets encapsidated by newly synthesized HBcAg
- ❑ Within the core particle, the pregenomic RNA serves as template to form the minus strand of the DNA; mediated by the reverse transcriptase activity of the polymerase gene
- ❑ Next, the RNA template is removed from the negative-strand DNA; mediated by the RNase H activity of the polymerase gene
- ❑ Then, the polymerase starts to synthesize the positive DNA strand, but the process is not completed
- ❑ As a result, the dsDNA which is formed has a full-length minus-strand DNA and a variable-length (50–80%) positive-strand DNA
- ❑ Core particles containing these dsDNA bud from pre-Golgi membranes (acquiring the envelope HBsAg) and may either exit the cell or re-enter the intracellular infection cycle.

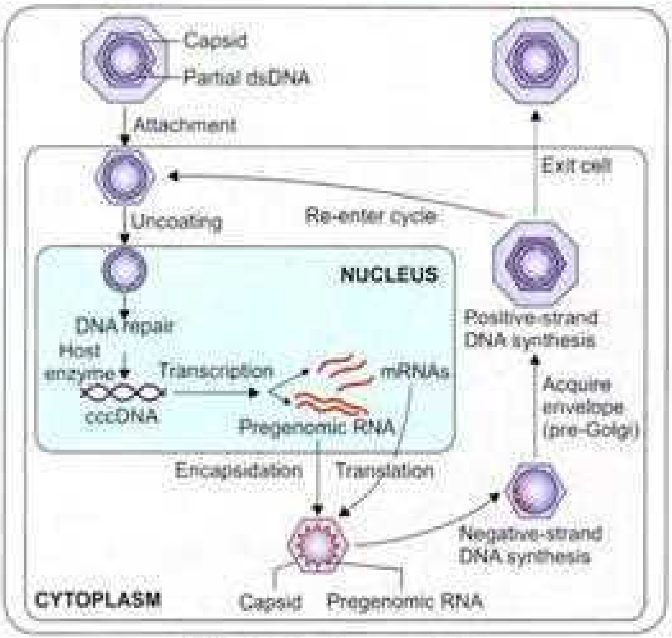


Fig. 50.4: Replication of HBV

Concept adapted and modified from Jawetz, Melnick, & Adelberg's Medical Microbiology; McGraw-Hill Education (with permission).

Transmission

Hepatitis B virus transmission occurs via multiple routes.

- ❖ **Parenteral route:** In developing countries, the most common mode of transmission is via blood and blood products transfusion and needle prick injuries
  - ■ Transmission also occurs by inoculation during surgical or dental procedures or percutaneous inoculation via shared razors and tooth brushes
  - ■ HBV is more infectious than HIV and HCV. As little as 0.00001 mL of blood can be infectious
  - ■ Chance of transmission of HBV following a contaminated needle prick injury is nearly 30% as compared to 3% and 0.3% with HCV and HIV respectively
  - ■ HBV can be transmitted in the absence of visible blood and remains infectious, on environmental surfaces for at least 7 days.
- ❖ **Sexual transmission** is found to be the most common route in most developed countries; particularly homosexual males being at higher risk
- ❖ **Vertical (perinatal) transmission:** The spread of infection from HBV carrier mothers to babies appears to be an important mode of transmission particularly in China and South East Asia
  - ■ Transmission occurs at any stage; in utero, during delivery (maximum risk) and during breastfeeding
  - ■ Risk is maximum if the mother is HBeAg positive.
- ❖ **Direct skin contact** with infected open skin lesions may trasmit the virus, e.g. impetigo (especially in children)
- ❖ Although HBV can survive in mosquitoes; no transmission has been observed.

High-risk groups which are more prone for acquiring infection are:

- Surgeons (maximum risk)
- Paramedical workers
- Sex workers especially homosexual males
- Recipients of blood transfusion and organ transplantation
- Drug addicts.

Epidemiology

Hepatitis B virus infection occurs throughout the world; usually sporadic, but occasional outbreaks can occur in hospitals.

- **Reservoir of infection:** Humans are the only reservoir of infection who can be either cases or carriers
  - Cases may be either inapparent or symptomatic
  - Carriers may be temporary (harbor the virus for weeks to months) or persistent/chronic (harbor the virus for >6 months).
- **Carriers** can also be grouped into:
  - **Simple carriers** (or chronic inactive HBV infection): They are of low infectivity, transmit the virus at a lower rate. They possess low level of HBsAg and no HBeAg
  - **Super carriers** (or Immunotolerant chronic HBV infection): They are highly infectious and transmit the virus efficiently. They possess higher levels of HBsAg and also have HBeAg, DNA polymerase and HBV DNA.
- **HBV prevalence:** It is determined based on HBsAg carrier rates. There are three epidemiological patterns observed among various countries:
  1. *Low endemicity:* Carrier rate is less than 2%. It is observed in many countries of American, European regions and Australia. Lowest is recorded for UK and Norway (0.01%)
  2. *Intermediate endemicity:* Carrier rate is between 2 and 8%. It is observed in India, China and many countries of Eastern Mediterranean and Southeast Asian regions
  3. *High endemicity:* Carrier rate is more than 8%. It is observed in many countries of African and Western Pacific regions. Some countries exceed HBV prevalence of >15% such as South Sudan, Kiribati, Swaziland and Solomon Islands.
- **Situation in the world:** WHO estimates that in 2015, about 257 million population were living with chronic HBV infection with a global prevalence of 3.5%.
  - The African (6.1%) and Western Pacific (6.2%) regions accounted for 68% of global burden.
  - 2.7 million persons were coinfected with HBV and HIV
  - The widespread use of HBV vaccine in infants has led to a considerable reduction in the incidence of new chronic HBV infections.
- **Situation in India:** India is considered to have an intermediate level of HBV endemicity (3.7% prevalence); with over 40 million HBV carriers, which constitutes 11% of the estimated global burden, which is second highest to China (30%)
  - Highest prevalence recorded from Andamans and Arunachal Pradesh
  - Tribal areas, the prevalence is extremely high (19%) than non-tribal populations
  - HBV accounts for 40–50% of hepatocellular carcinoma (HCC) and 10–20% of cirrhosis in India.
- HBV followed by HCV are the most common cause of:
  - Chronic hepatitis
  - Cirrhosis: HBV accounts for 30% of cirrhosis
  - Hepatocellular carcinoma (HCC): HBV accounts for 45% of HCC
  - Post-transfusion hepatitis: HBV (1:220,000)>HCV (1:1800,000).
- **Elimination of viral hepatitis:** The WHO has introduced Global health sector strategy on viral hepatitis (2016-2021) which aims at the elimination of viral hepatitis as a public health threat by 2030 (defined by reducing new infections by 90% and mortality by 65%). **World Hepatitis Day** is celebrated on 28th July every year.
- **Resistance:** HBV can be destroyed by hypochlorite and heat (by autoclaving)
- **Period of infectivity:** People infected with HBV are said to be infectious as long as the HBsAg is present in blood, i.e. during incubation period (a month before jaundice) up to several months thereafter (occasionally years for chronic carriers)
  - Patients become non-infectious once HBsAg disappears and is replaced by anti-HBs antibody
  - Maximum infectivity is observed when HBeAg is elevated in serum.
- **HBV and HIV Co-infection:**
  - The global HBV prevalence in HIV-infected persons is 7.4%. Conversely, about 1% of HBV-infected persons are also infected with HIV. The highest burden (71%) for HIV-HBV coinfection is found in sub-Saharan Africa
  - Although HBV does not alter the progression of HIV, the presence of HIV greatly enhances the risk of developing HBV-associated cirrhosis and liver cancer
  - Tenofovir, a drug recently included in the treatment regimen of HIV is also active against HBV. This may have a role in controlling HIV-HBV coinfection.
- **Age:** The outcome (Fig. 50.5) of HBV infection depends on the age. Following HBV infection:

- Chance of developing acute hepatitis is directly related to the age:
  - 1% (perinatal)
  - 10% (early childhood; 1–5 years of age)
  - 30% (late childhood; after 5 years of age).
- Chance of developing chronic hepatitis or carrier state is inversely related to age:
  - 80–90% (perinatal)
  - 30% (early childhood; 1–5 years of age)
  - 5% (late childhood; after 5 years of age).
- **Explanation:** Pathogenesis of HBV infection is immune mediated
  - Hepatocytes carrying viral antigen are subjected to natural killer cell mediated or CD8 T cell mediated cytotoxicity
  - Absence of an effective immune system (e.g. infants) leads to carrier state.

Clinical Manifestation

Hepatitis B has an incubation period of about 30–180 days. The onset of infection is slow and insidious.

- Patients may present with subclinical infection, acute or chronic hepatitis (Fig. 50.5)
- Clinically, HBV infection is indistinguishable from other hepatitis viruses; characterized by:
  - Pre-icteric phase (predominant gastrointestinal symptoms such as nausea and vomiting) followed by;
  - Icteric phase or jaundice.
- Clinical outcome may be either development of carrier state or complete recovery
- **Hepatic complications:** Very few cases may proceed to complications such as fulminant hepatitis or cirrhosis or hepatocellular carcinoma
- **Extrahepatic complications:** During the prodromal phase, a serum sickness-like syndrome characterized by arthritis, rash, angioedema, and rarely, hematuria and proteinuria may develop in 5–10% of patients. This is due to immune complex deposition.

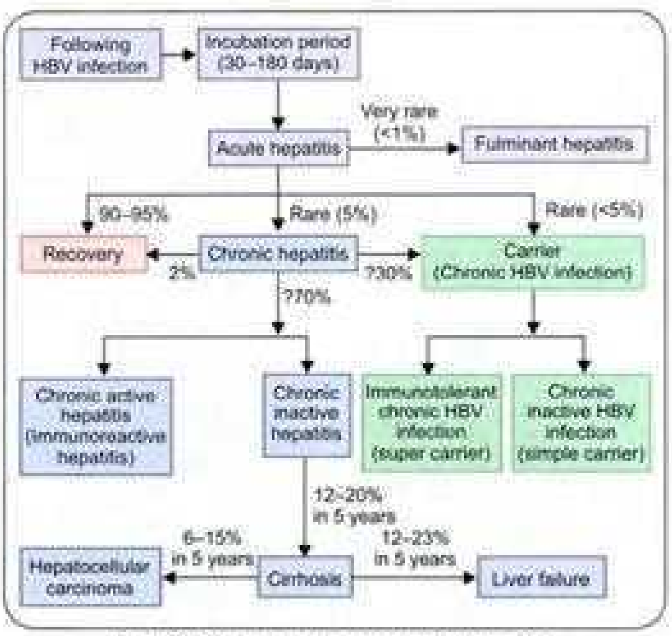


Fig. 50.5: Various outcomes of HBV infection

LABORATORY DIAGNOSIS	Hepatitis B virus

- **Antigen markers:** HBsAg, HBeAg and HBcAg
- **Antibody markers:** Anti-HBs, Anti-HBe and Anti-HBc
- **Molecular markers:** HBV DNA
- **Non-specific markers:** Elevated liver enzymes and serum bilirubin.

Laboratory Diagnosis (Viral Markers of HBV Infection)

Definitive diagnosis of hepatitis B depends on the serological demonstration of the viral markers (Figs 50.5 and 50.6 and Table 50.2, laboratory diagnosis synopsis box).

The most useful detection method for HBV antigens and antibodies is ELISA although various rapid test formats such as immunochromatographic test (ICT) are also available. Viral DNA can be detected by PCR (Polymerase chain reaction); but quantified by real time PCR. HBV does not grow in any conventional culture system.

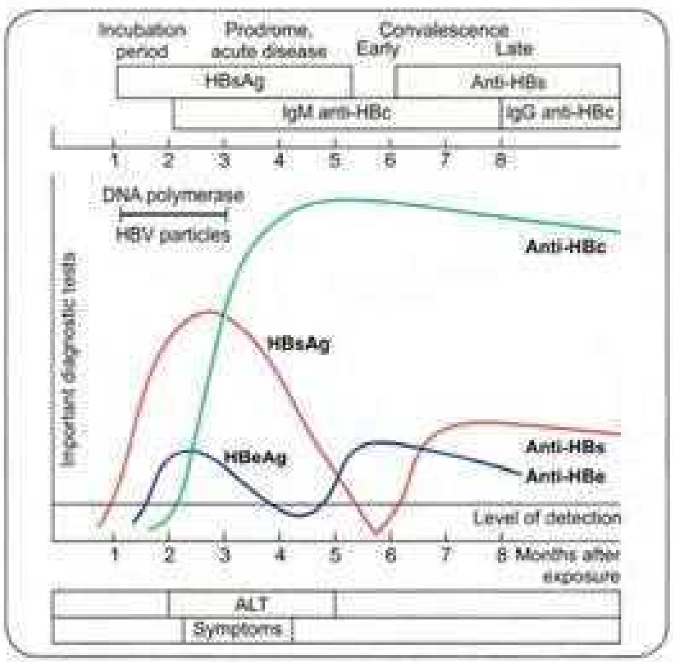


Fig. 50.6: Serological markers of hepatitis B virus in various stages of hepatitis B virus infection

Table 50.2: Interpretation of markers in various stages of hepatitis B infection

HBsAg	Anti-HBS	Anti-HBc	HBeAg	Anti-HBe	Symptoms	Liver enzymes	DNA	Interpretation
+	–	–	–	–	Absent	Normal	+	Early acute hepatitis (incubating)
+	–	IgM	++	–	Present	Elevated	++	Acute hepatitis B, high infectivity
+	–	IgG	++	–	Present	Elevated	++	Chronic hepatitis B, high infectivity (Immunoreactive chronic hepatitis)
+	–	IgG	–	+	Present	Elevated	+	Chronic hepatitis B, low infectivity (Chronic inactive hepatitis)
+	–	IgG	+	–	Absent	Normal	++	Immunotolerant chronic HBV infection (previously called as super carriers)
+	–	IgG	–	+/–	Absent	Normal	+	Chronic inactive HBV infection (previously called as simple carriers)
+	–	IgG	–	–	+/–	Normal/elevated	+	Precore-mutant hepatitis B infection
–	–	IgG	–/+	–/+	+/–	Normal/elevated	+	Escape mutant hepatitis B infection
–	+	IgG	–	+/–	Absent	Normal	–	Recovery
–	+	–	–	–	Absent	Normal	–	Post-vaccination
–	–	IgG (+/–)	–	–	Absent	Normal	+	Occult hepatitis B infection

Hepatitis B Surface Antigen (HBsAg)

HBsAg is the first marker to be elevated following infection; appears within 1-12 weeks (usually between 8 and 12 weeks of infection).

- It appears during incubation period; 2-6 weeks before the biochemical and clinical evidence of hepatitis
- Presence of HBsAg indicates onset of infectivity (i.e. patient is capable of transmission of HBV)
- It remains elevated in the entire duration of acute hepatitis; becomes undetectable 1-2 months after the onset of jaundice:
  - However, it persists rarely beyond 6 months if the disease progresses to chronic hepatitis or in carrier state
- HBsAg is used as an epidemiological marker of hepatitis B infection (i.e. to calculate prevalence of infection).

Hepatitis B Precore Antigen (HBeAg) and HBV DNA

HBeAg and HBV DNA appear concurrently with or shortly after appearance of HBsAg in serum.

- **They are the markers of:**
  - Active viral replication
  - High viral infectivity (i.e. high transmission rate).
- However, these markers can be present in either acute, chronic or carrier state; hence, they cannot differentiate between these stages. Their presence just indicates that the virus is actively multiplying, which could be either:
  - Acute active hepatitis
  - Chronic active hepatitis
  - Or a carrier in whom HBV is actively multiplying and is highly infectious (such carriers are called **super carriers**).

Hepatitis B Core Antigen (HBcAg)

- HBcAg is a hidden antigen due to its surrounding HBsAg coat. It is also non-secretory in nature; hence, it cannot be detected in blood
- However, HBcAg may be detected in hepatocytes by immunofluorescence test.

Anti-HBc IgM (Hepatitis B Core Antibody)

Anti-HBc IgM is the first antibody to be elevated following infection.

- It appears within first 1-2 weeks after the appearance of HBsAg and lasts for 3-6 months
- Its presence indicates acute hepatitis B infection
- It is probably the only marker (sometimes anti-HBcIgG) present during the period between appearance of anti-HBs antibody and disappearance of HBsAg.

Anti-HBc IgG (Hepatitis B Core Antibody)

Anti-HBc IgG appears in late acute stage and remains positive indefinitely whether the patient proceeds to:

- **Chronic stage** (with persistence of HBsAg, symptomatic and elevated liver enzymes)
- **Carrier state** (with persistence of HBsAg, but asymptomatic)
- Recovery (appearance of anti-HBs antibody).

It can also be used as epidemiological marker of HBV infection.

Anti-HBe (Hepatitis B Precore Antibody)

- Anti-HBe antibodies appear after the clearance of HBeAg and remain elevated for variable period
- Its presence signifies diminished viral replication and decreased infectivity.

Anti-HBs (Hepatitis B Surface Antibody)

It appears after the clearance of HBsAg and remains elevated indefinitely.

- Its presence indicates recovery, immunity and non-infectivity (i.e. stoppage of transmission)
- It is also the only marker of hepatitis B vaccination.

TREATMENT — Hepatitis B

Most acute hepatitis B infection is self-limiting; do not require any specific treatment. In contrast, treatment of chronic hepatitis B has perceived many recent advances. With the advent of newer antiviral drugs, now it is possible to contain the disease.

Indications*

The indications to initiate treatment for patients with hepatitis B infection are:

- Acute hepatitis with acute liver failure
- Chronic active hepatitis (immunoreactive hepatitis, HBeAg positive)
- Chronic inactive hepatitis (HBeAg –ve):
  - HBV DNA >2,000 IU/mL plus ALT ↑ (> normal) plus moderate degree of liver fibrosis
  - HBV DNA >20,000 IU/mL and ALT ↑↑ (>2 times normal); regardless of the degree of liver fibrosis.
- Associated cirrhosis (regardless of ALT level, viral load)
- Carriers (super or simple) with family history of HCC or cirrhosis.
- Super carriers or immunotolerant hepatitis (here, treatment is indicated if the person's age is >30 years)

Antiviral agents include (also refer Table 50.3):

- Antiviral agents (nucleoside analogues): Tenofovir and telbivudine are the agent of choice currently. Lamivudine, adefovir and entecavir can also be used, but they are less preferred because of high risk of development of resistance.
- Pegylated interferon alfa (used previously; now not in use because of adverse effects).

*Source: EASL guideline 2017 (European Association for the Study of the Liver).

Prophylaxis

Active Immunization (Hepatitis B Vaccine)

Hepatitis B vaccine is a recombinant subunit vaccine.

- The surface antigen (HBsAg) is used as vaccine candidate which is prepared in Baker's yeast by DNA recombinant technology by cloning the S gene into the yeast chromosome
- **Route of administration:** Vaccine is administered by intramuscular route over deltoid region (in infant-anterolateral thigh)
- **Dosage:** 10–20 µg/dose (half of the dose is given to children below 10 years)
- **Schedule:**
  - Recommended schedule for adults: Three doses are given at 0, 1 and 6 months
  - Under national immunization schedule: It is given at 6, 10, 14 weeks (along with DPT vaccine). Additional dose at birth may be given in areas with prevalence of HBV more than 8%
  - Minimum interval between the doses—4 weeks
  - If there is documentaion of partial vaccination (1 or 2 doses): Then do not restart; just complete the vaccine series regardless of when the last dose was taken.
- **Marker of protection:** Recipients are said to be protected if they develop seroconversion with an anti-HBsAg antibody titer of more than 10 mIU/mL.
- **Re-vaccination:** If titer remain <10 mIU/mL after first series of vaccination; the HCW is subjected to second series of vaccination (3 doses at 0, 1, 6 months)
- **Non/low responders:** About 5–10% of individuals mount an impaired immune response following vaccination. They may be either:
  - Non-responders (do not show seroconversion after two series of vaccination; i.e six doses of vaccination.) or;
  - Low responders (seroconversion occurs slowly after the second series of vaccination.).
- Seroconversion occurs in about 95% of infants, children and young adults. However, among older people (>60 years), the protection is about 65–75% only
- Protection may last for about 30 years or even longer
- **Booster doses are not needed:** The health care workers once protected, should not check their titer again or should not take booster vaccines. They remain protective even if the titer falls <10 mIU/mL. This is

Table 50.3: Treatment options available for chronic hepatitis B infection

	Interferon alfa	Antiviral agents*
Route and dosage	Subcutaneous, weekly	Oral, daily
Mode	Monotherapy	Monotherapy
Duration	48 weeks	Long-term until HBsAg loss
Side effects	High	Minimal
Decompensated disease, immuno-compromised patients	Cannot be given	Can be given
HBV DNA	<2000 IU/mL	Undetectable, i.e. <10 IU/mL**
HBsAg loss or HBeAg seroconversion	Occurs relatively earlier (1 year)	Occurs very slowly
Preference	Was preferred in past	First choice of treatment currently

*Telbivudine and tenofovir

**HBV DNA clearance from blood may occur early; however cccDNA may be present in hepatocytes quiescently; which may get activated later. Hence, HBeAg and HBsAg seroconversion (become negative) are considered as more reliable indicators of successful therapy.

because the memory cells get stimulated much faster and the titer rises very soon following an infection with HBV
- Newer vaccine containing whole HBsAg (i.e. product of *Pre S1 + Pre S2 + S genes*) is under development which may provide a better seroconversion.

Passive Immunization (Hepatitis B Immunoglobulin or HBIG)

- **Indications:** HBIG is used in the following situations where an **immediate protection** is warranted
  - Acutely exposed to HBsAg positive blood, e.g. surgeons, nurses, laboratory workers
  - Sexual contact of acute hepatitis B patients
  - Neonates borne to hepatitis B carrier mothers
  - Post-liver transplant patients who need protection against HBV infection.

  Following accidental exposure, HBIG should be started immediately (ideally within 6 hours, but not later than 48 hours).
- **Recommended dose** is 0.06 mL/kg (or 10-12 IU/kg) single dose, given IM
- HBIG provides a temporary protection for 3-6 months.

Combined Immunization

Combined immunization with **HBIG** and **vaccine** is more efficacious than HBIG alone. It is recommended for **neonates** born to HBV infected mother, where a single injection of 0.5 mL of HBIG is given to the neonate immediately after the birth, followed by full course of vaccine (the first dose being given within 12 hours of birth).

The guideline for post-exposure prophylaxis for hepatitis B is described in detail in Chapter 56.

General Prophylactic Measures

- Screening of blood bags, semen and organ donors
- Following safe sex practices (e.g. using condoms, avoiding multiple sex partners)
- Following safe injection practices—use of the disposable syringes and needles
- Following safe aseptic surgical practices
- Health education.

HEPATITIS C VIRUS

Hepatitis C virus (HCV) is the common cause of post-transfusion hepatitis in developing countries. It was discovered in 1989 and first labeled as "non-A, non-B hepatitis virus while performing the experiments in chimpanzees.

Morphology

Hepatitis C virus is classified under family Flaviviridae, genus *Hepacivirus*.
- It is spherical, 60 nm size and enveloped
- **Nucleic acid:** It contains a positive sense ssRNA
- **Proteins:** HCV possesses:
  - Three structural proteins: The nucleocapsid core protein C; two envelope glycoproteins (E1 and E2)
  - Six nonstructural (NS) proteins: NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Fig. 50.7)
  - One p7 membrane protein: It functions as an ion channel; was earlier considered as NS1.

Genetic Diversity of HCV

Similar to HIV, Hepatitis C virus displays diversity in the RNA genome that occurs because of high rates of mutations seen in the virus which inturn is due to (i) the high replicative activity and (ii) the lack of proofreading activity of the RNA polymerase.
- **Genotypes:** HCV is divided into six major genotypes or clades, which differ from each other by 31-33% in their RNA sequence
- **Subtypes:** Genotypes are further divided into more than 100 subtypes, which differ from each other by 20-25% in their RNA nucleotide sequence. Within any given patient, the subtypes of HCV circulate as complex closely related viral population known as **quasispecies**
- The **E2 envelope protein** is the most variable region of the entire HCV genome followed by the non-structural

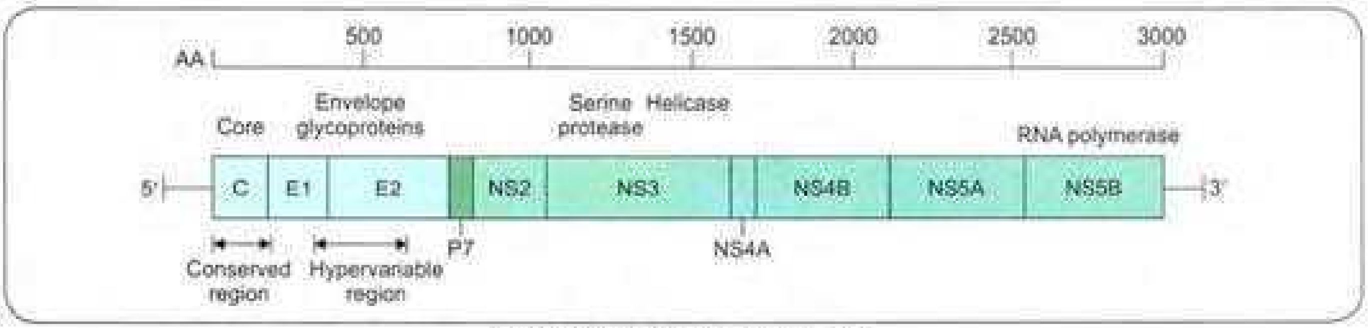


Fig. 50.7: Genome of Hepatitis C virus

proteins (especially, NS5B encoded RNA polymerase); hence they are more prone to undergo mutations

- Unfortunately, E2 protein happens to be the target against which most of the neutralizing (protective) antibodies are produced
- Thus, diversity in the gene coding E2 protein enables the emergent mutant virus strains to escape from host's humoral immunity, which in turn can result in:
  - Establishment of chronic infection
  - Failure of development of effective vaccine.
- HCV genotypes vary in their epidemiological distribution:
  - Genotype 1 and 2 represent the most common variants in Western countries
  - Genotype 3 is widely distributed in South and East Asia with subtype 3a common among IV drug users from Europe
  - Genotype 4 in North Africa and Middle-East
  - Genotype 5 in South Africa
  - Genotype 6 in Asia
  - In India, genotypes 1 and 3 are more prevalent.
- The genotypes also vary from each other in their (i) susceptibility to antiviral drugs; for example, patients with genotype-1b respond poorly to therapy than other genotypes and (ii) duration of treatment needed.

Transmission

Various modes of transmission of HCV are as follows:

- **Parenteral:** HCV is most commonly transmitted through exposure to infectious blood:
  - Recipients of contaminated blood transfusions, blood products or organ transplantations
  - Contaminated needles and sharps pricks
  - Injection drug users.
- **Vertical transmission** from infected mother to fetus may occur but at much lower rate (6%) than that of HBV (20%)
- **Sexual transmission** (rare).

Hepatitis C virus **does not spread** through breast milk, food or casual contacts including hugging or kissing.

Clinical Manifestations

Incubation period is about 15–160 days (average 50 days). Following an infection with HCV:

- **Acute hepatitis:** About 20% of people develop acute hepatitis; characterized by symptoms similar to that of other hepatitis viruses described earlier. Rest patients remain asymptomatic in early stage
- **Spontaneous clearance:** About 5–15% of infections, the virus gets cleared spontaneously within 12 weeks
- **Chronic disease:** About 75–85% directly develop chronic disease; out of which:
  - 60–70% develop chronic hepatitis
  - 5–20% develop cirrhosis
  - 1–5% develop hepatocellular carcinoma. (HCV accounts for 25% of total liver cancer patients).
- **Extrahepatic manifestations:** Due to deposition of circulating immune complexes (composed of HCV antigens and their antibodies) in extrahepatic sites, various manifestations can set in such as:
  - Mixed cryoglobulinemia
  - Glomerulonephritis
  - Arthritis and joint pain.

Epidemiology

Hepatitis C virus infection occurs worldwide. Every year, 3–4 million people are infected with HCV with more than 3.5 lakhs deaths.

Population prevalence rate:

- About 3% of the world population has been infected with HCV worldwide with more than 170 million chronic carriers
- Higher prevalence rates have been documented from Africa (up to 10%) followed by South America and Asia
- In India, the prevalence of HCV is about 1%.

Laboratory Diagnosis

HCV Antibody Detection Assay (ELISA)

The antibody detection tests have been the standard test for screening and primary testing for HCV diagnosis.

ELISA is the most common platform used; followed by rapid test and chemiluminescence formats.

- **First and second generation ELISA** were used previously. These assays were using limited HCV antigens (core, NS3 and NS4) for detection of antibodies. These assays are now obsolete as they are less sensitive (not able to detect few genotypes) and used to become positive only after 10 weeks of infection
- **Third generation ELISA:** This has been the standard method for HCV serology. It employs antigens from NS5 region in addition to core, NS3 and NS4 regions
  - *Advantages* include: (i) increased sensitivity and specificity (>99%); (ii) not influenced by HCV genotype, (iii) becomes positive in 5 weeks of infection
  - *Disadvantages:*
    - These assays detect IgG antibodies to HCV, hence, they do not discriminate between active or past infection; for which HCV RNA test is required
    - False positive result: Seen with autoimmune diseases, mononucleosis, and pregnancy
    - False negative result: Seen with immunosuppression such as HIV infection, transplant recipients, etc.

Table 50.4: Algorithm of HCV diagnosis (Adapted from CDC guideline, 2013)

Test outcome	Interpretation	Further actions to be taken
HCV antibody non-reactive	No HCV antibody detected	• It is reported as non-reactive. No further action is required • If history of recent exposure is present within 6 months, then: ➢ Do follow-up testing for HCV antibody (for immunocompetent patient) ➢ Test for HCV RNA (for immunocompromized patient)
HCV antibody reactive	Presumptive HCV infection	• *A repeatedly reactive result indicates either:* (i) current HCV infection, or (ii) past HCV infection that has resolved, or (iii) biologic false positivity for HCV antibody • HCV RNA PCR should be done—to identify current infection
HCV antibody reactive, HCV RNA detected	Current HCV infection	• Provide appropriate counseling to the patient and refer to HCV clinic • Treatment should be initiated (after repeating HCV RNA PCR for re-confirmation)
HCV antibody reactive, HCV RNA not detected	No current HCV infection	• No further action is required in most cases • If clinically suspected or history of exposure within 6 months: then follow up with HCV RNA testing and provide appropriate counseling

Recombinant Immunoblot Antibody Assay (RIBA)

RIBA was used in past as a supplementary test to confirm the ELISA result; not in use currently. It works based on the principle of western blot. It detects antibody against core, E2 hypervariable region and NS3, NS4A, NS4B and NS5A regions.

HCV Core Antigen Assay

Automated quantitative test detecting core antigen has been available recently (e.g. Abbott ARCHITECT HCV antigen assay).

- This test is less expensive and less time consuming than HCV RNA PCR
- *Advantages:* Antigen detection assays can be used as an alternative to HCV RNA testing for: (i) diagnosis of active/current infection, (ii) monitoring response to treatment
- *Disadvantage:* They are less sensitive than RT-PCR; hence not recommended for blood screening purpose

An ELISA format (Monolisa HCV Ag-Ab ULTRA, Biorad) has recently been available that detects simultaneously antigen (capsid Ag) and antibody (to NS3 and NS4 Ag).

Molecular Methods

Real time reverse transcriptase PCR detecting HCV RNA has been the gold standard method for:

- Confirmation of active HCV infection
- Quantification of HCV RNA for monitoring the response to treatment
- For determining HCV genotype and subtype (also done by sequence-based method).

Diagnosis of Acute HCV Infection

Most cases of HCV infections remain asymptomatic in early stage and are directly presented with chronic infection (i.e. after 6 months). Acute HCV infection (i.e. onset of symptoms within 6 months of exposure) occurs very rarely.

- Both IgM and IgG antibodies appear during acute stage; IgM may persist during chronic stage for variable time; whereas IgG persists for long duration both in chronic stage and during recovery
- Most assays available detect IgG antibodies. There is no IgM-based assay available currently
- The acute infection can be diagnosed by: (i) anti-HCV IgG seroconversion, (ii) HCV RNA positivity or (iii) IgG avidity assay (low avidity indicates acute infection, strong avidity-may indicate chronic/past infection).

The CDC recommended algorithm used for HCV diagnosis is depicted in Table 50.4.

TREATMENT — Hepatitis C

The goal of treatment in HCV infection is:

- To prevent development of complications such as cirrhosis and liver cancer
- To achieve **sustainable viral response (SVR)** which defined as undetectable HCV RNA in blood (≤15 IU/mL) at the end of treatment.

Interferon alfa plus ribavirin

Earlier, pegylated-interferon (PEG-IFN) alpha plus ribavirin (RBV) was used for treatment of HCV infection. This was associated with high adverse effects with a moderate degree of viral clearance (SVR 40–50%).

Direct-acting antiviral agents (DAAs)

After the discovery of direct-acting antiviral agents (DAAs), the treatment of HCV infection has been revolutionized. DAAs are the now the treatment of choice for HCV infection as they can produce SVR rate up to 90% with minimal side effects.

- **Three classes of DAAs** are available: NS3/4A (proteases) inhibitors, NS5B (polymerases) inhibitors and NS5A inhibitors (Table 50.5)
- **Combination therapy:** Currently, the treatment regimen for HCV includes combinations of different DAAs with or without PEG-IFN and ribavirin
  - *Genotype specific:* The drugs to be included in the regimen depends upon the genotype detected. Most DAAs are genotype specific; except sofosbuvir/velpatasvir which are active against all genotypes

Contd...

Table 50.5: Drugs used for hepatitis C virus infection

Class	Agents
Directly acting antiviral agents (DAAs)	
NS3/4A (proteases) inhibitors	*Ends with suffix 'previr'* Grazoprevir, Paritaprevir, Simeprevir
NS5B (polymerases) inhibitors	*Ends with suffix 'buvir'* Dasabuvir, Sofosbuvir
NS5A inhibitors	*Ends with suffix 'asvir'* Daclatasvir, Ledipasvir, Velpatasvir
Other classes	
Interferons	Pegylated interferon alfa-2a and 2b
Nucleoside inhibitor	Ribavirin

Contd...

TREATMENT **Hepatitis C**

- *Duration:* The duration of treatment regimen is either 12 or 24 weeks; depending up on the HCV genotype and presence or absence of liver cirrhosis.

Predictors of Treatment Response

- **Genotypes:** HCV genotype 1 and 4 are associated with poor prognosis than other genotypes
- **Subtype:** 1b shows poor prognosis compared to 1a
- Stage of disease (presence of cirrhosis) and associated co-infections such as HIV and HBV: associated with poor prognosis
- **Adherence:** Poor compliance to treatment, is associated with worse prognosis
- **Viral RNA load:** Higher the viral load (> 800,000 IU/mL), worse is the prognosis
- **Interleukin 28 B** is a strong inducer of interferon-α. People possessing a subtype of IL 28B (called CC genotype), produce a stronger immune response to HCV infection by inducing IFN-α release; hence have a better outcome
- **Race:** Caucasians and African Americans lack **CC genotype**, hence show a poor treatment response than that of Asians
- **Others:** Metabolic disorders such as insulin resistance, obesity metabolic syndrome, steatosis, lack of vitamin D supplement, older age, alcohol consumption—all reduce the chance of responding to HCV therapy.

Prevention

There is no effective vaccine available for HCV. General prophylactic measures are essentially same as that for HBV.

HEPATITIS D VIRUS

Hepatitis D virus is a defective virus; cannot replicate by itself; depends on Hepatitis B virus for its survival.

Morphology

Hepatitis D virus is taxonomically unclassified though resembles viroids. It is small in size (35 nm), consisting of:

- Circular, negative-sense ssRNA
- Protein coat is made up of single protein called **hepatitis D antigen (HDAg)**
- Surrounded by envelope protein derived from HBsAg from hepatitis B virus; hence, it is called **defective virus**.

Transmission

Transmission is similar to that of HBV and HCV. Parenteral route is the most common mode; followed by sexual and vertical routes.

HDV and HBV Association

The association of HDV with HBV is of two types (Table 50.6):

- **Co-infection:** It occurs when a person is exposed simultaneously to serum containing both HDV and HBV
  - Hepatitis B virus infection sets in first so that HBsAg becomes available for HDV
  - This is usually a transient and self-limited condition; clinically indistinguishable from acute hepatitis B infection
  - It rarely progresses to chronic stage (1–10%); at a rate similar to that of HBV infection alone
  - Vaccination against hepatitis B can prevent against HDV.
- **Super-infection:** It occurs when a chronic carrier of HBV is exposed to serum containing HDV. This results in disease 30–50 days later which may have two phases:
  - **Acute phase:** This is the first stage, in which HDV replicates actively with high transaminase levels with suppression of HBV
  - **Chronic phase:** All (100%) patients progress to chronic stage, in which HDV replication decreases, HBV replication increases, transaminase levels fluctuate, and the disease progresses to cirrhosis and hepatocellular carcinoma (HCC). Mortality rate is much higher (>20%).

The order of frequency of chronicity of viral hepatitis in decreasing order is: HDV superinfection (100%)> Perinatal HBV (90%) > HCV (85%) > HBV and HBV-HDV coinfection (1–10%). However, in terms of absolute number of chronic cases/carriers; HBV accounts for the maximum.

Laboratory Diagnosis

- **In co-infection:** IgM against both HDAg and HBcAg are elevated, although IgM anti-HDV appears late and is frequently short-lived
- **In super-infection:** As HBV infection is already established as carrier, IgG anti-HBc will be detected. Anti-HDV would be IgM type initially; but as patient progresses to chronic state, mixture of IgM and IgG would persist for months or longer
- Anti HBc antibody is the key to differentiate between co-infection and super-infection

Table 50.6: Differences between Hepatitis D and Hepatitis B virus (HDV-HBV) co-infection and super-infection

Features	Co-infection	Super-infection
Definition	HBV and HDV infection occurs simultaneously	HDV infection occurs to a carrier of HBV
Patient status	Healthy	HBV carrier
Risk of development of:		
Fulminant disease	More than that of HBV alone	More than that of co-infection
Chronic hepatitis	Rare	Much greater
Cirrhosis	Rare	More
HCC	Rare	More
Mortality	Rare	>20%
Diagnosis	• HBsAg • Anti-HBc (IgM) • Anti-HDV (IgM) • HDV RNA	• HBsAg • HBeAg • Anti-HBc (IgG) • Anti-HDV: ➢ In acute phase-IgM ➢ In Chronic phase-IgG and IgM • HDV RNA

Abbreviation: HCC, hepatocellular carcinoma.

- **IgM anti-HBc + IgM anti-HDV:** Indicates co-infection
- **IgG anti-HBc + mixture of IgM and IgG anti-HDV:** Indicates super-infection.

❖ HDV RNA is detectable in the blood and liver just before and in the early days of acute phase of both co-infection and super-infection

❖ HBeAg, the marker of active HBV replication may be present in super-infection.

Epidemiology

Globally, about 15 million people are infected with HDV (about 5% of 350 million of HBV infected persons).

Hepatitis D virus infection occurs worldwide, but prevalence varies greatly. Surprisingly, HDV is not prevalent in Southeast Asia including India; where HBV carriers are maximum.

Two epidemiologic patterns have been identified:

1. **In endemic areas**, such as Mediterranean countries (Northern Africa, Southern Europe, and Middle East) HDV is endemic among persons with hepatitis B and is transmitted predominantly by non-percutaneous means, especially by close personal contact.
2. **In non-endemic areas**, such as the USA and Northern Europe, HDV infection is confined to persons frequently exposed to blood and blood products, primarily injection drug users and hemophiliacs who are infected with HBV.
    - HDV infection can be introduced into a non-endemic population through IV drug users or by migration of persons from endemic to non-endemic areas.
    - Introduction of HDV into non-endemic areas where HBV infection is common may lead to explosive outbreaks of severe hepatitis with high mortality.

TREATMENT — **Hepatitis D virus**

Patients with HDV infection can be treated with IFN-α. Treatment for HBV should be continued as described earlier.

Prevention

Vaccination for HBV can also prevent HDV infection. General prophylactic measures are essentially same as that for HBV.

HEPATITIS E VIRUS

Hepatitis E virus (HEV) causes an enterically transmitted hepatitis primarily occurring in young adults which occurs as epidemics in developing countries.

Morphology

Although HEV resembles caliciviruses, taxonomically, it is distinct from them; hence has been assigned to a unique genus, *Hepevirus*, under the family Hepeviridae.

❖ HEV is small (30–32 nm size), non-enveloped with icosahedral symmetry

❖ It contains positive-sense, ssRNA and a specific antigen (HEV-Ag)

❖ **Genotypes:** HEV has single serotype; however, five genotypes exist in nature, which differ up to 25% in their RNA sequence
- Only four genotypes have been detected in humans
- Genotypes 1 and 2 appear to be more virulent
- Genotypes 3 and 4 are more attenuated and account for subclinical infections.

Clinical Manifestation

Incubation period is about 14–60 days (average 40 days).

❖ Most of the patients present as self-limiting acute hepatitis lasting for several weeks followed by complete recovery

❖ Fulminant hepatitis may occur rarely in 1–2% of cases; except for the **pregnant women** who are particularly at higher risk **(20%)** of developing fulminant hepatitis

❖ There is no chronic infection or carrier state.

Epidemiology

Hepatitis E virus is a zoonotic pathogen affecting various animals such as monkeys, cats, pigs and dogs

❖ **Transmission:** It is fecal-orally transmitted via sewage contamination of drinking water or food

- **Epidemics** of HEV infections have been reported primarily from India, Asia, Africa and Central America; HEV is the most common cause of acute hepatitis in this zone
- Other parts of the world (temperate climate), HEV is uncommon and usually occurs in travelers coming from endemic zone. China reported more than 1 Lakh cases of jaundice during an outbreak in 1986–88
- **In India,** HEV infection accounts for maximum (30–60%) cases of sporadic acute hepatitis and epidemic hepatitis. The first major epidemic of HEV was reported from New Delhi (1995) where 30,000 people were affected due to sewage contamination of the city's drinking water supply following a flood that occurred in Yamuna river
- Though it resembles to HAV, the striking features that differentiate HEV from that of HAV are:
  - Secondary attack rate (transmission from infected persons to their close contacts) is rare (1–2%) in HEV, compared to 10–20% in HAV
  - Age: Young adults (20–40 years age) are commonly affected in HEV infection compared to children in HAV infection.

Laboratory Diagnosis

- **HEV RNA** (by reverse transcriptase PCR) and **HEV virions** (by electron microscopy) can be detected in stool and serum even before the onset of clinical illness
- **Serum antibody** detection by ELISA:
  - **IgM anti-HEV** appears in serum at the same time with the appearance of liver enzymes and indicates acute infection
  - **IgG anti-HEV** replaces IgM in 2 to 4 weeks (once the symptoms resolve) and persists for years; indicates recovery or past infection.

TREATMENT **Hepatitis E virus**

There is no specific antiviral drug available.

Prevention

General measures for prevention and containment of infection are the same as described for HAV. China has produced and licensed first HEV vaccine called **'HEV 239'** using recombinant HEV proteins. However, it is not yet available globally.

HEPATITIS G VIRUS

Hepatitis G virus (HGV, also referred to as GB virus C) was discovered in 1995.

- It is related to Hepatitis C virus, belongs to family Flaviviridae, under the genus *Pegivirus*
- HGV is transmitted by contaminated blood or blood products, or via sexual contact
- Hepatitis G virus is wrongly named as it is not hepatotropic and does not cause hepatitis. Instead, it replicates in the bone marrow and spleen
- HGV is associated with acute and chronic liver disease, but it has not been clearly implicated as an etiologic agent of hepatitis
- It has been classified into six genotypes, each has its own geographical distribution
- HGV co-infection is observed in 6% of chronic HBV and in 10% of chronic HCV infections
- **HIV co-infection:** HGV commonly co-infects people infected with HIV (prevalence 35%); but surprisingly this dual infection is **protective against HIV** and patients survive longer.

EXPECTED QUESTIONS

I. Essay:

1. Raju, a 40-year-old male presented with history of loss of appetite, malaise and jaundice of 2 months duration. On examination, there was icterus, hepatomegaly and tenderness in the right hypochondriac region. He gave a history of blood transfusion in the past. On laboratory examination, he was found to be positive for HBsAg.
   a. What is the most probable etiological diagnosis?
   b. Discuss in detail about the various laboratory diagnosis of this condition.
   c. How will you prevent the transmission of this infection?

II. Write short notes on:

a. Hepatitis A virus.
b. Laboratory diagnosis of hepatitis C virus.
c. Hepatitis D virus.
d. Hepatitis E virus.

III. Multiple Choice Questions (MCQs):

1. **Perinatal hepatitis B transmission is maximum in:**
   a. 1st trimester b. 2nd trimester
   c. 3rd trimester d. During delivery
2. **Which hepatitis virus is associated with highest mortality in pregnancy?**
   a. Hepatitis A b. Hepatitis B
   c. Hepatitis C d. Hepatitis E
3. **Hepatitis virus that spreads by fecal-oral route:**
   a. Hepatitis A b. Hepatitis B
   c. Hepatitis C d. Hepatitis D
4. **Which is known as Australia antigen?**
   a. HBsAg b. HBeAg
   c. HBcAg d. HBV DNA

5. **Acute hepatitis B is best diagnosed by:**
   a. HBsAg b. HBeAg
   c. IgM Anti HBc antibody
   d. HBcAg
6. **Hepatitis B vaccine should be given as per which schedule:**
   a. 0, 1, 6 days b. 0, 1, 6 weeks
   c. 0, 1, 6 months d. 0, 1, 6 years
7. **Which of the following hepatitis virus is non-enveloped?**
   a. HAV b. HBV
   c. HCV d. HDV
8. **Which of the following hepatitis virus is a DNA virus?**
   a. HAV b. HBV
   c. HCV d. HDV
9. **Which of the following is the most common cause of transfusion associated hepatitis?**
   a. HAV b. HBV
   c. HCV d. HDV
10. **Which of the following hepatitis virus is the most common cause of hepatocellular carcinoma?**
    a. HAV b. HBV
    c. HCV d. HDV
11. **Which of the following hepatitis virus is the most common cause of acute viral hepatitis in children?**
    a. HAV b. HBV
    c. HCV d. HDV
12. **Which of the following hepatitis virus has the maximum potential for chronicity?**
    a. HAV b. HBV
    c. HCV d. HDV
13. **Which of the following hepatitis virus is the most common cause of chronic hepatitis?**
    a. HAV b. HBV
    c. HCV d. HDV
14. **Which of the following genotype(s) of hepatitis B virus is more common in India?**
    a. A and B b. A and C
    c. A and D d. B and C
15. **Which of the following hepatitis virus, the genome has reverse transcriptase activity?**
    a. HAV b. HBV
    c. HCV d. HDV
16. **YMDD mutation in hepatitis B leads to development of resistance to which of the following drug?**
    a. Lamivudine b. Interferon alfa
    c. Ribavirin d. Zidovudine
17. **Hepatitis B virus remains infectious on environmental surfaces for at least___days?**
    a. 7 days b. 14 days
    c. 1 month d. Never
18. **Serological testing of a patient complaining of jaundice for 1 week revealed HBsAg (+ve), anti-HBs (-ve), anti-HBcIgM (+ve), HBeAg (+ve) and anti-HBe (-ve). The provisional diagnosis is:**
    a. Acute hepatitis with high infectivity
    b. Acute hepatitis with low infectivity
    c. Chronic hepatitis with high infectivity
    d. Chronic hepatitis with low infectivity
19. **Serological testing of a patient complaining of jaundice for 6 months revealed HBsAg (+ve), anti-HBs (-ve), anti-HBcIgG (+ve), HBeAg (+ve) and anti-HBe (-ve). The provisional diagnosis is:**
    a. Acute hepatitis with high infectivity
    b. Acute hepatitis with low infectivity
    c. Chronic hepatitis with high infectivity
    d. Chronic hepatitis with low infectivity
20. **In India, which HCV genotypes are more prevalent?**
    a. 1 and 3 b. 2 and 4
    c. 1 and 2 d. 1 and 4
21. **NS3/4A (proteases) inhibitors include all, *except*?**
    a. Grazoprevir b. Paritaprevir
    c. Simeprevir d. Daclatasvir
22. **In hepatitis D superinfection, which markers will be elevated?**
    a. Anti-HDV only
    b. HBsAg, anti-HBc IgG and anti-HDV
    c. Anti-HBc IgG and anti-HDV
    d. HBsAg, anti-HBc IgM and anti-HDV
23. **Which hepatitis virus dual infection is protective against HIV and patients survive longer?**
    a. HBV b. HCV
    c. HDV d. HGV
24. **Following hepatitis B vaccination, which of the following marker will rise?**
    a. HBsAg b. Anti-HBs
    c. Anti-HBc d. Anti-HBe
25. **In precore-mutant hepatitis B infection, which marker will be characteristically absent?**
    a. HBsAg b. Anti-HBc
    c. HBeAg d. HBV DNA
26. **In escape mutant hepatitis B infection, which marker will be characteristically absent?**
    a. HBsAg b. Anti-HBc
    c. HBeAg d. HBV DNA
27. **Presence of HBsAg, in the absence of any other marker indicates:**
    a. Acute hepatitis b. Chronic hepatitis
    c. Supercarrier d. Incubation period
28. **Currently, the first choice for treatment of hepatitis B infection is:**
    a. Tenofovir b. Lamivudine
    c. Interferon alfa d. Ribavirin
29. **In hepatitis B vaccination, non-responder is one who does not show seroconversion after___doses of vaccination.**
    a. 3 b. 4
    c. 6 d. 9

Answers

1. d **2.** d **3.** a **4.** a **5.** c **6.** c **7.** a **8.** b **9.** b **10.** b **11.** a **12.** c **13.** b **14.** c **15.** b **16.** a
17. a **18.** a **19.** c **20.** a **21.** d **22.** b **23.** d **24.** b **25.** c **26.** a **27.** d **28.** a **29.** c

Oncogenic Viruses

51
CHAPTER

Chapter Preview

- Viral oncogenesis
- Oncogenic RNA viruses
- Oncogenic DNA viruses

CLASSIFICATION

Viruses account for 15% of all human malignancies. There are several oncogenic viruses found worldwide, which include the agents of two major malignancies—Human papillomavirus causing carcinoma cervix and Hepatitis B virus causing liver cancer (Table 51.1).

In addition to the list of human oncogenic viruses given in Table 51.1, there are certain other viruses that can cause cancers in animals but not in humans such as:

- Poxviruses such as Yaba virus and rabbit fibroma viruses
- Adenoviruses (types 12, 19, 21) produce sarcomas in newborn rodents
- Herpesviruses:
  - Marek's disease virus
  - Lucke's frog tumor virus.
- Animal retroviruses:
  - Avian leukosis viruses (e.g., Rous sarcoma virus)
  - Murine leukosis viruses
  - Murine mammary tumor virus
  - Leukosis sarcoma virus of various animals.

Table 51.1: Human oncogenic viruses and associated malignancies

Virus Family	Human Cancer
DNA oncogenic viruses	
Papillomaviridae/ Polyomaviridae	
Human papillomaviruses	• Cervical carcinoma • Other genital tract carcinoma ➢ Anal ➢ Vulval/vaginal ➢ Penile • Esophageal carcinoma • Laryngeal carcinoma • Oropharyngeal carcinoma
Merkel cell virus	• Merkel cell carcinoma of skin
Herpesviridae	
Epstein-Barr virus	• Burkitt's lymphoma • Hodgkin's disease • Nasopharyngeal carcinoma • B cell lymphoma
Human herpesvirus-8	• Kaposi's sarcoma • Castleman's disease • Primary effusion lymphoma
Hepadnaviridae	
Hepatitis B	Hepatocellular carcinoma
RNA oncogenic viruses	
Retroviridae	
HTLV-I	Adult T cell leukemia/lymphoma
HIV	AIDS-related malignancies
Flaviviridae	
Hepatitis C	Hepatocellular carcinoma

Note: The association of Herpes simplex virus-2 with cervical cancer and Cytomegalovirus with prostate cancer have not been proved yet. Molluscum contagiosum virus is not an oncogenic virus as the lesion produced (molluscum contagiosum) is a benign condition.

VIRAL ONCOGENESIS

Before understanding the detailed mechanism of viral oncogenesis, knowledge about oncogenes and normal host genes regulating cellular growth is essential.

Oncogenes

Oncogenes encode certain proteins (oncoproteins) that trigger the transformation of normal cells into cancer cells.

- **V-onc (viral oncogenes):** Oncogenes present in the viral genome are called as **viral oncogenes (V-onc)**. They are essential for the replication of the virus. Viral oncogenes are expressed only by certain retroviruses (called as **acutely transforming retroviruses**)

- **C-onc (cellular oncogenes):** They are the cellular counter part of viral oncogenes present in the cancer cells
- **Proto-oncogenes:** They are the cellular counter part of viral oncogenes present in the normal host cells.

Genes Regulating Host Cell Growth

There are four categories of genes present in the host cell, which regulate the cellular growth and proliferation. Defect in any of these regulatory genes would lead to transformation of the normal host cells into abnormal tumor cells.

1. **Proto-oncogenes:** They promote the host cell growth and proliferation that are essential for life. However, over activation of proto-oncogenes may lead to transformation of host cells.
2. **Anti-oncogenes or tumor suppressor genes**: They continuously check cellular growth and proliferation, and supress any abnormal proliferation of cells. Inactivation of tumor suppressor genes permits the abnormal event to occur resulting in cell transformation.
3. **Apoptosis-regulatory genes:** They control the programmed cell death by either upregulating or downregulating apoptosis depending on the requirement. Hence, they may act as proto-oncogenes or tumor suppressor genes. Mutations in apoptosis-regulatory genes are another mechanism by which the cellular transformation is accelerated.
4. **DNA repair genes:** They are the normal host genes that repair any mutations occurring during the cell growth. Failure of DNA repair genes lead to inability to repair the damaged DNA and may lead to persistent mutation.

Events that Must Occur Before Oncogenesis

- **Establishing persistent infection:** Prolonged interaction between the tumor virus and the host cell is essential for oncogenesis to develop and this is possible only when the oncogenic virus establishes a long-term persistent infection in host cells
- **Evades host immune response:** Host immune response plays an important role in viral clearance. The oncogenic virus follows various evasion mechanisms to bypass the host immune response, which are as follows:
  - By restricting the expression of viral genes which go unnoticed by the immune cells [e.g. Epstein-Barr virus (EBV) in B cells]
  - Infecting the sites that are relatively inaccessible to immune responses [e.g. human papillomavirus (HPV) infecting epidermis]
  - Undergoing mutation of certain genes that allows the virus to escape from the host cellular and humoral responses (e.g. HIV)
  - Infection and suppression of essential immune cells (e.g. CD4 T cell by HIV).
- **Immunosuppression** of the host allows the cancer cells to proliferate and escape the host immune response. Immunosuppressed organ transplant recipients and HIV-infected individuals are at increased risk of EBV and HPV associated malignancies
- **Host cell susceptibility**: Host cells may be permissive or non-permissive for replication of a given virus
  - Permissive cells support viral growth and replication of a progeny virus; non-permissive cells do not
  - Non-permissive cells refer to the host cells that either do not have surface receptors for viral attachment or do not support the viral replication or the release of virus progeny
  - Host cells permissive for one virus may be non-permissive for another
  - Though oncogenicity can occur both in permissive and non-permissive cells, but the risk is more when a non-permissive cell is infected by a tumor virus as the virus tries different ways to maintain its survival in a non-permissive cell and by doing so it may undergo some changes, which makes the cell immortal
  - This holds true especially for DNA tumor viruses. In a permissive cell, the DNA tumor viruses are released by host cell lysis. Hence, the DNA tumor viruses are not oncogenic to a permissive cell, unless the viral replicative cycle that normally results in death of the host cell is blocked in some way; and grow indefinitely
  - In contrast, RNA tumor viruses do not cause cell lysis, hence, they can be oncogenic to both permissive and non-permissive cells.
- **Retention of viral nucleic acid inside the host cells** is essential to maintain a stable genetic change that occurs in a tumor cell
  - The DNA copies of DNA tumor viruses are integrated within the host cell chromosome
  - RNA of retroviruses gets reverse transcribed into DNA
  - Hepatitis C virus is an exception, its RNA is neither reverse transcribed, nor integrated into the host chromosome; but are maintained in the tumor cells.

Mechanism of Viral Oncogenesis

Viral oncogenesis is a complex and multistep process requiring prolonged time (years to decades) and occur only in a small percentage of the infected individuals. There are multiple oncogenic events that take place to transform the host cells into cancer cells. Viruses contribute to only a portion of those oncogenic events. In addition, other factors are necessary such as host immunity and host genetic susceptibility, etc.

Oncogenic viruses transform the host cells into tumor cells mainly by two broad mechanisms (see the box below).

1. **Direct-acting oncogenic viruses**: Certain animal retroviruses (called as **acute transforming retroviruses**) possess viral oncogenes (*V-onc*), which they directly insert into the host cell chromosomes.
2. **Indirect-acting oncogenic viruses:** Most of the human oncogenic viruses possess certain transforming genes, which they insert into the host DNA leading to altered expression of pre-existing cellular genes (which regulate host cell growth) such as:
   - Proto-oncogenes
   - Tumor suppressor gene
   - Apoptosis regulatory genes
   - DNA repair genes.

ONCOGENIC RNA VIRUSES

Retrovirus

Retroviruses possess two copies of ssRNA that get reverse transcribed to DNA (proviral DNA) and get inserted into host chromosome:

- The proviral DNA of any retrovirus contains three important genes—*gag, pol,* and *env* in that order from the 5′ to the 3′ end with long terminal repeat (LTR) sequences present at either ends (Fig. 51.1)
- The LTRs exert regulatory control on the provirus gene functions and are linked directly to the host DNA
- In addition, certain retroviruses possess additional genes. The acutely transforming oncogenic retroviruses possess viral oncogenes (*V.onc*). The slow transforming oncogenic retroviruses possess additional regulatory gene (e.g. *tax* gene for HTLV-1 and *tat* gene for HIV)
- Oncogenic retroviruses belong to exogenous type; (i.e. spread horizontally between host cells). Endogenous retroviruses spread vertically from parent host cells to offspring) and they are mostly non-pathogens.

Oncogenicity of HTLV-I

Oncogenicity of HTLV-I are locked in the *tax* gene. It is transcription activator gene, essential for viral replication. At the same time, it modulates the host cell functions as well.

Oncogenic Retroviruses

They are classified into two groups:

1. Acute transforming or direct acting retroviruses:

They are certain animal retroviruses (e.g. Rous sarcoma virus) that carry **viral oncogene** which they directly insert into host chromosome.

- They are highly oncogenic and cause malignancy faster (within weeks or months)
- They can cause different types of malignancies such as, sarcoma, carcinoma, leukemia
- They are capable of transforming cells in culture as well
- **Replication defective:** Most acute transforming retroviruses are unable to replicate normally because the viral oncogene replaces some of the essential genes for viral replication. They require a standard helper retrovirus to replicate in host cells. Rous sarcoma virus is an exception as it is **replication competent**, contain full-length genome and replicate normally in the host cells.

2. Slow transforming or indirect acting retroviruses:

Most human oncogenic retroviruses, such as HTLV lare slow transforming viruses.

- They are **replication competent**, but replicate slowly
- Require a long latent period to develop malignancy
- Viral genome can insert anywhere in the host chromosomes randomly and not necessarily adjacent to proto-oncogenes
- **Low oncogenic potential:** They do not have viral oncogenes, but possess an additional regulatory gene (e.g. *tax* gene for HTLV-I)
- They have restricted tissue tropism for malignancies; induce malignant change only of blood cells
- They do not transform cultured cells.

- *Tax* gene is capable of activating the transcription of several cellular genes involved in T cells proliferation. These include:
  - Genes coding interleukin-2 (IL-2) and its receptor
  - Gene for myeloid growth factor, granulocyte-macrophage colony-stimulating factor.
- **Inhibit cell growth cycle:** *Tax* protein inactivates the cell cycle inhibitor p16/INK4a and activates cyclin D (a cell cycle enhancer), thus promoting the host cell growth cycle by accelerating the transition between G_1 and S phase
- *Tax* gene activates nuclear factor κβ (NF-κβ), a transcription factor that regulates certain host anti-apoptotic genes

Fig. 51.1: Proviral DNA of retrovirus

- *Tax* gene also interferes with **DNA-repair pathways** (base excision repair and nucleotide excision repair) which leads to sustained DNA mutation.

Hepatitis C

Hepatitis C is the only oncogenic virus that does not get integrated with host chromosome but its RNA remains in the host cell. It is also strongly linked to the pathogenesis of liver cancer. The oncogenic mechanisms of HCV are less well defined than those of HBV.

- Similar to HBV, chronic liver cell injury and compensatory regeneration seems to be the main mechanism
- In addition, components of the HCV genome, such as the HCV core protein, may activate a number of growth-promoting signal transduction pathways.

ONCOGENIC DNA VIRUSES

Epstein-Barr Virus

Epstein-Barr virus (EBV) is associated with several malignancies:

- Burkitt's lymphoma (tumor of jaw, mostly seen in African children)
- Nasopharyngeal carcinoma
- Hodgkin's lymphoma (mixed-cellularity type)
- Non-Hodgkin lymphoma (NHL).

Mechanism of Oncogenesis

Epstein-Barr virus infects B lymphocytes and possibly pharyngeal epithelial cells by attaching to the complement receptor (CR2) or CD21.

- EBV does not actively replicate inside the B cells thus does not cause lysis of B cells, but such latently infected B cells with EBV become immortalized and acquire the ability to grow indefinitely in cell lines
- Persistent EBV infection can induce malignant transformation of infected B cells and epithelial cells by expressing latent EBV antigens such as latent membrane protein (LMP) and EBNA (EBV nuclear antigen)
- Latent membrane protein-1 **(LMP-1)** is the most important viral oncogene
  - It is coated on the surface of the infected cells and behaves as active CD40 receptor, a key recipient of helper T-cell signals that stimulate B-cell growth
  - LMP-1 also activates the NF- κβ and JAK/STAT signalling pathways and promotes B-cell survival and proliferation
  - LMP-1 prevents apoptosis by activating anti-apoptotic factor BCL2
  - It induces the expression of pro-angiogenic factors such as vascular endothelial growth factor (VEGF) which may contribute to the oncogenesis of nasopharyngeal carcinoma
- **Viral EBNA-2** activates host cell **cyclin-D**, and the proto-oncogene *src*, thus promotes cell proliferation
- **VIL-10 (viral interleukin 10):** It is a viral cytokine, which modulates the transformation of B cells.

Role of Host Immune Response and c-MYC

- Effective host immune response is crucial for preventing cell transformation. Oncogenicity is kept under control by anti-LMP-1 antibodies
- Thus, oncogenicity is markedly enhanced in immunosuppressed individuals who are not able to produce anti-LMP-1 antibodies
- More so, B cells in immunocompetent individuals can still undergo malignant transformation in presence of another pre-existing mutation (8:14) that in turn activates the growth promoting MYC oncogene.

Human Papillomavirus

More than 100 types of human papillomaviruses have been recognized. However, certain types (e.g. 16 and 18 being most common; followed by 31, 33, 35, 39, 45, 51–53, 56, 58, 59, 66, 68, 73 and 82) have high oncogenic potential. They are associated with important malignancies such as:

- Squamous cell carcinomas of cervix
- Carcinoma of other genital mucosa (penis, vulva, vagina)
- Oropharyngeal carcinoma
- Laryngeal carcinoma
- Carcinoma of esophagus.

Mechanism of Oncogenesis (Fig. 51.2)

Human papillomavirus genome consists of an early (E) region, a late (L) region. The early region consists of seven genes (E1–E7), which code for early non-structural proteins. Products of early genes E6 and E7 have oncogenic potential.

- E6 enhances p53 degradation, thus inhibiting the activation of apoptosis promoting gene *bax*. It leads to inhibition of apoptosis and also inhibition of the p53 induced activation of tumor suppressor gene p 21

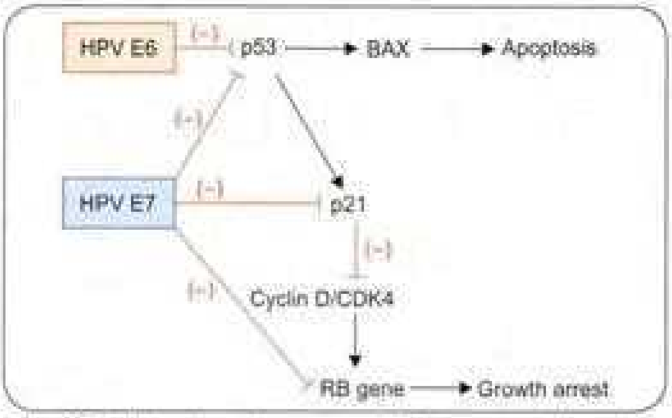


Fig. 51.2: Mechanism of oncogenesis by human papillomavirus

- E7 inhibits the tumor suppressor gene RB (retinoblastoma gene) either by:
  - Inhibiting p53
  - Inhibiting p21
  - Directly Inhibiting RB gene.

However, HPV infection alone is not sufficient for carcinogenesis. Co-transfection with *v-fos* DNA results in full malignant transformation. There are several other factors that also have been implicated in the pathogenesis of HPV-induced malignancies such as:

- Presence of other genetic co-factors like mutated *RAS* gene
- Cigarette smoking
- Coexisting microbial infections
- Dietary deficiencies
- Hormonal changes.

Hepatitis B Virus

Hepatitis B virus (HBV), in adjunction with hepatitis C is responsible for 70-85% of hepatocellular carcinomas worldwide.

Mechanism of Oncogenesis

Although not fully elucidated, there are several mechanisms proposed for the oncogenesis of HBV. The HBV genome does not contain any oncogenes, however; it gets integrated with the host genome randomly in the target cells.

- **Immunologically mediated chronic inflammation** appears to be the most dominant mechanism in the pathogenesis of viral-induced hepatocellular carcinoma
  - In chronic viral infection, hepatocellular injury occurs which is compensated by proliferation of hepatocytes. During the regenerative process, a plethora of growth factors, cytokines, chemokines, and other bioactive substances are produced by the activated immune cells which promote cell survival, tissue remodeling and angiogenesis
  - The activated immune cells also produce reactive oxygen species, that are genotoxic and mutagenic
  - One key molecular step seems to be activation of the NF-κβ pathway in hepatocytes which in turn blocks apoptosis, allowing the dividing hepatocytes to incur genotoxic stress and to accumulate mutations.
- **Hepatitis B X gene (HBx),** a regulatory gene in HBV genome, can activate the transcription of cellular and viral genes
- **Deletion of tumor suppressor genes**: Integration of viral DNA with the host genome can cause secondary rearrangements of chromosomes which may lead to deletion of tumor suppressor genes.

Kaposi's Sarcoma

Kaposi's sarcoma is caused by human herpesvirus 8 (HHV8). It usually infects the endothelial cells and/or hematopoietic progenitor cells.

- The transformation of malignant cells is directly related to the expression of early lytic genes of HHV-8 such as viral G protein-coupled receptor K1, **viral interleukin-6 (vIL-6)** and K15
- These genes induce the host cells to secrete the angiogenic, inflammatory and proliferative factors such as; vascular endothelial growth factor (VEGF), platelet-derived growth factor-β, angiopoietin 2, IL-6 and IL-8 that amount to continuous growth and transformation of cells.

EXPECTED QUESTIONS

I. Write short notes on:

1. List the human oncogenic viruses.
2. Mechanism of oncogenesis of HTLV-I.
3. Mechanism of oncogenesis of HPV.
4. Mechanism of oncogenesis of EBV.

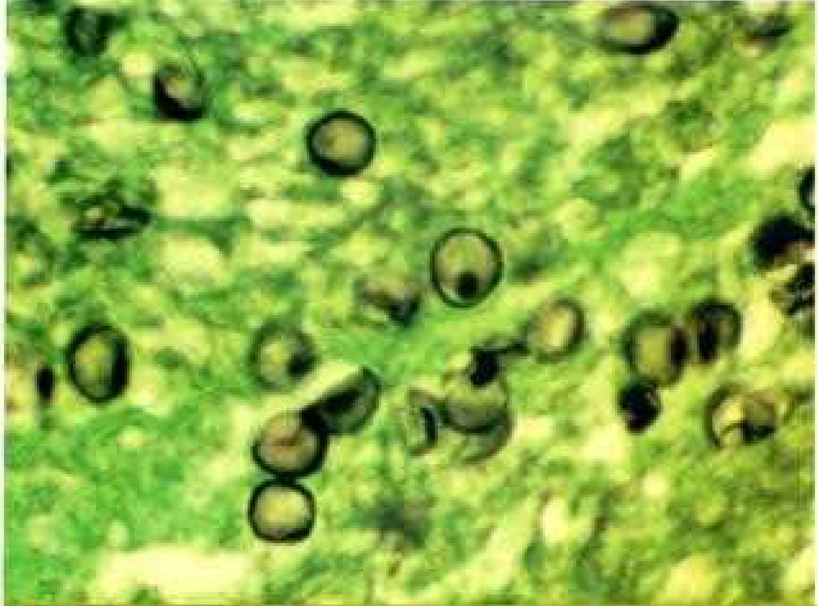
II. Multiple Choice Questions (MCQs):

1. Which of the following is not an oncogenic virus?
a. Hepatitis B virus b. Hepatitis C virus
c. HIV d. Varicella-zoster virus

2. Epstein-Barr virus is associated with the following malignancies except:
a. Nasopharyngeal carcinoma
b. Burkitt's lymphoma
c. Carcinoma of cervix
d. Non Hodgkin lymphoma

3. All of the following are oncogenic RNA viruses except:
a. HTLV-I b. HIV
c. Hepatitis B virus d. Hepatitis C virus

Answers
1. d **2.** c **3.** c



Mycology

SECTION 5

Section Outline

Medical Mycology

52
CHAPTER

Chapter Preview

- General mycology
  - Classification
  - Laboratory diagnosis
- Superficial mycoses
  - Tinea versicolor
  - Tinea nigra
  - Piedra
  - Dermatophytosis
- Subcutaneous mycoses
  - Mycetoma
  - Sporotrichosis
  - Chromoblastomycosis
  - Rhinosporidiosis
  - Phaeohyphomycosis
- Systemic mycoses
  - Histoplasmosis
  - Blastomycosis
  - Coccidioidomycosis
  - Paracoccidioidomycosis
- Opportunistic mycoses
  - Candidiasis
  - Cryptococcosis
  - Zygomycosis
  - Aspergillosis
  - Penicilliosis
  - *Pneumocystis* pneumonia
  - Fusariosis
- Mycotoxicoses
  - Mycotoxicosis
  - Mycetism

GENERAL MYCOLOGY

Medical mycology is the branch of medical science that deals with the study of medically important fungi. The name 'fungus' is derived from Greek '*mykes*' meaning mushroom (a type of edible fungus). Fungi differ from bacteria and other eukaryotes in many ways.

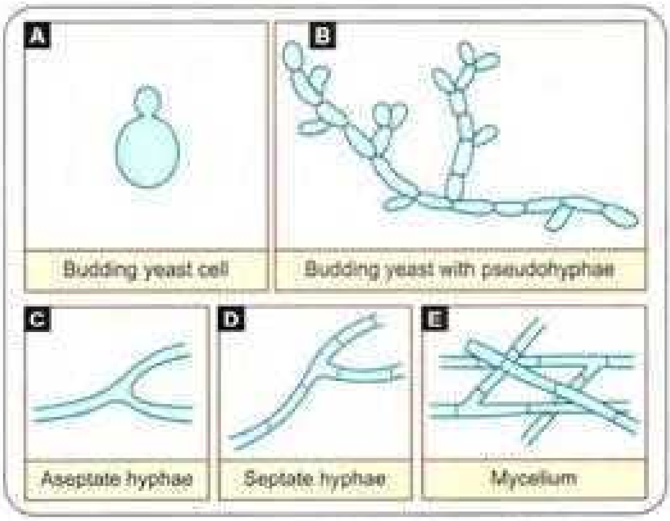
- Fungi are eukaryotic and they possess all the eukaryotic cell organelles such as mitochondria
- They possess a rigid cell wall, composed of chitin, β-glucans and other polysaccharides
- Fungal cell membrane contains ergosterol instead of cholesterol
- Fungi may be unicellular or multicellular
- They lack chlorophyll and divide by asexual and/or sexual means by producing spores.

CLASSIFICATION OF FUNGI

Morphological Classification

Based on the morphological appearance, there are four main groups of fungi given as follows: (Fig. 52.1):

1. **Yeast:** They grow as round to oval cells that reproduce by an asexual process called **budding** in which cells form protuberances which enlarge and eventually separate from the parent cells. Examples include:
   - *Cryptococcus neoformans* (pathogenic)
   - *Saccharomyces cerevisiae* (non-pathogenic).
2. **Yeast-like:** In some yeasts (e.g. *Candida*), the bud remains attached to the mother cell, elongates and undergoes repeated budding to form chains of elongated cells known as **pseudohyphae**. They can be differentiated from true hyphae as they have constrictions at the septa.
3. **Molds:** They grow as long branching filaments of 2–10 µm width called **hyphae**.
   - Hyphae are either septate (i.e. form transverse walls) or nonseptate (there are no transverse walls and they are multinucleated, i.e. coenocytic)
   - Hyphae grow continuously and form a branching tangled mass of growth called **mycelium**



Figs 52.1A to E: Morphological forms of fungi

- Based on the growth pattern in culture medium, the mycelia may be categorized into two types:
  1. **Aerial mycelium:** It is the part of the mycelium which projects above the surface of culture medium.
  2. **Vegetative mycelium:** It is the part of the mycelium that grows on the surface of the culture medium.
- Molds reproduce by formation of different types of sexual and asexual spores
- Examples of true molds include—Dermatophytes, *Aspergillus, Penicillium, Rhizopus, Mucor*, etc.

4. **Dimorphic fungi:** They exist as molds (hyphal form) in the environment at ambient temperature (25°C) and as yeasts in human tissues at body temperature (37°C). Several medically important fungi are thermally dimorphic such as:
   - *Histoplasma capsulatum*
   - *Blastomyces dermatitidis*
   - *Coccidioides immitis*
   - *Paracoccidioides brasiliensis*
   - *Penicillium marneffei*
   - *Sporothrix schenckii.*

Taxonomical Classification

Based on the production of sexual spores, the Kingdom Fungi has been divided into four medically important phyla. They are as follows:

1. Phylum zygomycota: They are lower fungi, produce sexual spores known as **zygospores** and possess aseptate hyphae, e.g. *Rhizopus* and *Mucor*.
2. Phylum ascomycota: They produce sexual spores known as **ascospores** and possess septate hyphae, e.g. *Aspergillus*.
3. Phylum basidiomycota: They produce sexual spores known as **basidiospore** e.g. *Cryptococcus*.
4. Phylum deuteromycota **(Fungi imperfecti):** In majority of the medically important fungi, the sexual state is either absent or unidentified yet. Hence, they are traditionally grouped as fungi imperfecti.

Types of fungal spores produced are given in Table 52.1.

Table 52.1: Types of fungal spores

Sexual spore	Observed in
Zygospores	*Zygomycetes*
Ascospores	*Aspergillus*
Basidiospores	*Cryptococcus*
Asexual spore	**Observed in**
Vegetative asexual spore	
Arthrospore	*Coccidioides and Trichosporon*
Blastospore	*Candida*
Chlamydospore	*Candida albicans*
Aerial asexual spore	
Conidiospore or conidia	*Aspergillus*
Sporangiospore	Zygomycetes
Microconidia	Dermatophytes
Macroconidia	Dermatophytes

CLASSIFICATION OF FUNGAL DISEASES

Although more than 25,000 species of fungi are known, most of them are saprophytes in soil and decaying plant materials. Only few are medically important. Fungal infections (or mycoses) can be categorized into the following clinical types (Table 52.2):

- **Superficial mycoses:** These are the fungal infections involving the skin, hair, nail and mucosa
- **Subcutaneous mycoses:** These are the mycotic infections of the skin, subcutaneous tissue and sometimes bone, resulting from inoculation of saprophytic fungi of soil or decaying matter. They are mainly confined to the tropics and subtropics
- **Systemic mycoses:** They involve multiple organs. Mostly they are caused by the saprophytic fungi, which spread by inhalation of spores leading to pulmonary infection. From lungs, they disseminate to cause various systemic manifestations
- **Opportunistic mycoses:** They are caused by the fungi that are normally found as human commensals or in environment; but can act as human pathogen in presence of opportunities such as low immunity

Table 52.2: Classification of fungal diseases

Fungal disease	Agents
Superficial mycoses*	
Tinea versicolor	*Malassezia furfur*
Tinea nigra	*Hortaea werneckii*
Piedra	*Trichosporon beigelii* *Piedraia hortae*
Dermatophytosis	*Trichophyton* *Microsporum* *Epidermophyton*
Subcutaneous mycoses	
Mycetoma	*Madurella mycetomatis, Pseudallescheria boydii* Others
Sporotrichosis	*Sporothrix schenckii*
Chromoblastomycosis	*Phialophora verrucosa* *Fonsecaea pedrosoi*
Rhinosporidiosis	*Rhinosporidium seeberi*
Systemic mycoses*	
Histoplasmosis	*Histoplasma capsulatum*

Contd...

Contd...

Blastomycosis	*Blastomyces dermatitidis*
Coccidioidomycosis	*Coccidioides immitis*
Paracoccidioidomycosis	*Paracoccidioides brasiliensis*
Opportunistic mycoses	
Candidiasis	*Candida albicans* Other *Candida* species
Cryptococcosis	*Cryptococcus neoformans*
Zygomycosis	Mucorales • *Rhizopus* • *Mucor* • *Absidia* (new name *Lichtheimia*) Entomophthorales** • *Basidiobolus ranarum* • *Conidiobolus coronatus*
Aspergillosis	*Aspergillus flavus* *Aspergillus fumigatus* *Aspergillus niger*
Penicilliosis	*Penicillium marneffei* (new name *Talaromyces marneffei*) Other *Penicillium* species
Pneumocystosis	*Pneumocystis jirovecii*
Fusariosis	*Fusarium* species
Mycotoxicoses	Fungi producing toxins (see Table 52.12)

*Superficial and systemic manifestations are also seen in candidiasis, cryptococcosis, aspergillosis and zygomycosis

**Entomophthorales cause subcutaneous infection.

❖ **Mycotoxicoses:** They refer to the manifestations produced due to direct ingestion of fungal toxins.

LABORATORY DIAGNOSIS OF FUNGAL DISEASES

The laboratory diagnosis of fungal diseases comprises of the following:

Specimen Collection

It depends on the site of infection such as skin scraping, hair, nail, sputum, etc. For systemic mycoses, blood sample may also be collected. Cerebrospinal fluid (CSF) is collected for cryptococcal meningitis.

Microscopy

Fungal elements can be detected in the clinical specimens by direct microscopic examination of material from the lesion.

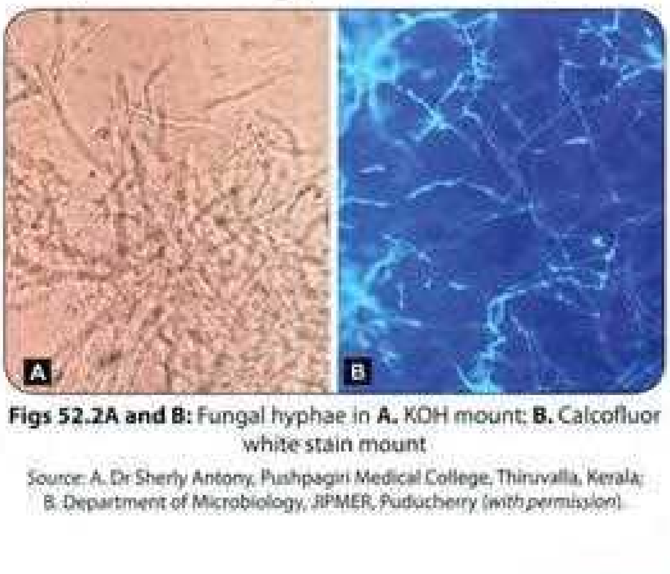
❖ **Potassium hydroxide (KOH) preparation**: Keratinized tissue specimens such as skin scrapings and plucked hair samples are treated with 10% KOH which digests the keratin material so that the fungal hyphae will be clearly seen under the microscope. Heat the slide gently over the flame and leave it aside for 5–10 minutes before examination (Fig. 52.2A)
  - About 10% is the usual concentration of KOH used
  - About 20–40% KOH is needed for the specimens such as nail that otherwise takes longer time to dissolve
  - Biopsy specimens as they take longer time to dissolve, are usually dissolved in 10% KOH in a test tube and examined after overnight incubation
  - Glycerol (10%) can be added to prevent drying
  - DMSO (dimethyl sulfoxide) can be added to help in tissue digestion
  - Caution should be maintained while interpretation of hyphae, which may be confused with collagen fiber, cotton fiber or hair present in the clinical specimens.

❖ **Gram stain:** It is useful in identifying the yeasts (e.g. *Cryptococcus*) and yeast like fungi (e.g. *Candida*). They appear as gram-positive budding yeast cells (see Fig. 52.20A)

❖ **India ink and nigrosin stains:** They are used as negative stains for demonstration of capsule of *Cryptococcus neoformans* (see Fig. 52.21A)

❖ **Calcofluor white stain:** It is more sensitive than other stains; binds to cellulose and chitin of fungal cell wall and fluoresce under UV light (Fig. 52.2B)

❖ **Histopathological stains:** They are useful for demonstrating fungal elements from biopsy tissues. This is useful for detecting invasive fungal infection
  - Periodic acid schiff (PAS) stain: It is the recommended stain for detecting fungi. PAS positive fungi appear magenta/deep pink, whereas the nuclei stain blue
  - Gomori methenamine silver (GMS) stain: It is used as an alternative to PAS for detecting fungi. It stains both live and dead fungi, as compared to PAS which stains only the live fungi. GMS stains the polysaccharide component of the cell wall. Fungi appear black whereas the background tissue takes pale green color (see Figs 52.23 and 52.32)
  - Mucicarmine stain: It is used for staining the carminophilic cell wall of *Cryptococcus* and *Rhinosporidium*



Figs 52.2A and B: Fungal hyphae in **A.** KOH mount; **B.** Calcofluor white stain mount

Source: A. Dr Sherly Antony, Pushpagiri Medical College, Thiruvalla, Kerala; B. Department of Microbiology, JIPMER, Puducherry (*with permission*).

- Masson fontana stain: It is used for pigmented (or pheoid) fungi
- Hematoxylin and Eosin (H and E) stain.

❖ **Lactophenol cotton blue (LPCB):** It is used to study the microscopic appearance of the fungal isolates grown in culture. It contains:
- Phenol acts as disinfectant
- Lactic acid preserves the morphology of fungi
- Glycerol prevents drying
- Cotton blue stains the fungal elements blue.

Culture

Fungal culture is frequently performed for isolation and correct identification of the fungi.

Culture Media

❖ **Sabouraud's dextrose agar (SDA):** It is the most commonly used medium in diagnostic mycology. It contains peptone (1%), dextrose (4%) and has a pH of 5.6. This may not support some pathogenic fungi

❖ **Neutral SDA** (Emmons' modification): It differs from original SDA in having neopeptone (1%) and dextrose (2%) and pH of 7.2

❖ **Corn meal agar and rice starch agar:** They are the nutritionally deficient media used for stimulation of chlamydospore production

❖ **Brain heart infusion (BHI) agar and blood agar:** They are the enriched media, used for growing fastidious fungi like *Cryptococcus* and *Histoplasma*

❖ **Niger seed agar** and **bird seed agar:** They are used for the selective growth of *Cryptococcus*

❖ **CHROMagar *Candida* medium:** It is used as selective as well as differential medium for speciation of *Candida*.

Culture Condition

❖ **Temperature:** Most of the fungi grow well at 25–30°C except the dimorphic fungi that grow at both 25°C and 37°C

❖ **BOD incubators** (biological oxygen demand): It is a special incubator used in diagnostic mycology, which is capable of maintaining low temperature

❖ **Incubation time:** Culture plates should be incubated for 2–3 weeks

❖ **Antibiotics** such as cycloheximide (actidione), chloramphenicol and gentamicin can be added to the culture media to inhibit bacterial growth.

Culture Identification

The correct identification of the fungus is based on the macroscopic appearance of the colonies grown on culture and microscopic appearance (LPCB mount of colonies).

Macroscopic Appearance of the Colony

❖ **Rate of growth:**
- Rapid growth (<5 days): It is seen in saprophytes, yeasts and agents of opportunistic mycoses
- Slow growth (1–4 weeks): It is observed in dermatophytes, agents of subcutaneous and systemic mycoses.

❖ **Pigmentation:** It can be seen on the reverse side of the culture media

❖ **Texture:** It refers to how the colony would have felt if allowed to touch. It may be of various types such as—glabrous (waxy/leathery), velvety, yeast like, cottony or granular/powdery

❖ **Colony topography:** Colony surface may be rugose (radial grooves), folded, verrucose or cerebriform (brain-like).

Microscopic Appearance of Fungi

❖ **Teased mount:** A bit of fungal colony is teased out from the culture tube and the LPCB mount is made on a slide and viewed under microscope. If proper teasing is not done, then the intact morphology may not be identified properly. Identification is based on the following:
- Nature of hyphae (such as septate or aseptate, hyaline or phaeoid, narrow or wide) and
- Type of sporulation (conidia or sporangia).

❖ **Slide culture:** Though this is a tedious procedure, it gives the most accurate *in situ* microscopic appearance of the fungal colony. A sterile slide is placed on a bent glass rod in a sterile petri dish. Two square agar blocks measuring around 1 cm^2 (smaller than the coverslip) are placed on the slide. Bits of fungal colony are inoculated onto the margins (at the center) of the agar block. Then the coverslip is placed on the agar block and the petri dish is incubated at 25°C. After sufficient growth occurs, LPCB mounts are made both from the coverslip and the underneath slide (Fig. 52.3)

❖ **Cellophane tape mount:** The impressions are taken by placing the cellophane tape on the colonies present on the surface of SDA plate, then LPCB mount is made from the cellophane tape. This is easy to perform than slide culture and *in-situ* fungal morphology is also maintained.

Other Methods of Identification

❖ **For *Candida*:** Germ tube test, Dalmau plate culture, carbohydrate fermentation and carbohydrate assimilation tests are done

❖ **For dermatophytes:** Hair perforation test, dermatophyte test medium and dermatophyte identification medium are used

❖ Urease test can be done for the fungi that produce urease enzyme, e.g. *Cryptococcus*.

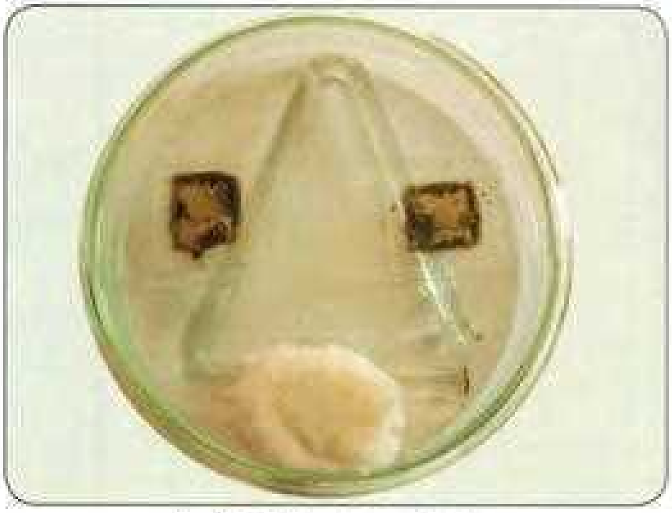


Fig. 52.3: Slide culture technique

Source: Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (with permission).

Immunological Methods

These tests are available to detect the antibody or antigen from serum and/or other body fluids.

- **Antibody detection** can be done by ELISA, immunodiffusion test, agglutination test, and complement fixation test (CFT)
- **Antigen detection:** Example includes latex agglutination test for detecting cryptococcal antigen from CSF
- **Immunohistochemistry:** It refers to detecting antigens (e.g. proteins) on the cells of a tissue section by using fluorescent tagged antibodies that bind specifically to the antigens. It is useful in deep mycoses.

Tests for Metabolites

An alternative approach for the diagnosis of fungal infections is detection of specific fungal metabolites in body fluids by gas liquid chromatography.

Tests to Demonstrate Delayed Hypersensitivity

Skin tests are available for demonstrating delayed type of hypersensitivity for pathogens like *Histoplasma, Blastomyces, Coccidioides, Paracoccidioides, Dermatophyte, Sporothrix* and *Candida*.

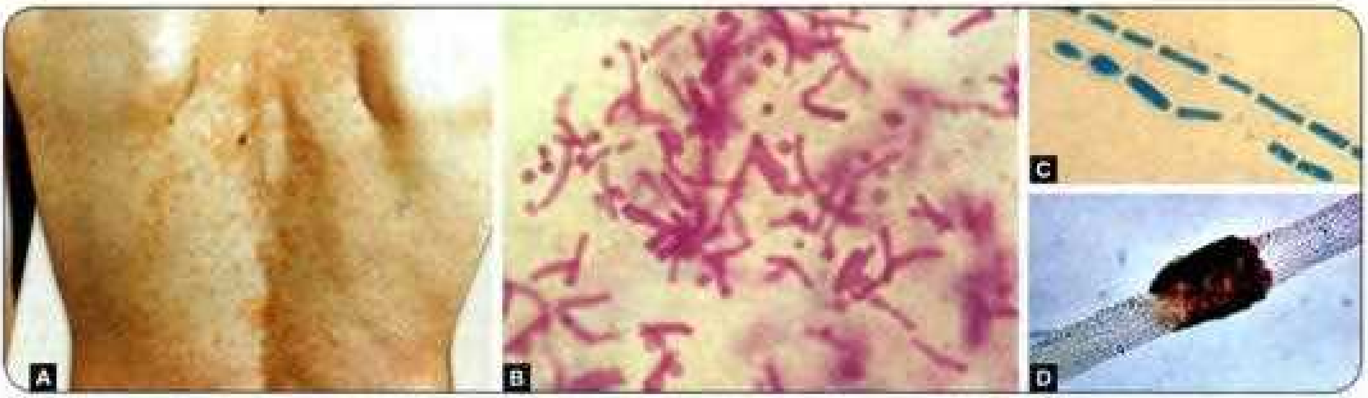
Molecular Methods

Polymerase chain reaction (PCR) and its modifications such as multiplex PCR, nested PCR and the most advanced real time PCR and DNA sequencing methods have been developed for accurate identification of fungi from culture as well as from the specimens.

Antifungal agents, their mechanisms of action and their use have been described in Table 52.3.

Table 52.3: Antifungal agents, their mechanisms of action and uses

Class	Drug	Use	Mechanisms
Antifungal antibiotics			
Polyenes	Amphotericin B	Systemic mycoses (all invasive fungal infections)	Bind to ergosterol and disrupts fungal cell membranes
	Nystatin, hamycin	Topical use (skin infection)	
Echinocandins	Caspofungin, micafungin, anidulafungin	Systemic mycoses mainly due to Candida and *Aspergillus*	Inhibits β-glucan synthesis in fungal cell wall
Benzofurans	Griseofulvin	Dermatophytoses	Disrupts mitotic spindle by binding to fungal cell tubulin
Synthetic antifungal agents			
Azoles	Imidazoles: Clotrimazole, miconazole, ketoconazole, oxiconazole	Topical use (except, ketoconazole can be used for both topical and systemic use)	Inhibits ergosterol synthesis of fungi
	Triazoles	Systemic mycoses (invasive fungal infections)	
	Fluconazole	*Cryptococcus and Candida* (except *C. krusei, C. glabrata*)	
	Itraconazole	Sporothrix, chromoblastomycosis, dermatophytes, *Cryptococcus, Candida* (except *C. krusei, C. glabrata*) and *Trichosporon*	
	Voriconazole	All invasive fungal infections except mucormycosis	
	Posaconazole and isavuconazole	All invasive fungal infections including mucormycosis	
Antimetabolite	Flucytosine (5-FC) (converted to fluorouracil inside body)	Systemic mycoses Used in combination with amphotericin B.	Fluorouracil inhibits thymidylate synthetase, thus inhibiting DNA synthesis
Allylamines	Terbinafine	Topical use	Inhibits ergosterol synthesis
Other topical agents	Tolnaftate, Benzoic acid, Whitfield's ointment, Undecylenic acid, Ciclopirox olamine		



Figs 52.4A to D: A. Tinea versicolor (hypopigmented patches); **B.** *Malassezia furfur* (yeast cells and hyphae with spaghetti and meatballs appearance); **C** and **D.** Piedra; **C.** Arthrospores of *Trichosporon beigelii* (white piedra); **D.** Black nodule on hair shaft (black piedra)

Source: Public Health Image Library/Dr. Lucille KG A. ID# 12534; B. ID# 2916; C. ID# 3936 and D. ID#3937/Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

SUPERFICIAL MYCOSES

TINEA VERSICOLOR

Tinea versicolor (or pityriasis versicolor) is a chronic recurrent condition involving the superficial layer (stratum corneum) of skin, caused by a lipophilic fungus *Malassezia furfur*.

Clinical Manifestation

It is characterized by flat-round scaly patches of hypo- to hyperpigmentation of skin (Fig. 52.4A).

- Lesions are non-inflammatory and non-pruritic (or rarely pruritic)
- Lesions can be mistaken for vitiligo, but the latter is not scaly
- Areas rich in sebaceous glands are commonly involved such as neck, chest, or upper arms
- Disease is more common in moist humid areas.

Other manifestations caused by *Malassezia furfur* include:

- **Seborrheic dermatitis:** It manifests as erythematous pruritic scaly lesions called **dandruff in adults** and cradle cap in babies. It can be severe in patients with advanced AIDS
- Atopic dermatitis
- Folliculitis (hair follicle infection)
- Disseminated infection can occur rarely.

Laboratory Diagnosis

Diagnosis of tinea versicolor is largely made clinically. The laboratory diagnostic methods are as follows:

- **Direct microscopy:** Skin scrapings are examined microscopically after treating with 10% KOH. Mixture of budding yeasts and short septate hyphae are seen, described as **spaghetti and meatballs** appearance (Fig. 52.4B)
- **Culture:** *Malassezia furfur* being lipophilic, SDA with olive oil overlay is the ideal media for culture. Typical '**fried egg**' colonies appear after incubating for 5–7 days at 32–35°C
- **Urease test:** It gives a positive urease test
- **Wood's lamp examination:** Under Wood's lamp, the scaly lesions show golden yellow fluorescence.

TREATMENT **Tinea versicolor**

Topical lotions like selenium sulfide shampoo, ketoconazole shampoo or cream, terbinafine cream should be used for 2 weeks.

TINEA NIGRA

It is characterized by painless, black, non-scaly patches present on palm and sole; more commonly in females. It is caused by *Hortaea werneckii*. It is a black-colored yeast like fungus.

PIEDRA

Piedra is characterized by nodule formation on hair shaft, which may be either black or white in color.

White Piedra

Here, white nodules are formed on the hair shaft, which are less firmly attached.

- **Agent:** *Trichosporon beigelii*
- **Identifying feature:** *T. beigelii* is an urease positive, yeast like fungus; produces creamy white colonies, containing hyaline septate hyphae intervening with rectangular **arthrospores** (Fig. 52.4C).

Black Piedra

It is characterized by formation of black nodules, which are firmly attached to the hair shaft (Fig. 52.4D).

- **Agent:** *Piedraia hortae*
- **Identifying feature:** It is a phaeoid fungus; produces reddish brown colonies; containing dark brown thick septate hyphae with ascus containing ascospores.

DERMATOPHYTOSES

Dermatophytoses (or tinea or ringworm) is the most common superficial mycoses affecting skin, hair and nail; caused by a group of related fungi (called **dermatophytes**) that are capable of infecting keratinized tissues. These include:

- ***Trichophyton* species:** Infect skin, hair and nail
- ***Microsporum* species:** Infect skin and hair
- ***Epidermophyton* species:** Infect skin and nail.

Depending on the usual habitat (humans, animals, or soil), dermatophytes are classified as follows (Table 52.4):

- **Anthropophilic:** These are the fungal species exclusively infecting humans
- **Zoophilic:** They infect animals as well as birds
- **Geophilic:** These fungal species are frequently isolated from soil.

Pathogenesis

Dermatophyte infection is acquired by direct contact with soil, animals or humans infected with fungal spores. Then the spores are carried to different areas due to scratching of the inoculated site. Predisposing factors include moist humid skin and tight ill-fitting underclothing.

- **Skin:** Dermatophytes grow in a centrifugal pattern in the stratum corneum; leading to formation of characteristic well-demarcated **annular-** or **ring-**shaped pruritic scaly skin lesions with central clearing and raised edges. Scaling, erythema, and rarely blister formation may occur
- **Nails:** They invade the nails through the lateral or superficial nail plates and then spread throughout the nails
- **Hair shafts:** Dermatophytes can invade within the hair shaft or may be found surrounding it. Hairs become brittle and areas of alopecia may appear. A deep and persistent suppurative folliculitis may be produced; called as **Majocchi granuloma**
- Lesions are not produced by the tissue invasion by the fungi per se; but in response to the host's inflammatory reaction elicited by fungal antigens
- Males are more commonly infected than females as progesterone is inhibitory to dermatophyte growth.
- Severity depends on the infecting fungi, immune status of the host and the site of lesion
  - Anthropophilic dermatophytes are the most common dermatophytes affecting humans. They cause relatively mild and chronic lesions but respond poorly to treatment
  - In contrast, geophilic and zoophilic species, being less adapted to human hosts, produce more acute

Table 52.4: Classification of dermatophytes based on their usual habitat

Habitat	*Trichophyton*	*Microsporum*	*Epidermophyton*
Anthropophilic	*T. rubrum* *T. mentagrophytes* *T. schoenleinii* *T. tonsurans* *T. violaceum*	*M. audouinii*	*E. floccosum*
Zoophilic	*T. equinum* *T. verrucosum*	*M. canis* *M. equinum*	
Geophilic	*T. ajelloi*	*M. gypseum*	*E. stockdaleae*

Table 52.5: Clinical types of dermatophytoses

Clinical types	Area involved
Tinea capitis (infection of the scalp)	Scaly patches are produced on scalp, in which hair shafts are broken off right above the skin. It is of various types
1 Kerion	It is a painful inflammatory reaction, producing boggy lesions on scalp Agent: *Trichophyton verrucosum*
2 Favus	Cup like crust (scutula) is formed around the infected hair follicle with minimal hair shaft involvement (Fig. 52.5A) Agent: *Trichophyton schoenleinii*
3 Ectothrix	Arthrospores are formed on the surface of hair shaft (Fig. 52.6A) Agents: *M. audouinii, M. canis,* and *T. mentagrophytes*
4 Endothrix	Arthrospore are formed within the hair completely filling the hair shaft; thus, can result in alopecia (Fig. 52.6B) Agents: *T. tonsurans* and *T. violaceum*
Tinea corporis	Infection of the non-hairy skin of the body (trunk and limbs) (Fig. 52.5D)
Tinea pedis (athlete foot)	Infection of the web space between the toes, which then spreads to the sole in a "**moccasin**" pattern (Fig. 52.5C)
Tinea cruris (or jock itch)	Infection of the groin area
Tinea barbae	Infection of the beard and moustache area of face
Tinea faciei	Infection of the non-bearded area of face (Fig. 52.5B)
Tinea imbricata	Concentric lesions of the skin Agent: *T. concentricum*
Tinea unguium (nail plate infection)	Infection of nail beds Agents: *T. mentagrophytes* and *E. floccosum*
Tinea manuum	Infection of the palmar aspect of hands

inflammatory response and severe infections; but they tend to resolve more quickly.

Clinical Types

Depending on the site of involvement, various clinical types of dermatophytic or tinea or ring worm infections are produced (Table 52.5). Incubation period is about 1–2 weeks.

Dermatophytid or Id Reaction

Occasionally, hypersensitivity to dermatophyte antigens may occur, which leads to appearance of secondary eruption in sensitized patients because of circulation of allergenic products. However, these lesions are distinct from the primary ringworm lesions as they occur distal to primary site and fungal culture often turns negative.

Figs 52.5A to D: Ring worm infections (Tinea). **A.** Tinea capitis (favus); **B.** Tinea faciei; **C.** Tinea pedis; **D.** Tinea corporis

Source: Public Health Image Library, A. ID#: 2936, B.ID#: 4807, C. ID#:2939 and D. ID#: 2938 Centers for Disease Control and Prevention (CDC), Atlanta. *(with permission)*

Laboratory Diagnosis

Woods Lamp Examination

Certain dermatophytes fluoresce when the infected lesions are viewed under Wood's lamp. It is usually positive for various *Microsporum* species and *Trichophyton schoenleinii*. Other dermatophytes do not fluoresce under Wood's lamp. Fluorescence is due to the presence of pteridine pigment in cell wall.

Specimen Collection

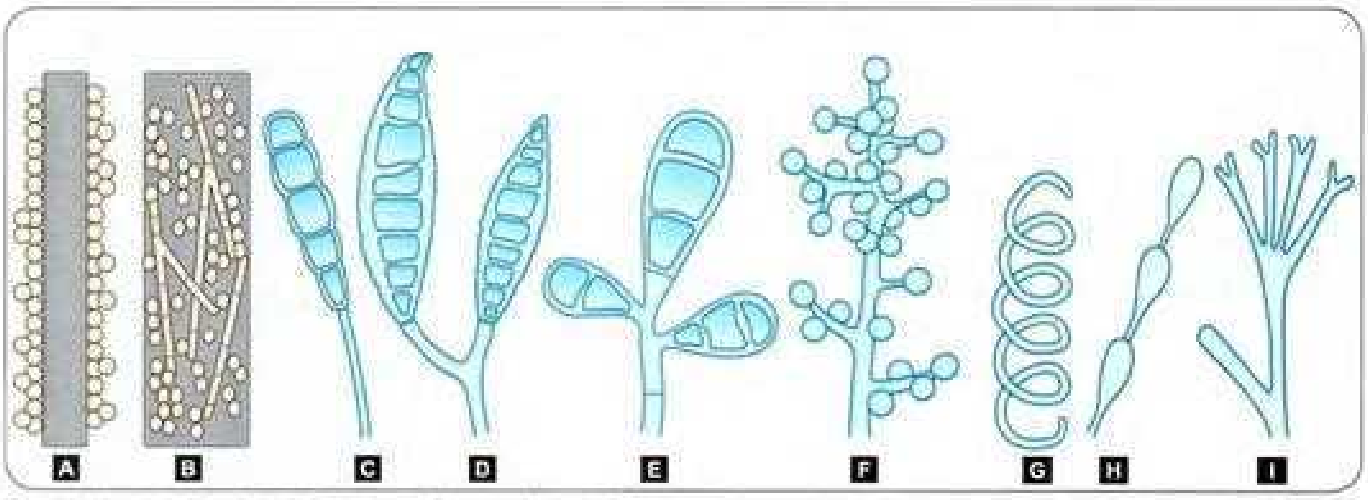
Skin scrapings, hair plucks (broken or scaly ones) and nail clippings are obtained from the active margin of the lesions and are kept in folded black paper. Hairs should be plucked, but not cut.

Direct Examination

The specimen is mounted in KOH (10% for skin scrapings or hair, 20–40% for nail clippings) or calcofluor white stain and is examined for the presence of thin septate hyaline hyphae with arthroconidia (see Figs 52.2A and B). When hair is involved, the arthroconidia may be found on the surface of the hair shaft (ectothrix) or within the shaft (endothrix) (Figs 52.6A and B).

Culture

Specimens should be inoculated onto SDA containing cycloheximide and incubated at 26–28°C for 4 weeks.



Figs 52.6A to I: A. Ectothrix; **B.** Endothrix infection of hair shaft by dermatophytes; **C to E.** Macroconidia of various dermatophyte species; **C.** *Trichophyton mentagrophytes*; **D.** *Microsporum canis*; **E.** *Epidermophyton floccosum*; **F.** Microconidia of *Trichophyton mentagrophyte*; **G to I.** Special types of hyphae seen in dermatophytes. **G.** Spiral hypha; **H.** Racquet hypha; **I.** Favic chandelier

Potato dextrose agar is used to stimulate the sporulation. Identification is made by:

Table 52.6: Distribution of conidia of dermatophytes

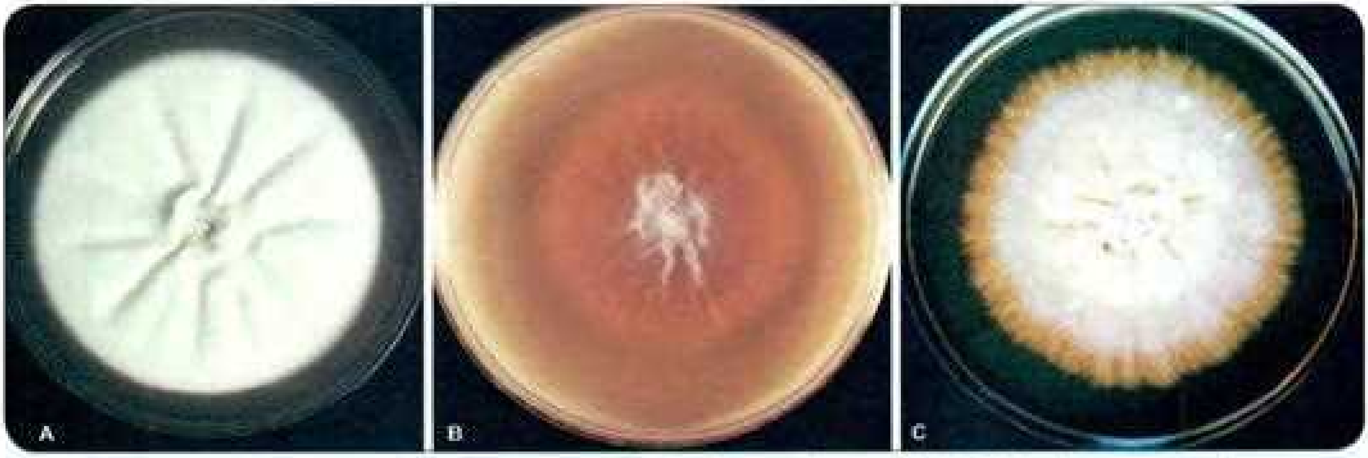
Dermatophytes*	Macroconidia	Microconidia
Trichophyton	Rare, thin walled, smooth, pencil shaped	Abundant
Microsporum	Numerous, thick walled, rough, spindle shaped	Rare
Epidermophyton	Numerous, smooth walled, club shaped	Absent

*Appearance of microconidia and macroconidia may vary depending on the dermatophyte species.

- **Macroscopic appearance** of the colonies such as—rate of growth, texture, pigmentation, colony topography
- **Microscopic appearance:** The colonies are teased and LPCB mount is made to demonstrate the hyphae and spores (or conidia):
  - **Conidia:** Two types of spores or conidia are observed such as small unicellular **microconidia,** and large septate **macroconidia**; both are used for identification of species (Table 52.6 and Figs 52.6C to F)
  - **Special hyphae:** Dermatophytes possess thin septate hyaline hyphae; some species have specialized

Table 52.7: Identification features of commonly encountered dermatophyte species

Dermatophytes	Macroscopic appearance	Microscopic appearance
T. rubrum	Velvety, red pigment on reverse	Microconidia—tear drop shaped, plenty Macroconidia—few, long, pencil shaped
T. mentagrophytes (Figs 52.7A, 52.8A)	White to tan Powdery Pigment variable	Microconidia—numerous, round to pyriform Macroconidia—cigar shaped Spiral hyphae seen
T. schoenleinii	Smooth, waxy	Microconidia and macroconidia—rare or absent Chlamydospores seen Hyphae—hyphal swelling and favic chandelier seen
T. violaceum	Slow growing, waxy Violet pigment on reverse	Microconidia and macroconidia—rare or absent Distorted hyphae seen Chlamydospores seen
M. audouinii	Slow growing, velvety, brownish	Thick-walled chlamydospores seen Macroconidia and microconidia—rare
M. gypseum	Buff colored, powdery	Macroconidia—abundant, thick walled, spiny, spindle shaped, 4–6 septa, rounded ends Microconidia—rare
M. canis (Figs 52.7B, 52.8B)	Cottony, orange pigment on reverse	Macroconidia—abundant, thick walled, spiny, spindle shaped, up to 15 septa, pointed ends Microconidia—rare
E. floccosum (Figs 52.7C, 52.8C)	Powdery, folded, yellowish green	Macroconidia club or clavate shaped in clusters, 4–6 septa Microconidia—absent



Figs 52.7A to C: Macroscopic (colony) appearance of various dermatophytes on SDA. **A.** *Trichophyton mentagrophytes*; **B.** *Microsporum canis*; **C.** *Epidermophyton floccosum*

Source: Public Health Image Library/ A. ID#: 14717, B. ID#:15474, C. ID#:2937/Dr Lucille KG/Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*).

hyphae such as spiral hyphae, racquet hyphae and favic chandeliers (Figs 52.6G to I).

Identification features (macroscopic and microscopic) of commonly encountered dermatophyte species is given in Table 52.7.

Other Methods of Diagnosis

Apart from culture, there are several other methods available for identification of dermatophytes such as:

- **Hair perforation test:** It is positive for *Trichophyton mentagrophytes* and *Microsporum canis*. The test is performed by inoculating a colony into a petri dish containing water, yeast extract, and hair. Fungi pierce the hair producing wedge-shaped perforations
- **Urease test:** *Trichophyton mentagrophytes* is urease positive
- **Dermatophyte test medium** and **Dermatophyte identification medium:** They are used for presumptive identification. These tests are based on color change in the medium due to production of alkali metabolites
- **Molecular methods:** PCR can be used to detect species specific genes (e.g. chitin synthase gene)
- **Skin test:** It is done for detecting hypersensitivity to dermatophyte antigen (trichophytin).

TREATMENT — Dermatophytoses

- **Oral terbinafine or itraconazole** are the drugs of choice for treatment of dermatophytosis. Duration of treatment depends on the affected site (1–2 weeks for skin lesions, 6 weeks for hair infection, 3 months for onychomycosis). They can be given as pulse therapy
- Alternate: Oral griseofulvin and ketoconazole may be given
- Topical lotion such as whitfield ointment or tolnaftate can be applied.

SUBCUTANEOUS MYCOSES

The agents of subcutaneous mycoses usually inhabit the soil. They enter the skin by traumatic inoculation with contaminated material and tend to produce the granulomatous lesions in the subcutaneous tissue.

MYCETOMA

Mycetoma is a chronic, slowly progressive granulomatous infection of the skin and subcutaneous tissues.

- Clinically, it is manifested as a triad of swelling, discharging sinuses and presence of granules in the discharge
- Mycetoma is also known as **Maduramycosis** or **Madura foot,** as it was first described in Madurai, South India, by John Gill (1842).

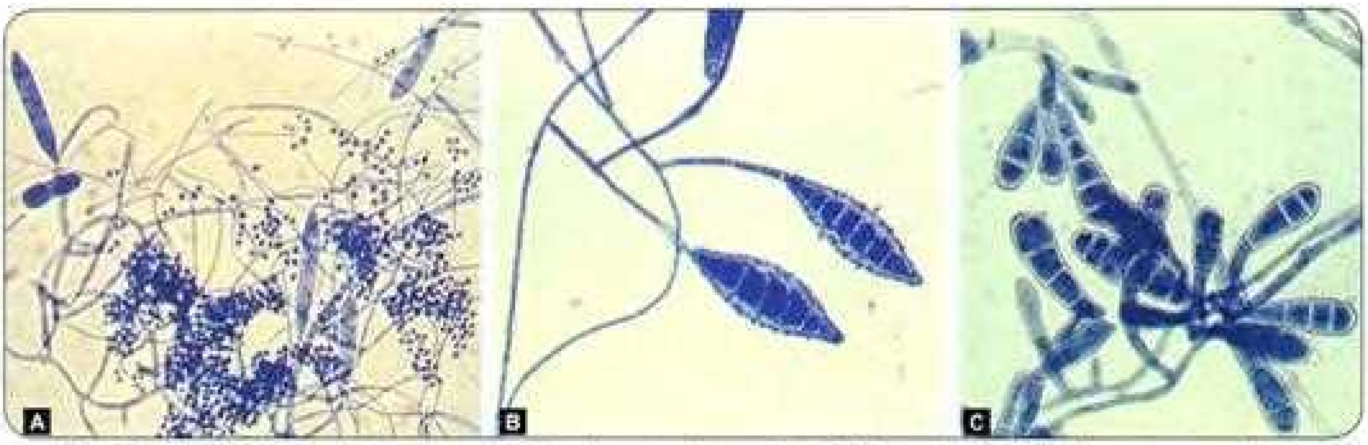
Types of Mycetoma and Causative Agents

Mycetoma can be of two types. It can be caused by either fungal agents (eumycetoma) or bacterial agents (actinomycetoma). They differ from each other by various properties like color of granules/grains and in clinical manifestations, etc. (Tables 52.8 and 52.9).

There is a third category called **botryomycosis** which refers to a mycetoma like condition caused by some bacteria such as *Staphylococcus aureus*.

Pathogenesis

The causative agents enter the skin or subcutaneous tissue from the contaminated soil, usually by the accidental trauma such as thorn prick or splinter injury. Then the disease evolves slowly; initially micro abscesses are formed by the polymorphs, replaced later by chronic granulomatous tissue in skin and subcutaneous tissues.



Figs 52.8A to C: Microscopic appearance of various dermatophyte species (LPCB mount)—**A.** *Trichophyton mentagrophytes*; **B.** *Microsporum canis* and **C.** *Epidermophyton floccosum*

Source: Public Health Image Library/ A. ID#: 15105, B. ID#: 15472, C. ID#: 14588, Centers for Disease Control and prevention (CDC), Atlanta (with permission).

Table 52.8: Agents of mycetoma and types of grains they produce

Eumycetoma	Actinomycetoma
Black granules: • *Madurella mycetomatis* • *Madurella grisea** • *Exophiala jeanselmei* • *Curvularia* species **White granules:** • *Pseudallescheria boydii* • *Aspergillus nidulans* • *Acremonium* species • *Fusarium* species	**White to yellow granules:** • *Nocardia* species • *Streptomyces somaliensis* • *Actinomadura madurae*: It is the most frequent cause, significantly out numbering the cases caused by *Nocardia* **Pink to red granules:** *Actinomadura pelletieri*

*Recently renamed as *Trematosphaeria grisea*

Table 52.9: Clinical manifestations of eumycetoma and actinomycetoma

Clinical manifestations	Eumycotoma	Actinomycotoma
Tumor	Single, well-defined margins	Multiple tumor masses with ill-defined margins
Sinuses	Appear late, few in number	Appear early, numerous with raised inflamed opening
Discharge	Serous	Purulent
Grains	Black/white	White/ red
Bone	Osteosclerotic lesions	Osteolytic lesions
Grains contain	Fungal hyphae (> 2 um)	Filamentous bacteria (< 2 um)

Clinical Manifestations

Hallmark of mycetoma is presence of clinical triad consisting of (Fig. 52.9):

- Tumor like swelling, i.e. tumefaction
- Discharging sinuses
- Discharge oozing from sinuses containing granules.

Eumycetoma and actinomycetoma vary clinically (Table 52.9). Feet are the most common site affected, although any site can be involved. There may be involvement of underlying fasciae and bones, producing osteolytic or osteosclerotic bony lesions. Lesions are usually painless.

Epidemiology

Mycetoma is endemic in Africa, India, the Central and South America, and has a non-uniform distribution.

- Overall, actinomycetoma is more common (60%) than eumycetoma (40%) globally, whereas eumycetoma is more common in Africa
- However, within a country, the distribution may vary in different regions
- A meta-analysis done in 2013 showed that most of the cases are reported globally from Mexico, Sudan and India
- In India, Rajasthan reports the maximum cases of mycetoma per year followed by Tamil Nadu and West

Fig. 52.9: Mycetoma of foot

Source: Public Health Image Library/ ID#: 14816/Centers for Disease Control and prevention (CDC), Atlanta (with permission).

Bengal. Actinomycetoma predominates in India (65%), except in Rajasthan where eumycetoma is more common.

Laboratory Diagnosis

Specimen Collection

The lesions should be cleaned with antiseptics and the grains should be collected on sterile gauze by pressing the sinuses from periphery or by using a loop.

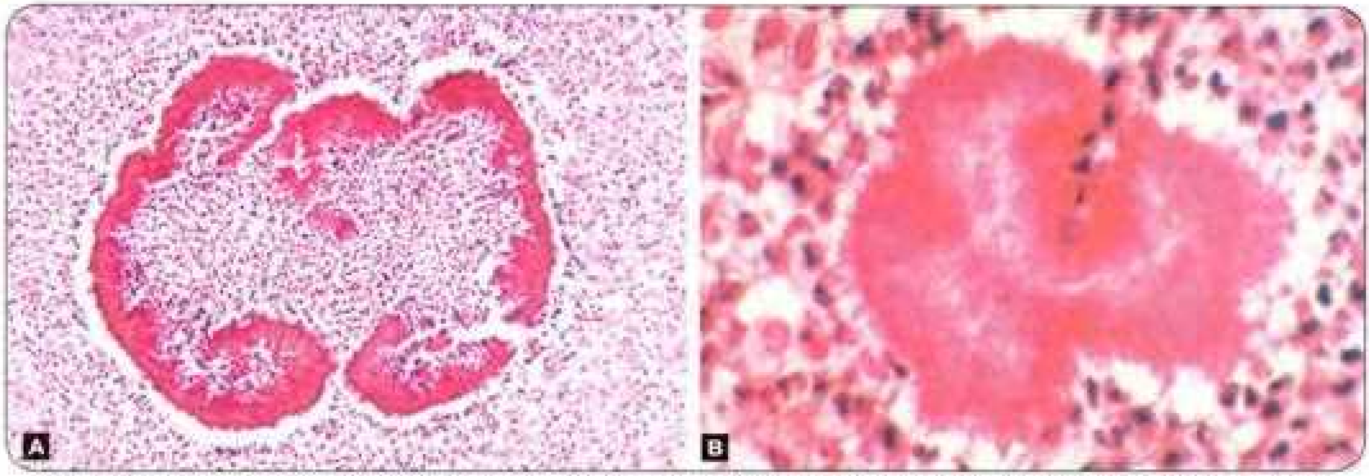
Direct Examination

Granules are thoroughly washed in sterile saline; crushed between the slides and examined.

- **Macroscopic appearance of granules** such as color, size, shape, texture may provide important clue to identify the etiological agent
- **If eumycetoma is suspected:** Grains are subjected to KOH mount, which reveals hyphae of 2–6 µm width along with chlamydospores at margin
- **If actinomycetoma is suspected:** Grains are subjected to Gram staining which reveals filamentous gram-positive bacilli (0.5–1 µm wide). Modified acid fast stain is performed if *Nocardia* is suspected, as it is partially acid fast
- **Histopathological staining of the granules:**
  - Eumycetoma: Reveals granulomatous reaction with palisade arrangement of hyphae in the cement substance (Fig. 52.10A)
  - Actinomycetoma: Shows granulomatous reaction with filamentous bacteria at the margin (Fig. 52.10B).

Culture

Granules obtained from deep biopsies are the best specimen for culture as they contain live organisms. Both



Figs 52.10A and B: A. Eumycetoma (black grain and cement like substance); **B.** Actinomycetoma caused by *Nocardia brasiliensis* (hematoxylin-eosin staining)

Source: Public Health Image Library/ A. ID#: 4331, B. ID#: 15055/Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

fungal (e.g. SDA) and bacteriological media (such as Lowenstein Jensen media) should be included in the panel.

- Identification of the eumycetoma agents is usually carried out by observation of the growth rate, colony morphology, production of conidia and their sugar assimilation patterns
- Agents of actinomycetoma can be identified by their growth rate, colony morphology, urease test, acid fastness and decomposition of media containing casein, tyrosine, xanthine, etc.

TREATMENT — Mycetoma

Treatment of mycetoma consists of surgical removal of the lesion followed by use of:

- Antifungal agents for eumycetoma (itraconazole or amphotericin B for 8–24 months) or
- Antibiotics for actinomycetoma such as Welsh regimen (amikacin plus cotrimoxazole).

SPOROTRICHOSIS

Sporotrichosis or Rose Gardner's disease is presented as subcutaneous noduloulcerative lesions; caused by a thermally dimorphic fungus, *Sporothrix schenckii*.

Pathogenesis

Spores of *S. schenckii* are introduced into skin following minor trauma caused by thorn prick or splinter injury. Enzymes secreted by the fungus, such as serine proteinase and aspartic proteinase help in local invasion. *S. schenckii* has a typical tendency to spread along the lymphatics.

Clinical Manifestations

Sporotrichosis is a chronic subcutaneous pyogranulomatous disease. Incubation period is about 3 weeks. Various clinical types have been observed.

- **Lymphocutaneous type:** It is the most common type (80%) and is characterized by:
  - Noduloulcerative lesions (painless) occur along the lymphatics; called as sporotrichoid pattern of spread, which is also seen in other conditions (see highlight box below).
  - Lymph nodes become enlarged, suppurative, indurated and have cord like feeling on palpation.
- **Other clinical types** are rare such as:
  - Osteoarticular type: It is seen among alcoholics
  - Pulmonary type: It occurs following spore inhalation, seen in people with underlying chronic obstructive pulmonary disease (COPD)
  - Disseminated sporotrichosis: It occurs in immunocompromised patients (such as AIDS)
  - Fixed cutaneous type: Single nodule is found, that is less progressive and does not spread by lymphatics. It is more common in endemic areas such as Mexico where people show strong immunity against the fungi.

Sporotrichoid lymphocutaneous infection

It is a syndrome characterized by the development of superficial cutaneous lesions that progress along dermal and subcutaneous lymphatics

- **Common causes are:** *Sporothrix schenckii, Nocardia brasiliensis, Mycobacterium marinum* or *Leishmania brasiliensis*
- **Rare causes are:** Coccidioidomycosis, cryptococcosis, blastomycosis, histoplasmosis, anthrax, *Burkholderia pseudomallei*, lepromatous leprosy, lupus vulgaris, *Francisella tularensis* and cowpox virus.

Epidemiology

Sporotrichosis is prevalent in tropical countries with high humidity.

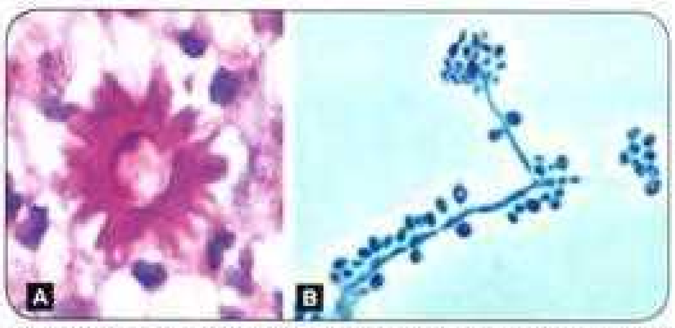
- **World:** It has been reported frequently from Central South America, South Africa and India
- **In India**, sporotrichosis is prevalent in sub Himalayan hilly areas of northeast states ranging from Himachal Pradesh to Assam. Other endemic foci are northern Karnataka and southern Maharashtra
- **Source:** *S. schenckii* has been isolated from decaying vegetations (such as wood, bark, leaves), and soil
- **Risk factors** include people walking bare foot, certain occupations such as farmers and gardeners.

Laboratory Diagnosis

- **Direct microscopy:** Specimens such as pus, aspirate from nodules, curettage or swabbing from ulcers are subjected to KOH mount or calcofluor staining which demonstrate elongated yeast cells of 3-5 µm in diameter. But the sensitivity is very low
- **Histopathological staining** (e.g. hematoxylin and eosin) of tissue sections reveals cigar-shaped asteroid bodies

Asteroid body consists of a central basophilic yeast cell surrounded by radiating extensions of eosinophilic mass, composed of antigen-antibody complexes (Fig. 52.11A). Such eosinophilic halo is described as **Splendore-Hoeppli phenomenon**; which is also observed in zygomycosis, candidiasis, aspergillosis and blastomycosis.

- **Culture:** It is the most definitive tool for diagnosis. Specimens are inoculated onto SDA and blood agar in duplicate and incubated at 25°C and 37°C simultaneously, because *S. schenckii* is a dimorphic fungus
  - **At 25°C:** It produces mycelial form, consisting of slender delicate hyphae with conidia arranged in **flower-like pattern** (Fig. 52.11B)
  - **At 37°C:** It produces yeast form, characterized by moist creamy white colonies which turn brown black in 10-14 days.



Figs 52.11A and B: *Sporothrix schenckii*. **A.** Yeast form (asteroid body); **B.** Mold form showing thin septate hyphae with flower-like sporulation

Source: A. Dr Manoj Singh and Dr M Ramam, AIIMS, New Delhi; B. Public Health Image Library/Dr Libero Ajello B. ID#: 4208/Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*).

- **Serology:** Latex agglutination test detects serum antibodies in patients with extracutaneous form of the disease, but is not always diagnostic
- **Skin test:** It may demonstrate delayed type of hypersensitivity reaction against sporotrichin antigen.

TREATMENT — Sporotrichosis

Itraconazole is the drug of choice for all forms of sporotrichosis; except for disseminated form where amphotericin B is recommended. Treatment is given until 2-4 weeks after the lesions resolve.

CHROMOBLASTOMYCOSIS

Chromoblastomycosis refers to slow growing chronic subcutaneous lesions caused by group of dematiaceous or phaeoid fungi (i.e. darkly pigmented fungi) that produce a characteristic morphology called **sclerotic body**.

- **Agents** of chromoblastomycosis include:
  - *Fonsecaea pedrosoi* and *F. compacta*
  - *Phialophora verrucosa*
  - *Cladosporium carrionii*
  - *Rhinocladiella aquaspersa*.
- **Lesions** are typically slow growing and polymorphic, such as verrucose (most common type), crusted, ulcerative and nodular or tumor like
- Most commonly seen in tropical or subtropical climates, often in rural areas
- **Sclerotic bodies:** Histopathological appearance of these fungi is characterized by formation of brown thick walled round cells (5-12 µm size) with multiple internal transverse septa. They are also called **Medlar bodies** or **muriform cells** or golden-brown septate "copper pennies." (Fig. 52.12A)

TREATMENT — Chromoblastomycosis

It consists of surgical removal (cryosurgery or laser therapy) of the lesion followed by antifungals (itraconazole).

PHAEOHYPHOMYCOSIS

Phaeohyphomycosis refers to chronic subcutaneous lesions, caused by dematiaceous or phaeoid fungi other than that are described in chromoblastomycosis (i.e. they do not produce sclerotic bodies). They exist in mycelial form. Agents include:

- *Alternaria* species
- *Bipolaris* species
- *Curvularia* species
- *Exophiala jeanselmei*
- *Cladophialophora bantiana* (it is neurotropic, produces brain abscess, frontal lobe being the most common site affected).

RHINOSPORIDIOSIS

Rhinosporidiosis is a chronic granulomatous disease, characterized by **large friable polyps in the nose** (most common site), conjunctiva and occasionally in ears, larynx, bronchus and genitalia.

- **Agent:** It is caused by *Rhinosporidium seeberi*. Its taxonomic status is controversial. Previously classified under fungi, now, it is considered to be an aquatic protistan parasite
- **Source:** Stagnant water is the main source of infection. Spores are inhaled while taking bath in contaminated ponds and rivers
- **Distribution:** Rhinosporidiosis is common in tropical countries, especially in Sri Lanka and India (Tamil Nadu, Kerala, Odisha and Andhra Pradesh)
- **Diagnosis** is made by histopathology of the polyps that demonstrates **spherules** (large sporangia up to 350 µm size, that contain numerous endospores, each 6–9 µm in size) (Fig. 52.12B). It is stained better with mucicarmine stain. *R. seeberi* has not been cultivated yet.

TREATMENT **Rhinosporidiosis**

Radical surgery with cauterization is the mainstay of treatment. Dapsone has been found to be affective. Recurrence is common.

SYSTEMIC MYCOSES

HISTOPLASMOSIS

Histoplasmosis is a systemic granulomatous disease caused by a dimorphic fungus, *Histoplasma capsulatum*. The species name is a misnomer as it is not capsulated. It was first reported by Samuel Darling (1905), hence is also known as **Darling's disease**. It has three varieties:

1. *H. capsulatum* var. *capsulatum:* It causes classical histoplasmosis (most common type).
2. *H. capsulatum* var. *duboisii:* It causes African histoplasmosis.
3. *H. capsulatum* var. *farciminosum:* It causes epizootic histoplasmosis.

The description below is confined to *H. capsulatum* var. *capsulatum*. The other two varieties are described thereafter.

Epidemiology

Histoplasmosis occurs worldwide, but is endemic in USA, particularly in states bordering the Ohio River valley and the lower Mississippi River. In India, it is reported frequently from the region of West Bengal along the Ganga River. The fungus inhabits in the humid and acidic soil that contains large amount of bird or bat droppings.

Pathogenesis

H. capsulatum is transmitted by inhalation of spores (i.e. microconidia) which usually circulate in the air after the contaminated soil is disturbed.

- After the spores enter into the lungs, they are engulfed inside the alveolar macrophages and then transform into yeast forms
- The yeasts survive within the phagolysosome of the macrophage by producing alkaline substances, such as bicarbonate and ammonia
- Then, the intracellular yeasts travel to the lymph nodes and spread to the other parts of the body through bloodstream
- Majority of the infected people show strong cell-mediated immune response (CMI) within 2 weeks. Granulomas are formed which later get healed with fibrosis and calcification. Unlike latent tuberculosis, histoplasmosis once healed, rarely reactivates
- However, in patients with impaired CMI, the disseminated infection sets in.

Clinical Manifestations

Clinically, the classical histoplasmosis ranges from asymptomatic infection (in immunocompetent people) to life-threatening illness seen in people with low CMI. The various clinical types include:

- **Pulmonary histoplasmosis:** It is the most common form.
  - Acute form presents with mild flu like illness, pulmonary infiltrates in chest X-ray with hilar or mediastinal lymphadenopathy
  - Chronic cavitary histoplasmosis may be seen in smokers with underlying structural lung disease.

Figs. 52.12A and B: A. Sclerotic body; **B.** *Rhinosporidium seeberi*—spherules containing sporangia filled with endospores (H and E stain)

Source: A. Claudio Guedes Salgado, Associate Professor, Dermato-Immunology Laboratory, Institute of Biological Sciences, Para Federal University, Brazil; B. Public Health Image Library/Dr Martin Hicklin/ID#: 3107/Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*).

- **Mucocutaneous histoplasmosis:** Skin and oral mucosal lesions may develop secondary to pulmonary infection. Oral lesions are particularly seen in Indian patients (Fig. 52.13)
- **Disseminated histoplasmosis:** It develops if CMI is very low (e.g. untreated HIV-infected people or following organ transplantation). The common sites affected are bone marrow, spleen, liver, eyes and adrenal glands.

Laboratory Diagnosis

- **Specimens:** Useful specimens include sputum, aspirate from bone marrow and lymph node, blood and biopsies from skin and mucosa
- **Direct microscopy:** Histopathological staining (such as PAS, Giemsa or GMS stain) of the specimens reveals tiny oval yeast cells (2–4 µm size) with narrow-based budding within the macrophages with an underlying granulomatous response (Figs 52.14A and C)

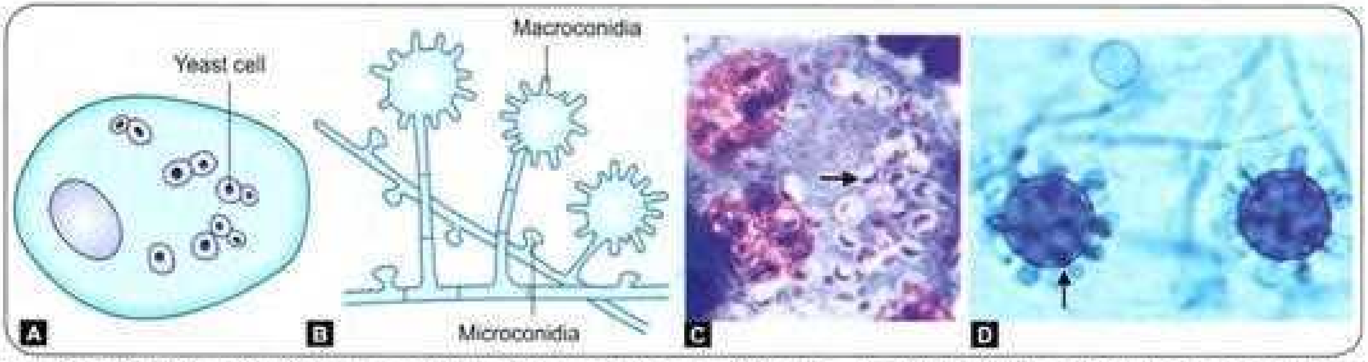
Fig. 52.13: Oral lesions of histoplasmosis (*arrow showing*)

Source: Public Health Image Library/Dr Lucille K Georg/ID#:15363/Centers for Disease Control and Prevention (CDC), Atlanta. (*with permission*).

- **Culture:** It is the gold standard method of diagnosis. Specimens should be inoculated onto media such as SDA, blood agar and BHI agar in duplicate and incubated simultaneously at 25°C and 37°C. *Histoplasma* is a dimorphic fungus, hence:
  - **At 25°C:** It forms mycelial phase; produces white to buff brown colonies that consist of two types of conidia or spores:
    1. Tuberculate macroconidia, with typical thick walls and finger-like projections which is a characteristic feature of this fungus (Figs 52.14B and D).
    2. Microconidia are smaller, thin, and smooth-walled.
  - **At 37°C:** It gets converted into yeast form (creamy white colonies), which is best developed in special media like Kelley's media.
- **Serology:** Antibodies in serum can be detected by CFT and immunodiffusion test
  - Antibodies appear after 1 month of infection; hence are more useful in chronic stage; but are often negative in early course and in disseminated stage
  - False positive result may occur due to past infection or cross infection with *Blastomyces*.
- **Skin test:** It may be done to demonstrate delayed type hypersensitivity response to histoplasmin antigen, which indicates prior exposure.
- **Molecular test:** PCR targeting specific ITS D1/D2 gene (variable region of 28S rRNA) is available.

TREATMENT — Histoplasmosis

Liposomal amphotericin B is the antifungal agent of choice in acute pulmonary and disseminated histoplasmosis. Itraconazole is recommended for chronic cavitary pulmonary histoplasmosis.



Figs 52.14A to D: A and **B.** *Histoplasma capsulatum* (schematic diagram). **A.** Yeast form; **B.** Mycelial form; **C** and **D.** *Histoplasma capsulatum.* **C.** 2–4 µm yeast cells with narrow-based budding (Giemsa stain) (arrow showing); **D.** Mold form, septate thin hyphae with tuberculate macroconidia (arrow showing)

Source: Public Health Image Library/C. Dr Lucille K. Georg/ID#:15365, D. Dr Libero Ajello/ID#:15364/Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*).

H. capsulatum var. duboisii

It causes African histoplasmosis which is clinically distinct; characterized by frequent skin and bone involvement. Its yeast form exists as large thick walled oval yeast cells (7-15 µm) with prominent narrow based budding.

H. capsulatum var. farciminosum

It causes epizootic histoplasmosis. It is a form of lymphangitis in horses and mules.

BLASTOMYCOSIS

Blastomycosis (also known as North American blastomycosis or Gilchrist's disease or Chicago disease) is a fungal infection of humans and other animals, notably dogs and cats, caused by the dimorphic fungus, *Blastomyces dermatitidis*.

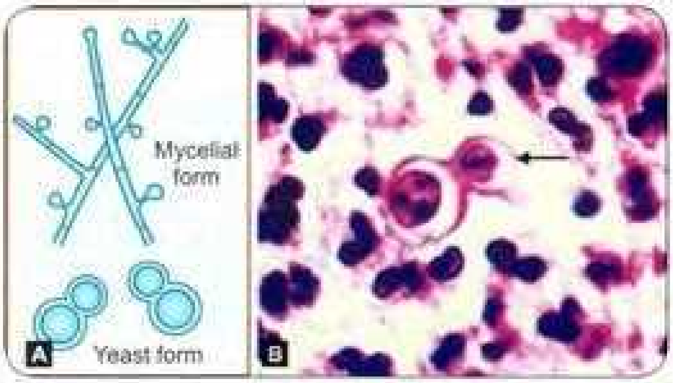
Pathogenesis

Blastomycosis is transmitted by inhalation of the conidia of *B. dermatitidis*. The spores enter into the lungs, and are engulfed by alveolar macrophages, where they get converted into yeast phase. This yeast expresses a 120-kDa glycoprotein called **BAD-1** (*B. dermatitidis* adhesin-1) which is an essential virulence factor and also a major inducer of cellular and humoral immune responses.

Clinical Manifestations

Acute pulmonary blastomycosis is the most common form. Extrapulmonary manifestations may also occur such as:

- Skin involvement is the most common extrapulmonary form; characterized by either verrucous (more common) or ulcerative type of skin lesions
- Osteomyelitis may develop along with contiguous soft-tissue abscesses and draining sinuses



Figs 52.15A and B: *Blastomyces* **A.** Schematic (Mycelial and yeast form); **B.** Histopathological stain (*arrow showing*) broad-based budding yeast cells (Figure of 8 appearance)

Source: B. Public Health Image Library/ID#493/Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*).

- Prostate and epididymis involvement in men
- Central nervous system (CNS) involvement has been reported in ~40% of AIDS patients. Brain abscess is the usual presentation, followed by cranial or spinal epidural abscess and meningitis.

Epidemiology

Like histoplasmosis, blastomycosis is also endemic in North America, particularly in states bordering the Ohio River and Mississippi River.

Laboratory Diagnosis

- **Histopathological staining** of the tissue biopsy specimens reveals thick-walled round yeast cells of 8-15 µm size with single broad-based budding **(figure of 8** appearance) (Figs 52.15A and B)
- **Culture media** such as SDA, blood agar and BHI agar are inoculated. At 25°C, mycelial form containing hyphae with small pear-shaped conidia are produced; whereas at 37°C mold to yeast conversion takes place
- **Skin test:** It is done to demonstrate delayed type hypersensitivity to blastomycin antigen
- **Antibody detection:** Immunodiffusion test specific for *B. dermatitidis* has been developed against yeast phase antigens such as antigen—A, BAD-1 and ASWS antigen (alkali soluble water soluble)
- **Antigen detection assay** to detect *Blastomyces* antigen in urine (more sensitive) and in serum is commercially available
- **Molecular methods,** including DNA probe hybridization and real time PCR are available.

TREATMENT — Blastomycosis

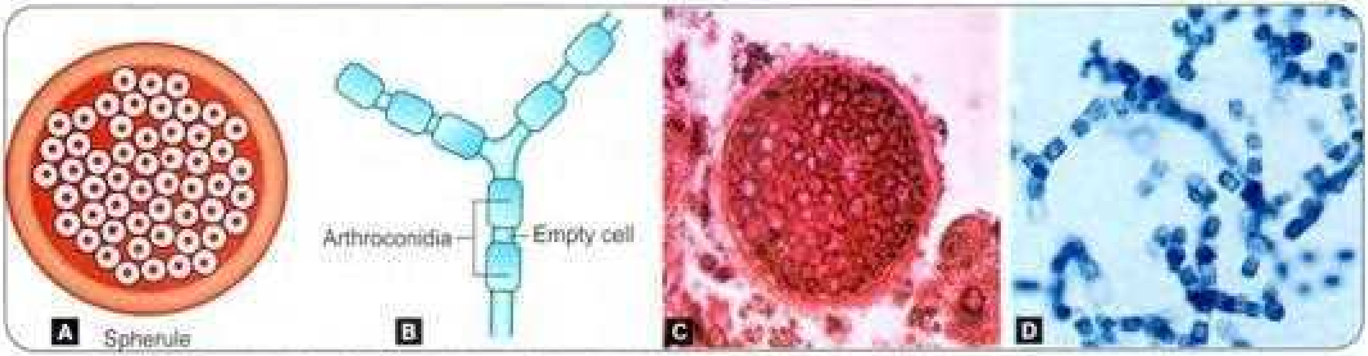
Liposomal amphotericin B is the drug of choice in most of the cases. Itraconazole can be given in immunocompetent patients with mild pulmonary or non-CNS extrapulmonary blastomycosis.

COCCIDIOIDOMYCOSIS

Coccidioidomycosis (also called desert rheumatism or Valley fever or California fever), is a systemic fungal disease caused by a dimorphic soil dwelling fungus—*Coccidioides* which has two species, *C. immitis and C. posadasii*.

Pathogenesis

Coccidioides is transmitted by inhalation of arthroconidia. In lungs, they enlarge, become rounded, and develop internal septations to form large sac like structures of size up to 200 µm called **spherules,** that encompass numerous endospores. Spherules may rupture and release packets of endospores that can disseminate and develop into new spherules. If returned to artificial media or the soil, spherules revert back to the mycelial stage.



Figs 52.16A to D: *Coccidioides* **A.** Spherules (schematic); **B.** Hyphae with arthroconidia (schematic); **C.** Spherules (PAS staining); **D.** Hyphae with arthroconidia (LPCB mount)

Source: Public Health Image Library/C. ID#:14499, D. ID#:12196/Centers for Disease Control and Prevention (CDC) Atlanta (*with permission*).

Clinical Manifestations

Most patients are asymptomatic (60%). In remainders, pulmonary coccidioidomycosis is the most common form; presents as pneumonia, cavities, pleural effusion or nodule formation.

- Skin lesions such as rashes or erythema nodosum and arthritis with joint pain may appear secondary to pulmonary infection particularly in women
- Disseminated form: Males and persons with low CMI (HIV infected patients with CD4+ T cell count <250/µL) are at higher risk. Common sites for dissemination include skin, bone, joints, soft tissues, and meninges.

Epidemiology

It is endemic in certain parts of Arizona, California, Nevada, New Mexico, Texas, Utah and northern Mexico.

Laboratory Diagnosis

- **Histopathological staining** (H and E stain, PAS or GMS) of sputum or tissue biopsy specimens demonstrates spherules which are large sac like structures (20–80 µm size), have thick, double refractile wall, and are filled with endospores (Figs 52.16A and C)
- **Cultures** on SDA produces mycelial growth, described as fragmented hyphae consisting of **barrel-shaped arthrospores** with alternate cells distorted (empty cells) (Figs 52.16B and D):
  - *Coccidioides* differs from other dimorphic fungi as it grows as mold at both 25°C and 37°C in usual culture media. It forms spherules at 37°C in certain special culture media only
  - Cultures are highly infectious; may lead to accidental inhalation of spores in laboratories, require biosafety level-3 precautions.
- **Serology:** Antibodies are detected by immunodiffusion test and CFT
- **Skin test:** It is done by using fungal extracts (coccidioidin or spherulin); if produces at least a 5 mm induration within 48 hours after injection (delayed hypersensitivity reaction) indicates past infection.

TREATMENT **Coccidioidomycosis**

Triazoles such as itraconazole are the drug of choice to treat most cases of coccidioidomycosis, except for diffuse pneumonia with pulmonary sequelae where amphotericin B is recommended.

PARACOCCIDIOIDOMYCOSIS

Paracoccidioidomycosis (also known as South American blastomycosis, Lutz-Splendore-de Almeida disease) is a systemic disease caused by the dimorphic fungus—*Paracoccidioides brasiliensis*.

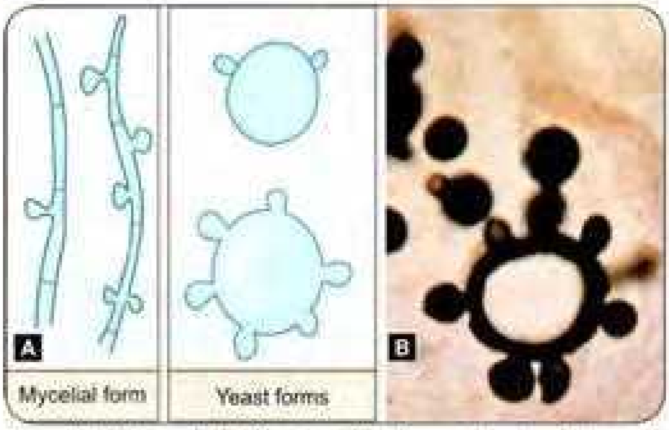
Pathogenesis and Clinical Manifestations

Transmission is by inhalation of spores, which then transform into the yeast phase in lungs. It occurs as two major forms.

1. **Acute form (or juvenile type):** It affects young adults under 30 years age. It is a less common variety, but more severe form, manifests as **disseminated infection** involving multiple viscera and is refractory to treatment.
2. **Chronic form (or adult form):** It accounts for 90% of cases and predominantly affects older men. It results from reactivation of quiescent lung lesions.
   - It is less severe form, manifested as progressive pulmonary disease affecting lower lobes, with fibrosis
   - Skin, oral mucosal lesions and cervical lymphadenopathy are the other features.

Epidemiology

Paracoccidioidomycosis is endemic in Brazil and other South American countries.



Figs 52.17A and B: Paracoccidioidomycosis. **A.** Schematic representation of mycelial and yeast forms; **B.** Methenamine silver staining shows yeast from (pilot wheel appearance)

Source: B. Public Health Image Library/Dr Lucille K Georg/ID#:527/Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

Laboratory Diagnosis

- **Histopathological staining** of pus, tissue biopsies or sputum reveals round thick-walled yeasts, with multiple narrow-necked buds attached circumferentially giving rise to **Mickey mouse or pilot wheel** appearance (Figs 52.17A and B)
- **Culture** on SDA yields mycelial form at 25°C which converts into yeast phase at 37°C when grown in BHI agar supplemented with blood and glutamine
- **Serology:** Antibodies are detected by immunodiffusion, and most recently by ELISA, using gp43 antigen of *P. brasiliensis*
- **Skin test:** It demonstrates delayed type hypersensitivity response against paracoccidioides antigen.

TREATMENT **Paracoccidioidomycosis**

Itraconazole is the treatment of choice for paracoccidioidomycosis, except for the seriously ill patients where amphotericin B is recommended. Sulfonamides are effective, but the response is slow with frequent relapses.

OPPORTUNISTIC MYCOSES

Opportunistic mycoses are caused by a group of fungi, which are normally a part of human anatomical flora (e.g. *Candida*) or found in nature and frequently isolated as laboratory contaminants (e.g. *Aspergillus, Rhizopus* and *Penicillium*). However, they are capable of causing infection in presence of opportunities such as low immunity.

CANDIDIASIS

Candidiasis is the most common fungal disease in humans, affecting the skin, mucosa, and various internal organs; caused by *Candida*, a yeast like fungus that produces pseudohyphae. Various species of *Candida* include:

- *Candida albicans:* It is the most common and most pathogenic species of *Candida* infecting humans
- Other *Candida* species which can occasionally cause infection such as—*C. tropicalis, C. glabrata, C. krusei, C. parapsilosis, C. dubliniensis, C. kefyr, C. guilliermondii* and *C. viswanathii.*

Pathogenesis

Candidiasis is worldwide in distribution, accounts for the most common fungal infection in humans, both in HIV and non-HIV infected people.

Predisposing Factors

Predisposing factors that are associated with increased risk of infection with *Candida* include:

- Physiological state: Extremes of age (infancy, old age), pregnancy
- Low immunity: Patients on steroid or immunosuppressive drugs, post-transplantation, malignancy, HIV-infected people
- Patients on broad spectrum antibiotics—suppress the normal flora
- Others: Diabetes mellitus, febrile neutropenia and zinc or iron deficiency.

Virulence Factors

Candida albicans possesses the following virulence factors that contribute to the pathogenesis:

- **Adhesins:** Help in adhesion to the skin and mucosa
- **Enzymes** such as aspartyl proteinases and serine proteinases-help in tissue invasion
- **Toxins:** Glycoprotein extracts of *Candida* cell wall are pyrogenic similar to bacterial endotoxins
- **Pseudohyphae:** Presence of pseudohyphae indicates active infection; phospholipase released from the hyphal tip may help in invasion, though not proved
    - *C. albicans* has a unique ability to transform frequently between three phenotypic forms in the tissue—yeast (blastospores), pseudohyphae, and true hyphae. This property is known as **phenotypic switching**
    - This enables adaptation to changing conditions in host and thereby assists the fungus in evading host defense system (Fig. 52.18).

Clinical Manifestations

Candida species produce a spectrum of infections ranging from skin and mucosal infection to invasive and allergic infections.

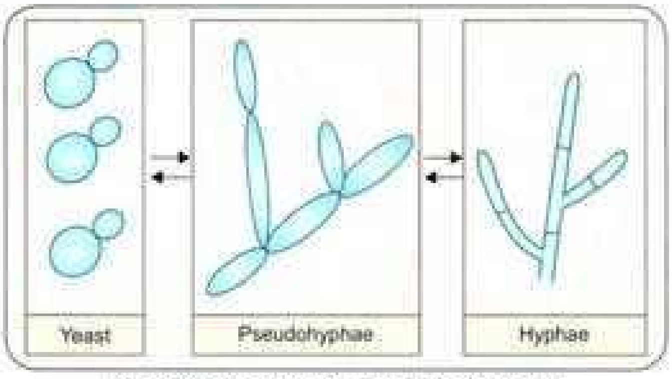
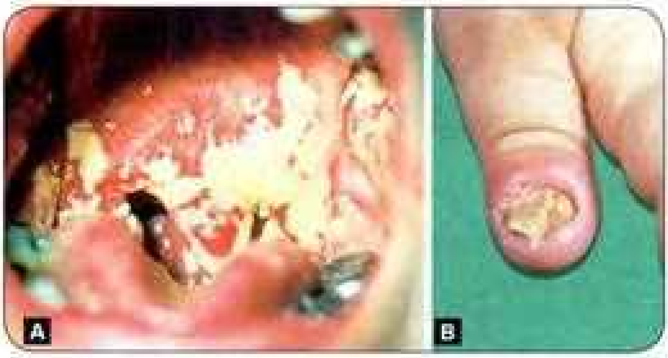


Fig. 52.18: Phenotypic switching of *Candida*



Figs 52.19A and B: Candidiasis. **A.** Oral thrush; **B.** Onychomycosis

Source: Public Health Image Library/ A. ID#:1217, B. Mr Gust, ID#:15669/Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*).

- **Mucosal candidiasis:** The various mucosal manifestations include:
  - Oropharyngeal candidiasis (oral thrush): It presents as white, adherent, painless patches in the mouth (Fig. 52.19A)
  - Vulvovaginitis: It is characterized by pruritus, pain, and vaginal discharge that is usually thin, but may become whitish curd like in severe cases
  - Balanitis and balanoposthitis (occurring in uncircumcised males)
  - Esophageal candidiasis
  - Angular stomatitis and denture stomatitis
  - **Chronic mucocutaneous candidiasis**
    - It is seen in infants and children with deficient CMI (T cell defect)
    - Lesions are produced involving hair, nail, skin, and mucous membrane; which are usually resistant to treatment
    - It is associated with other endocrine abnormalities.
- **Cutaneous candidiasis:** The following cutaneous manifestations are produced in candidiasis:
  - Intertrigo: It is characterized by erythema and pustules in the skin folds; associated with tight fitting undergarments and sweating
  - Paronychia (involving nail-skin interface) and onychomycosis (fungal infection of nail) (Fig. 52.19B)
  - Diaper candidiasis: Pustular rashes, associated with use of diapers in infants
  - Perianal candidiasis
  - Erosio interdigitalis blastomycetica: It is an infection affecting the web spaces of hands or toes
  - Generalized disseminated cutaneous candidiasis, seen in infants.
- **Invasive candidiasis:** It results from hematogenous or local spread of the fungi. Various forms are:
  - Urinary tract infection
  - Pulmonary candidiasis
  - Septicemia (mainly by *C. albicans* and *C. glabrata*)
  - Arthritis and osteomyelitis
  - Meningitis
  - Ocular—keratoconjunctivitis and endophthalmitis
  - Hepatosplenic candidiasis
  - Disseminated candidiasis
  - Nosocomial candidiasis (mainly by *C. glabrata*).
- **Allergic candidiasis** includes:
  - **Candidid:** This is an allergic reaction to the metabolites of *Candida*, characterized by vesicular lesions in the web space of hands and other areas, similar to that of dermatophytid reaction (both conditions are together called **'id'** reaction)
  - **Other allergic reactions** include: Gastritis, irritable bowel syndrome and eczema.

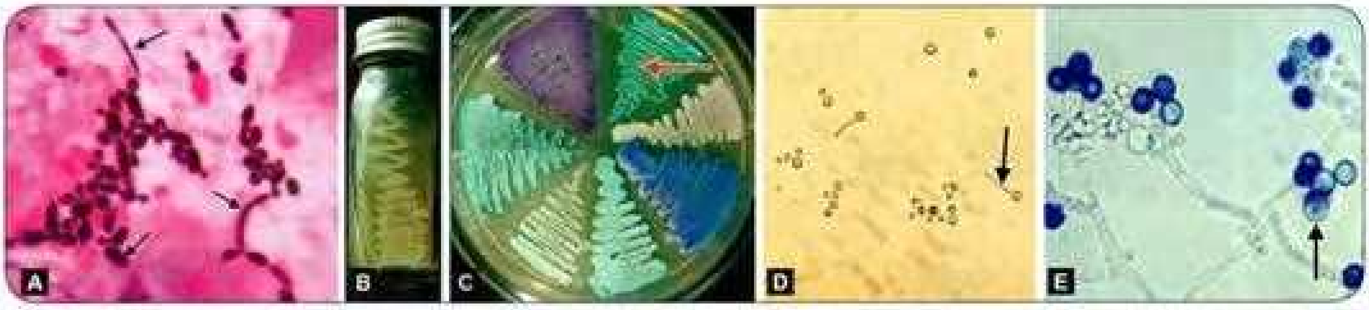
LABORATORY DIAGNOSIS *Candida albicans*

- **Direct microscopy:** Gram-positive oval budding yeast cells with pseudohyphae
- **Culture on SDA:** Produces creamy white and pasty colony
- **Tests for species identification:**
  - Germ tube test (positive for *C. albicans*)
  - Dalmau plate culture for chlamydospore production
  - CHROMagar
  - Growth at 45°C (positive for *C. albicans*)
  - Carbohydrate assimilation test and carbohydrate fermentation test
  - Molecular methods such as PCR.
- **Immunodiagnosis:**
  - Antibody detection against cell wall mannan antigen
  - Antigen detection such as cell wall mannan and cytoplasmic antigens.
- Enzyme detection, e.g. enolase
- Detection of metabolites, e.g. mannitol and arabinitol.

Laboratory Diagnosis

Specimen Collection

Depending on the site of infection, various specimens can be collected such as whitish mucosal patches, skin and nail scrapings, sputum, urine or blood.



Figs 52.20A to E: A. *Candida albicans*—gram-positive oval budding yeast cells with pseudohyphae; B. *Candida albicans* on SDA shows creamy white colonies; C. CHROMagar showing colonies of various *Candida* species producing different colors (e.g. light-green color by *C. albicans*, red arrow); D. *Candida albicans* shows positive germ tube test (arrow showing); E. *Candida albicans* shows thick walled chlamydospores (arrow showing)

Source: A to D. Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry; E. ID#2917/Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

Direct Microscopy

Gram staining reveals gram-positive oval budding yeast cells (4–6 µm size) with pseudohyphae (Fig. 52.20A). It has to be differentiated from true hyphae (Table 52.10).

Culture

Specimens can be inoculated onto SDA with antibiotic supplements and then incubated at 37°C. *Candida* can also grow in bacteriological culture media such as blood agar. Blood for culture can be inoculated in to blood culture bottles (conventional and automated blood culture bottles such as BacT/ALERT).

- Colonies appear in 1–2 days and described as creamy white, smooth, and pasty with typical yeasty odor (Fig. 52.20B)
- Gram staining of the colonies shows gram-positive budding yeast cells with pseudohyphae except for *C. glabrata* which does not show pseudohyphae.

Tests for Species Identification

- **Germ tube test:** It is a specific test for *C. albicans*; also called Reynolds Braude phenomenon
  - Colonies are mixed with human or sheep serum and incubated for 2 hours. Wet mount preparation is examined under microscope
  - Germ tubes are formed, described as long tube like projections extending from the yeast cells
  - It is differentiated from pseudohyphae as there is no constriction at the origin (Fig. 52.20D, Table 52.10)
  - Though the test is specific for *C. albicans*, it may also be positive for *C. dubliniensis*.
- **Dalmau plate culture:** Culture on cornmeal agar can provide clue for species identification. *C. albicans* produces thick walled chlamydospores (Fig. 52.20E)
- **CHROMagar:** Different *Candida* species produce different colored colonies on CHROMagar (Fig. 52.20C)
- **Growth at 45°C:** It differentiates *C. albicans* (grows) from *C. dubliniensis* (does not grow at 45°C)
- **Carbohydrate fermentation test** and **carbohydrate assimilation test** can differentiate between various *Candida* species
- **Molecular methods** such as PCR using species specific primers are useful for species identification.
  - Yeast specific universal primer and species specific primers are available targeting different genes from internal transcribed spacer region such as ITS-1 and ITS-2.
  - Assays have been developed for direct detection of *Candida* species from clinical specimens such as blood.

Table 52.10: Differences between pseudohyphae and true hyphae

Features	Pseudohyphae	True hyphae
Septa	Constricted	No constriction
Origin of branches	Constricted and septate	• No constriction • No septum present
Grows by	Budding	Apical elongation

Immunodiagnosis

- **Antibody detection:** Various formats like ELISA, latex agglutination tests are available detecting serum antibodies against cell wall mannan antigen
- **Antigen detection:** *Candida* specific antigen such as cell wall mannan and cytoplasmic antigens can be detected by ELISA
- **Enzyme detection:** Assays are available to detect enzymes specific for *Candida* such as enolase, aspartate proteinase, etc.
- **Test for metabolites:** Specific metabolites of *Candida* such as mannitol, arabinitol can be detected. G test is done for detection of β-1-3 -D-glucan.

TREATMENT **Candidiasis**

The antifungal drugs recommended depends upon the type of candidiasis.
- Cutaneous candidiasis or oral thrush: the drug of choice is topical azole
- Esophageal and vulvovaginal candidiasis: the drug of choice is oral fluconazole or caspofungin
- Disseminated candidiasis: the drug of choice is liposomal amphotericin B or caspofungin.

C. glabrata and *C. krusei* exhibit intrinsic resistance to azoles and are refractory to treatment with azoles.

CRYPTOCOCCOSIS

Cryptococcosis is caused by a capsulated yeast called *Cryptococcus neoformans*, which is capable of producing potentially fatal meningitis in HIV infected people.

Species and Serotypes

Cryptococcus has two species, *C. neoformans* and *C. gattii* and four serotypes A, B, C and D.
- *C. neoformans* occurs in two varieties—*C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans*; which correlate with serotypes A and D, respectively
- *C. gattii* is antigenically diverse and corresponds to the serotypes B and C
- Most laboratories do not routinely distinguish between the types, and report all isolates simply as *C. neoformans*.

Pathogenesis

Infection is acquired by inhalation of aerosolized forms of *Cryptococcus*. Both yeast cells as well as basidiospores (the sexual stage of *Cryptococcus*) are infectious.
- In immunocompetent individuals, the lungs exhibit defense mechanisms which usually limit the infection
- However, in people with low immunity, pulmonary infection occurs followed by dissemination through blood
- **CNS spread:** The unique feature of *Cryptococcus* is its ability to cross blood-brain barrier which occurs by the yeast cells either they migrate directly across the endothelium or carried inside the macrophages as "Trojan horse"
- **Virulence factors** of *Cryptococcus* that favor invasion and spread of infection include:
  - Polysaccharide capsule—It is the principal virulence factor of the fungus. It is antiphagocytic and also inhibits the host's local immune responses
  - Ability to make melanin by producing an enzyme called phenyl oxidase
  - Production of other enzymes such as phospholipase and urease.
- **Risk factors:** Individuals at high risk for cryptococcosis include:
  - Patients with advanced HIV infection with CD4 T cell counts less than 200/µL: They are at high risk of acquiring *C. neoformans* infection. However, *C. gattii* is not associated with HIV infection. It usually causes infection in immunocompetent individuals
  - Patients with hematologic malignancies
  - Transplant recipients
  - Patients on immunosuppressive or steroid therapy.

Clinical Manifestations

Various clinical manifestations of cryptococcosis include:
- Pulmonary cryptococcosis: It is the first and the most common presentation
- Cryptococcal meningitis: It presents as chronic meningitis, with headache, fever, sensory and memory loss, cranial nerve paresis and loss of vision (due to optic nerve involvement)
- Skin lesions: They are commonly seen with *C.neoformans* var. *neoformans* (serotype D)
- Osteolytic bone lesions.

Epidemiology

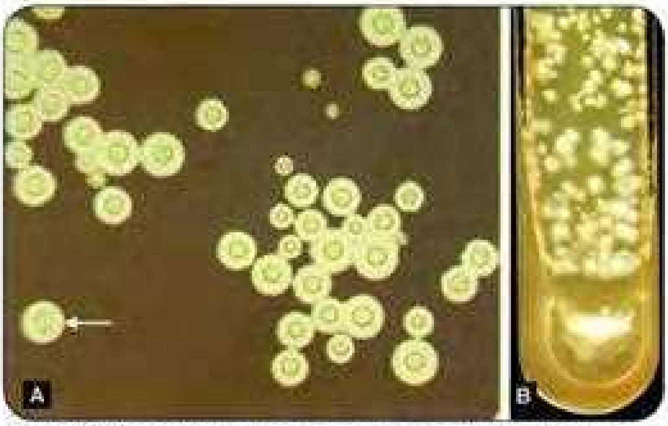
Worldwide, cryptococcosis accounts for nearly 1 million cases, with more than 600,000 deaths annually.
- **Geographical distribution:** *C. neoformans* var. *grubii* (serotype A) strains are found worldwide; however, *C. neoformans* var. *neoformans* (serotype D) strains are restricted to Europe and *C. gattii* is confined to tropics; outbreaks occurred in Vancouver in 1999
- **Habitat:** *C. neoformans* is frequently found in soils contaminated with avian excreta and pigeon droppings. In contrast, *C. gattii* inhabits in eucalyptus tree.

Laboratory Diagnosis

Specimens such as CSF, blood or skin scrapings can be collected.

Direct Detection Methods

- **Negative staining:** Modified India ink stain (added with 2% mercurochrome) and nigrosin stain are used to demonstrate the capsule, which appears as refractile delineated clear space surrounding the round budding yeast cells against black background
  - Capsules may be twice as thick as the diameter of yeast cells (Fig. 52.21A)
  - India ink stain is less sensitive (60–70%).
- **Gram staining** may show gram-positive round budding yeast cells



Figs 52.21A and B: *Cryptococcus neoformans*. **A.** India ink staining shows clear refractile capsules surrounding round budding yeast cells; (arrow showing) **B.** Growth on SDA at 37°C—shows creamy white mucoid colonies

Source: Public Health Image Library/A. Dr Leanor Haley, ID#:3771 B. Dr William Kaplan, ID#:3199/Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

- **Other stains include:**
  - Mucicarmine stain: It stains the carminophilic cell wall of *C. neoformans*
  - Masson-Fontana stain: It demonstrates the production of melanin
  - Alcian blue stain to demonstrate the capsule.
- **Antigen detection:** The capsular antigens can be detected from CSF or serum by latex agglutination test. It is a rapid and sensitive (95% sensitivity) and specific method.

Culture

CSF is inoculated onto SDA without antibiotics, blood agar or chocolate agar and incubated at 37°C. Blood is inoculated in biphasic blood culture bottles. Colonies appear as mucoid creamy white and yeast like (Fig. 52.21B).

Confirmation of *Cryptococcus* species is made by:

- Niger seed agar and bird seed agar: They are used to demonstrate melanin production (brown colored colonies)
- Growth at 37°C
- Urease test is positive
- Assimilation of inositol and nitrate
- Mouse pathogenicity test.

TREATMENT — Cryptococcosis

Treatment depends upon the type of cryptococcosis.

- Cryptococcosis without CNS involvement: Fluconazole is the drug of choice
- HIV-infected patients with CNS involvement: The recommended regimen is induction phase for two weeks (amphotericin B ± flucytosine) followed by oral fluconazole therapy till CD4 T cell count raises >200 /µL for 6 months.

ZYGOMYCOSIS

Zygomycosis represents group of life-threatening infections caused by aseptate fungi belonging to the phylum Zygomycota. Agents of zygomycosis fall into two orders:

1. Order mucorales (causes mucormycosis)
   - *Rhizopus* (*R. arrhizus* and *R. microsporus*)
   - *Mucor racemosus*
   - *Rhizomucor pusillus*
   - *Lichtheimia corymbifera* (previously called as *Absidia corymbifera*)
   - *Apophysomyces elegans*.
2. Order entomophthorales (causes entomophthoromycosis)
   - *Basidiobolus ranarum*
   - *Conidiobolus coronatus*.

Mucormycosis

Pathogenesis

Spores of fungi causing mucormycosis are found ubiquitously in the environment. Transmission occurs via inhalation, inoculation or rarely ingestion of spores. Spores develop into mycelial form containing wide aseptate hyphae which are angioinvasive in nature resulting in spread of infection.

Predisposing factors: Agents of mucormycosis require iron as growth factor. Hence conditions with increased iron load are at higher risk of developing invasive mucormycosis.

- Diabetic ketoacidosis (DKA) is the most important risk factor. Acidosis causes release of iron from the sequestered proteins in serum
- End stage renal disease
- Patients taking iron therapy or deferoxamine (iron chelator)
- Defects in phagocytic functions (e.g. neutropenia or steroid therapy).

Clinical Manifestations

Agents of mucormycosis are angioinvasive in nature. Mucormycosis has six types of clinical presentations.

1. **Rhinocerebral mucormycosis:** It occurs commonly in patients with diabetic ketoacidosis. It is the most common form; starts as eye and facial pain, may progress to cause orbital cellulitis, proptosis and vision loss (Figs 52.22A and B).
2. Pulmonary mucormycosis is the second most common form, occurs in patients with leukemia.
3. Cutaneous mucormycosis.
4. Gastrointestinal mucormycosis such as necrotizing enterocolitis; seen commonly in premature neonates.
5. Disseminated mucormycosis: Brain is the most common site of dissemination, but can affect any organ.
6. Miscellaneous forms: Anybody site may be randomly affected such as bones, trachea and kidneys, etc.

Laboratory Diagnosis

- **Histopathological staining** such as H&E or methenamine silver stain of tissue biopsies shows broad aseptate hyaline hyphae with wide angle branching (Fig. 52.23)
- **Culture on SDA at 25°C:** Reveals characteristic white cottony woolly colonies with tube filling growth (hence called **lid lifters**) (Figs 52.24A and B). In some species, e.g. *Rhizopus* the colonies become brown black later, due to sporulation giving rise to **salt and pepper** appearance (Fig. 52.24A)
- **Microscopic appearance:** LPCB mount of the colonies reveals broad aseptate hyaline hyphae, from which sporangiophore arises and then ending at sporangium which contains numerous sporangiospores (Figs 52.24C, D and 52.25)
- **Rhizoid:** Some species bear a unique root like growth arising from hyphae called rhizoid which provides initial clue for identification of the fungus. Species can be differentiated depending on the position of the rhizoids with respect to sporangiophore (Fig. 52.25A to C)
  - *Rhizopus* bears nodal rhizoids
  - *Lichtheimia* bears internodal rhizoids
  - *Mucor*: rhizoids absent.

TREATMENT — Zygomycosis

Amphotericin B deoxycholate remains the drug of choice for all forms of mucormycosis. Posaconazole or isavuconazole can be given alternatively. For mild localized skin lesions in immunocompetent patients, which can be removed surgically.

Figs 52.22A and B: Mucormycosis manifestations. **A.** Orbital cellulitis; **B.** Proptosis

Source: Public Health Image Library/A. Dr Thomas F Sellers, ID#:2831, B. Lucille K Georg, ID#:14554 /Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*).

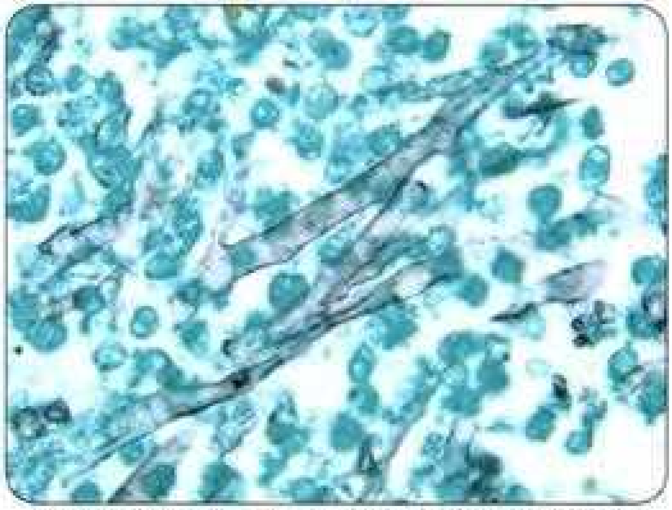
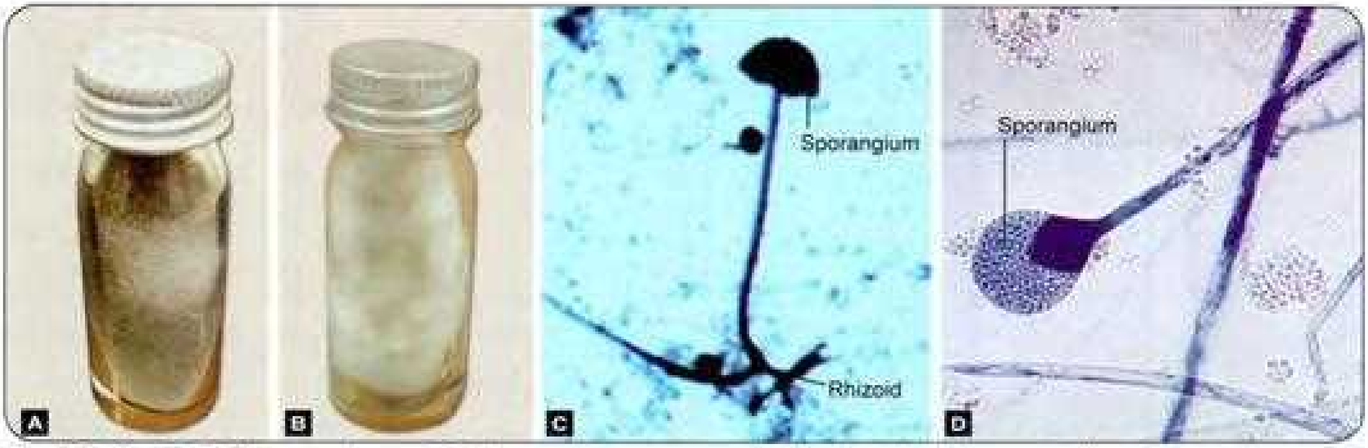


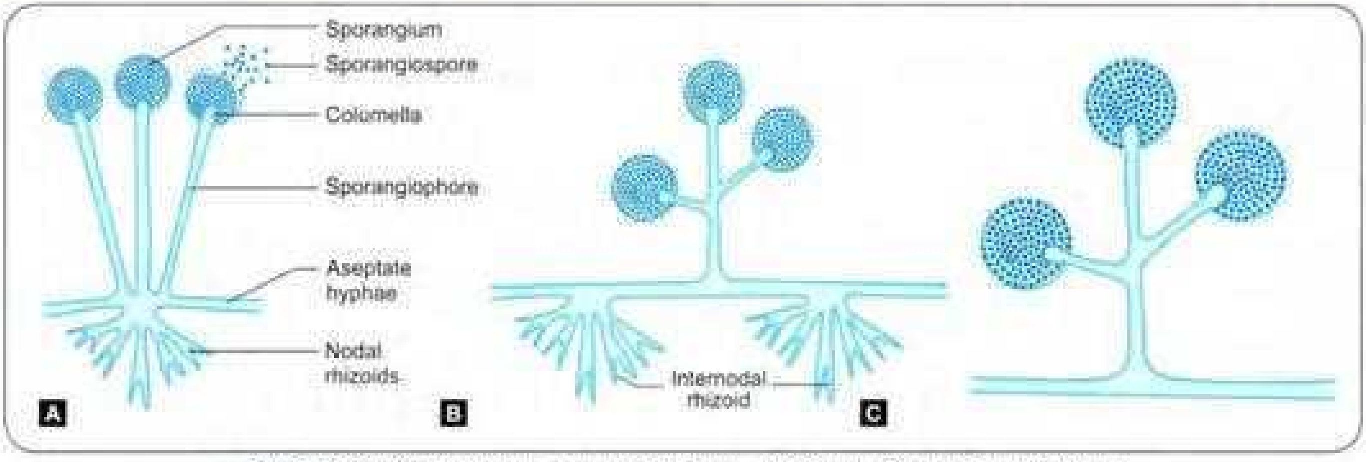
Fig. 52.23: Zygomycosis—histopathology of tissue section shows aseptate broad hyphae (Methenamine silver stain)

Source: Public Health Image Library/Dr Libero Ajello, ID#:4234/Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*).



Figs 52.24A to D: A. *Rhizopus* colony on SDA shows white cottony woolly colonies with black spores (salt and pepper appearance); **B.** *Mucor* on SDA—white cottony woolly colonies; **C.** LPCB mount of colonies of *Rhizopus* shows sporangium with rhizoid present; **D.** LPCB mount of colonies of *Mucor* shows sporangium with rhizoid absent

Source: A to C. Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry; D. Public Health Image Library/ Dr Lucille K Georg, ID#:3960/ Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*).



Figs 52.25A to C: Microscopic schematic diagram. **A.** *Rhizopus*; **B.** *Lichtheimia*; **C.** *Mucor*

Entomophthoromycosis

This includes the subcutaneous lesions produced by members of the order Entomophthorales, i.e. *Conidiobolus* and *Basidiobolus;* the latter is also associated with visceral involvement.

ASPERGILLOSIS

Aspergillosis refers to the invasive and allergic diseases caused by a hyaline mold named *Aspergillus*. There are nearly 35 pathogenic and allergenic species of *Aspergillus*, important ones being—*A. fumigatus*, *A. flavus* and *A. niger*.

Pathogenesis

Aspergillus species are widely distributed in nature, most commonly growing on decaying plants, producing chains of conidia. Transmission occurs by inhalation of airborne conidia.

Risk factors for invasive aspergillosis are:

- Glucocorticoid use (the most important risk factor)
- Profound neutropenia
- Neutrophil dysfunction
- Underlying pneumonia, chronic obstructive pulmonary disease, tuberculosis or sarcoidosis
- Anti-tumor necrosis factor therapy.

Clinical Manifestations

The incubation period varies from 2 to 90 days. Depending upon the site of involvement, *Aspergillus* produces various clinical manifestations such as:

- **Pulmonary aspergillosis:** It is the most common form of aspergillosis; includes various manifestations such as:
  - Allergic bronchopulmonary aspergillosis (ABPA)
  - Severe bronchial asthma
  - Extrinsic allergic alveolitis
  - Aspergilloma (fungal ball)
  - Acute angioinvasive pulmonary aspergillosis
  - Chronic cavitary pulmonary aspergillosis.
- **Other forms of aspergillosis include:**
  - Invasive sinusitis
    - Invasive sinusitis (acute and chronic form)
    - Chronic granulomatous sinusitis
    - Maxillary fungal ball
    - Allergic fungal sinusitis
  - Cardiac aspergillosis: Endocarditis (native or prosthetic) and pericarditis
  - Cerebral aspergillosis: Brain abscess, hemorrhagic infarction, and meningitis
  - Ocular aspergillosis: Keratitis and endophthalmitis
  - Ear infection: Otitis externa
  - Cutaneous aspergillosis: Direct invasion of the skin occurs in neutropenic patients at the site of IV catheter insertion and in burns patients
  - Nail bed infection: Onychomycosis
  - Mycotoxicosis: Various *Aspergillus* species produce several fungal toxins; e.g. *A. flavus* produces aflatoxin, which causes liver carcinoma (see Table 52.12).

Clinical manifestations also depend on the species involved:

- *A. fumigatus* accounts for most of the cases of acute pulmonary and allergic aspergillosis
- *A. flavus* is more common in hospitals and causes more sinus, skin and ocular infections than *A. fumigatus*
- *A. niger* can cause invasive infection but more commonly colonizes the respiratory tract and causes otitis externa.

Laboratory Diagnosis

Specimens such as sputum and tissue biopsies may be collected.

Direct Examination

KOH (10%) mount or histopathological staining (Fig. 52.26) of specimens reveals characteristic narrow septate hyaline hyphae with acute angle branching.

Culture

Specimens are inoculated onto SDA and incubated at 25°C. Species identification is done based on macroscopic and microscopic (LPCB mount) appearance of the colonies (Table 52.11).

- Colonies consist of hyaline septate hyphae from which conidiophores arise which end at vesicles. Vesicles are either tubular or globular in shape
- From the vesicle, finger-like projections of conidia producing cells arise called **phialides** or **sterigmata**. Phialides are arranged either in one or two rows, the first row is called **metulae**
- Conidia arise from the vesicles either on their entire surface or only on the upper half (Figs 52.27A to C and Table 52.11).

Fig. 52.26: Hematoxylin-eosin stained (H and E) lung section shows septate narrow hyphae with acute angle branching—confirms invasive aspergillosis

Source: Public Health Image Library/Armed Forces Institute of Pathology; Dr Hardin, ID#:15630/Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

Antigen Detection

ELISA (kinetic ELISA) detecting *Aspergillus* specific galactomannan antigen in patient's sera or urine is useful for establishing early diagnosis.

Antibody Detection

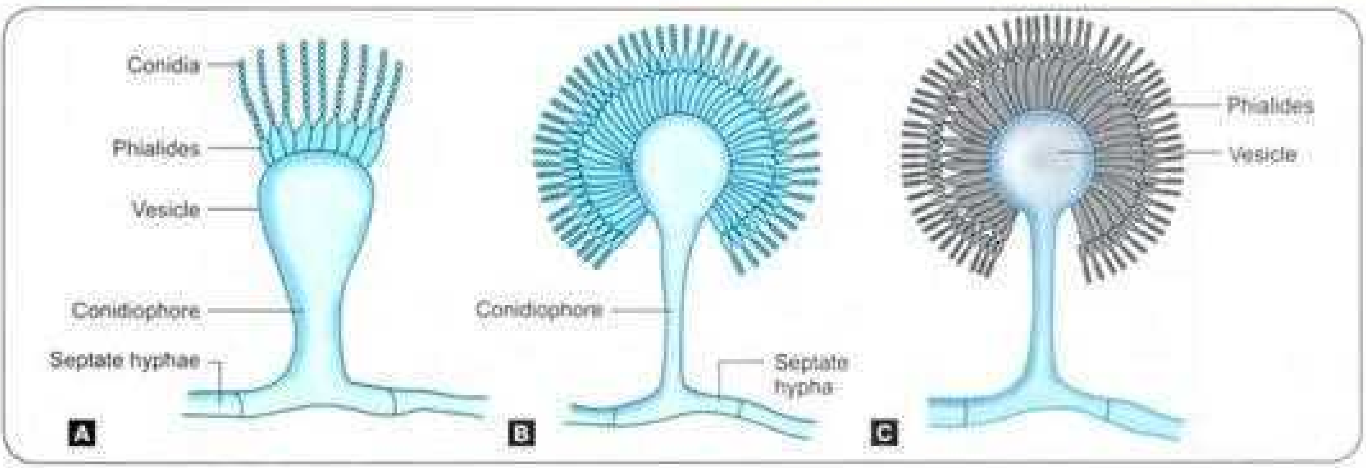
- Detection of serum antibodies is very useful for chronic invasive aspergillosis and aspergilloma, where the culture is usually negative. Titer falls rapidly following clinical improvement
- In allergic syndromes such as ABPA and severe asthma, specific serum IgE levels are elevated.

Detection of Metabolites

Detection of β-1-3-D-glucan (by G test) or mannitol (by gas liquid chromatography) is useful alternative for establishing the diagnosis, particularly when the culture is negative.

Table 52.11: Identification features of *Aspergillus* species

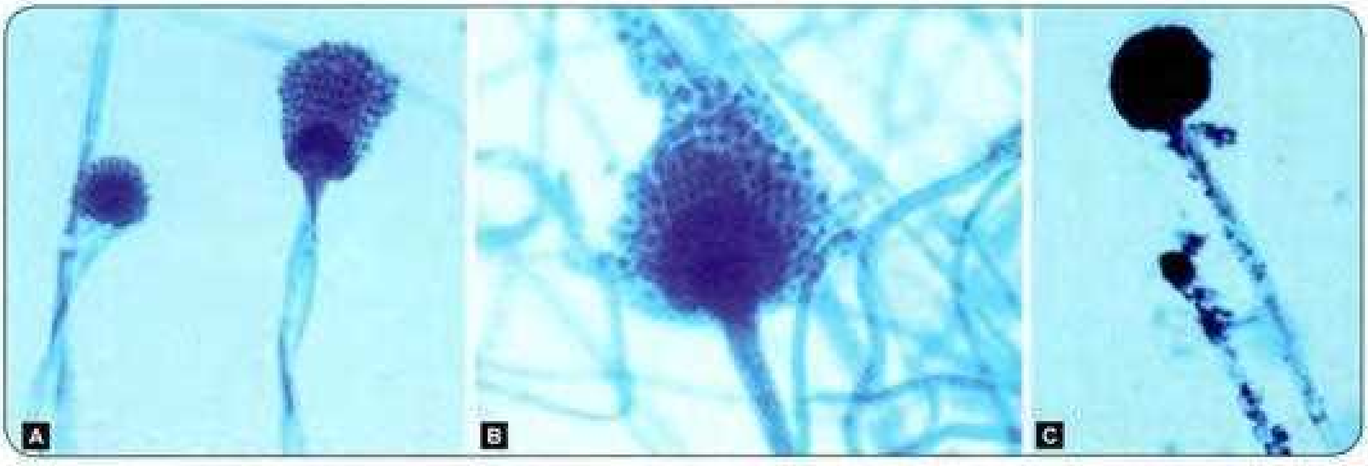
Aspergillus species	Macroscopic appearance of colony	Microscopic appearance of colony (LPCB mount)
A. fumigatus (Figs 52.27A, 52.28A, 52.29A)	Colonies—smoky green, velvety to powdery, reverse is white	• Vesicle is conical-shaped • Phialides are arranged in single row • Conidia arise from upper third of vesicle • Conidia are hyaline
A. flavus (Figs 52.27B, 52.28B, 52.29B)	Colonies—yellow green, velvety, reverse is white	• Vesicle is globular-shaped • Phialides in one or two rows • Conidia arise from entire vesicle • Conidia are hyaline
A. niger (Figs 52.27C, 52.28C, 52.29C)	Colonies—black, cottony type, reverse is white	• Vesicle is globular-shaped • Phialides in two rows • Conidia arise from entire vesicle • Conidia are black in color



Figs 52.27A to C: Conidiation of various *Aspergillus* species. **A.** *A. fumigatus*; **B.** *A. flavus*; **C.** *A. niger*

Figs 52.28A to C: *Aspergillus* (colonies on SDA). **A.** *Aspergillus fumigatus*; **B.** *Aspergillus flavus*; **C.** *Aspergillus niger*

Source: Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (with permission).



Figs 52.29A to C: *Aspergillus* microscopic picture (LPCB mount). **A.** *Aspergillus fumigatus*; **B.** *Aspergillus flavus*; **C.** *Aspergillus niger*

Source: A to C. Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry (with permission).

Table 52.12: Features of common mycotoxins

Mycotoxin	Produced by fungal species	Source	Clinical condition
Aflatoxin	*Aspergillus flavus* *Aspergillus parasiticus, A. nomius* *Penicillium puberulum*	Nuts, maize	Hepatoma, hepatitis Indian childhood cirrhosis Reye's syndrome
Fumonisins	*Fusarium moniliforme*	Maize	Equine leukoencephalomalacia Porcine pulmonary edema Carcinoma esophagus
Trichothecenes	*Fusarium graminearum*	Maize, wheat, sorghum	Alimentary toxic aleukia Biological warfare (yellow rain)
Ochratoxin	*Aspergillus ochraceus, A. niger* *Penicillium verrucosum*	Cereals, bread	Nephropathies (Balkan endemic nephropathy)
Cyclopiazonic acid	*Aspergillus flavus, A. versicolor, A. oryzae* *Penicillium cyclopium*	Groundnut, corn	Kodua poisoning Co-contaminant with aflatoxin
Zearalenones	*Fusarium graminearum*	Wheat, maize	Genital disorder in pigs

Skin Test

Positive skin test to various antigen extracts of *Aspergillus* indicates hypersensitivity response and is usually positive for various allergic type of aspergillosis.

TREATMENT — Aspergillosis

Following are the first line treatment recommended in different forms of aspergillosis.
- For invasive aspergillosis—voriconazole is the drug of choice.
- For ABPA—itraconazole is the drug of choice.
- For single aspergilloma—surgery is indicated.
- For chronic pulmonary aspergillosis—itraconazole or voriconazole is the drug of choice.
- For prophylaxis, posaconazole is indicated.

PENICILLIOSIS

Penicilliosis denotes the group of infections caused by pathogenic *Penicillium* species.

Clinical Significance

Penicillium has more than 250 species, most are found as saprophytes in the environment. However, some species are associated with human diseases such as:
- *Penicillium marneffei* (a dimorphic fungus, described later)
- Mycotoxicoses is caused by toxins released by certain species of *Penicillium* such as *P. cyclopium, P. verrucosum* and *P. puberulum* (Table 52.12)
- Other *Penicillium* species are usually found in environment and are isolated as common laboratory contaminants. Rarely they are associated with human diseases such as:
  - Invasive penicilliosis: For example, endophthalmitis and endocarditis
  - Superficial disease: Such as otomycosis, keratitis and onychomycosis
  - Allergic disease: For example, asthma and allergic pneumonitis.

Laboratory Diagnosis

Except for *P. marneffei* which is a dimorphic fungus, all other *Penicillium* species occur only as molds, can grow easily on SDA at 25°C.
- **Colonies** are rapid growing, flat with velvety to powdery texture and greenish in color (Fig. 52.30A)
- **Microscopic appearance:** LPCB mount of the colonies reveals hyaline thin septate hyphae, and the vesicles are absent. The conidiophore and its branches give rise to elongated metulae, from which flask-shaped phialides originate which bear chain of conidia. Such an arrangement is called as **brush border appearance** (Figs 52.30B and C).

Penicillium marneffei

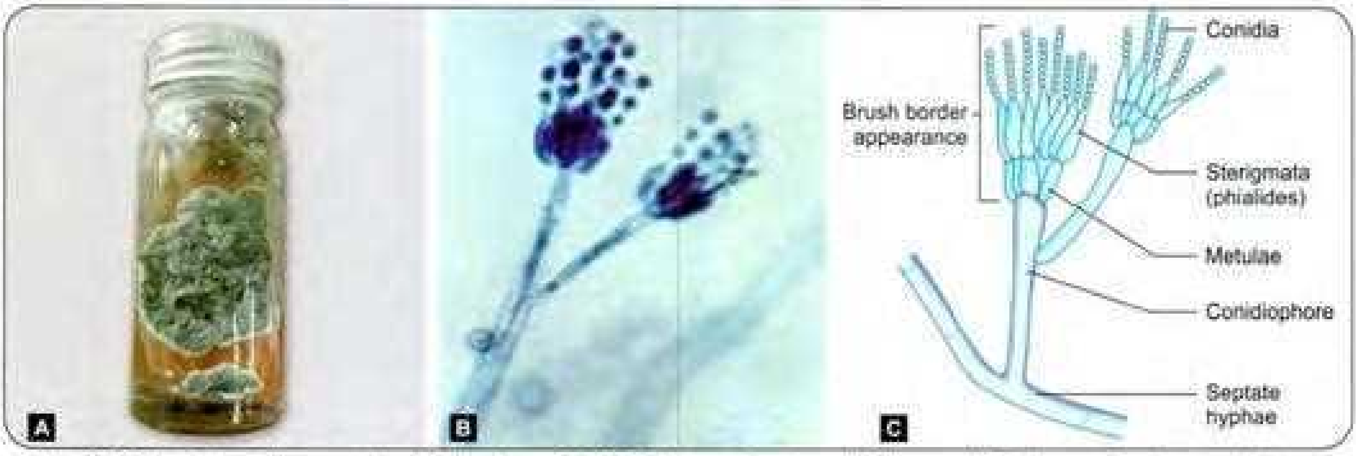
Penicillium marneffei is a thermally dimorphic fungus that causes opportunistic infection in HIV-infected patients. It is recently renamed as *Talaromyces marneffei*.

Epidemiology

P. marneffei is endemic in South East Asian countries including Thailand, Vietnam and India (Manipur).

Pathogenesis

P. marneffei is found mostly in rural areas where the bamboo rats are prevalent, which are the reservoirs of infection; however, there is no direct rat to man transmission.
- Immunocompromised hosts (e.g. patients with advanced AIDS) are at higher risk
- Transmission is by inhalation of conidia from the environment
- Mold to yeast conversion occurs in the lungs and then the yeast form spreads via blood to the reticuloendothelial system.



Figs 52.30A to C: *Penicillium* species. **A.** Colonies on SDA; **B.** Microscopic picture (LPCB mount); **C.** Schematic microscopic picture

Source: A. Department of Microbiology, Pondicherry Institute of Medical Sciences, Puducherry. B. Public Health Image Library/Lucille Georg, ID#:8398/Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

Clinical Manifestations

P. marneffei produces two types of clinical manifestations.

1. **Systemic infection:** The manifestations are similar to that of disseminated histoplasmosis such as fever, weight loss, dyspnea, lymphadenopathy and hepatosplenomegaly.
2. **Skin lesions:** Warty lesions mimicking that of molluscum contagiosum are seen.

Laboratory Diagnosis

- **Histopathological staining** of tissue sections, skin scrapings or blood smear shows **oval or elliptical yeast cells with central septation**, which indicates that these cells divide by transverse fission rather than budding (Fig. 52.31A)
- **Culture:** *P. marneffei* being dimorphic; produces yeast like colonies at 37°C and mold form at 25°C
- The mold form has a characteristic **brick red pigment** (Fig. 52.31B), but the microscopic appearance of mold form is similar to other *Penicillium* species.

TREATMENT **Penicilliosis**

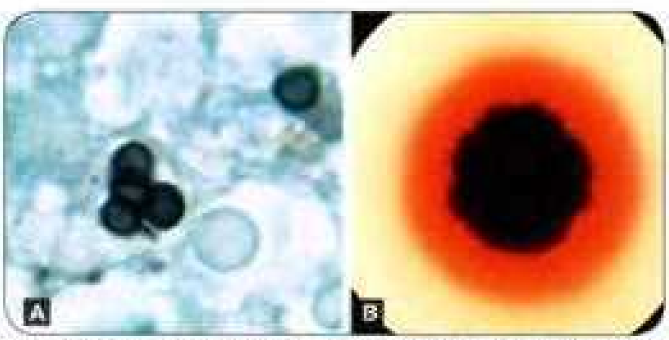
AIDS patients with severe penicilliosis are treated with amphotericin B till the condition improves followed by maintenance therapy with itraconazole for 12 weeks. In mild penicilliosis, itraconazole is recommended for 12 weeks.

PNEUMOCYSTIS PNEUMONIA

Pneumocystis pneumonia (PcP) has been increasingly reported after the discovery of HIV/AIDS.

Taxonomy

Recently, the taxonomy of *Pneumocystis* has been changed (2002). Once thought to be a protozoan, now it is classified under fungus based on nucleic acid sequence studies.



Figs 52.31A and B: *Penicillium marneffei*. **A.** Methenamine silver staining shows yeast cells with central septations; **B.** Red pigmented colony (mold form)

Source: Public Health Image Library/Dr Libero Ajello, A. ID#:11959 and B. ID#:11967/ Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

- Taxonomists renamed the human species of *Pneumocystis* as *Pneumocystis jirovecii*
- The previously used species name *P. carinii* has been assigned to describe the rat species of *Pneumocystis*.

Pathogenesis

Like protozoa, *Pneumocystis* exists in cyst and trophozoite forms. The cysts are found in environment, whereas in human tissues both cysts and trophozoites (containing 4–8 sporozoites) are found.

Once inhaled, the cysts are carried to the lungs where they transform into the trophozoite stage. The trophozoites induce an inflammatory response, that leads to recruitment of plasma cells resulting in formation of **frothy exudate** filling the alveoli. Hence, this condition is also called **plasma cell pneumonia**.

Laboratory Diagnosis

Histopathological examination of lung tissue or fluids obtained by bronchoscopy, bronchial lavage, or open lung biopsy reveals **cysts**.

- Gomori's methenamine silver (GMS) staining is the method of choice to demonstrate the cysts of *P. jirovecii*. The cysts resemble black colored **crushed ping-pong balls**, against the green background (Fig. 52.32)
- *Pneumocystis* is not cultivable and there is no serological test available
- PCR assay has been developed for detection of *P. jirovecii* specific genes.
- Detection of 1, 3 β-D-glucan in serum.

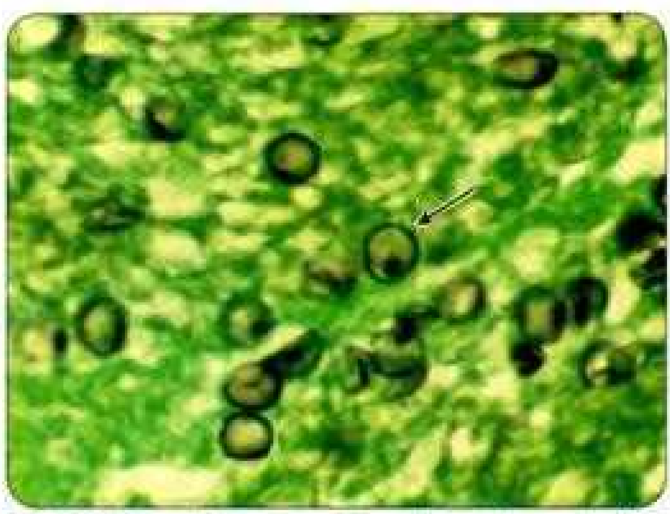


Fig. 52.32: Cysts of *Pneumocystis jirovecii* in lung tissue of an AIDS patient (methenamine silver stain) (arrow showing)

Source: Public Health Image Library/Dr Edwin P. Ewing, Jr. ID#: 960/Centers for Disease Control and Prevention (CDC), Atlanta (with permission).

TREATMENT ***Pneumocystis* pneumonia**

Cotrimoxazole (trimethoprim/sulfamethoxazole) is the drug of choice for *Pneumocystis* pneumonia. It is given for 14 days in non-HIV patients and 21 days in patients with HIV. It is also the recommended drug for primary and secondary prophylaxis in patients with HIV.

FUSARIOSIS

Pathogenesis

Fusarium species are soil and plant saprophytes found worldwide. They rarely cause human infections.

- In immunocompetent individuals, they cause:
  - Keratitis in contact lens wearers
  - Onychomycosis.
- In immunocompromised patients—they are angioinvasive and cause pulmonary and sinus infection
- In patients with neutropenia and hematologic malignancies, disseminated fusariosis occurs with frequent skin lesions
- Important species infecting humans are: *F. solani* (most common), followed by *F. oxysporum* and *F. verticillioides*.

Laboratory Diagnosis

Fusarium is a filamentous fungus, grows rapidly on SDA at 25°C and produces woolly to cottony, flat, spreading white to pink colonies. LPCB mount of the colony reveals hyaline septate hyphae bearing round microconidia, sickle-shaped large macroconidia and chlamydospores (Fig. 52.33).

TREATMENT **Fusariosis**

Fusarium species are resistant to many antifungal agents. Liposomal amphotericin B, voriconazole or posaconazole are recommended.

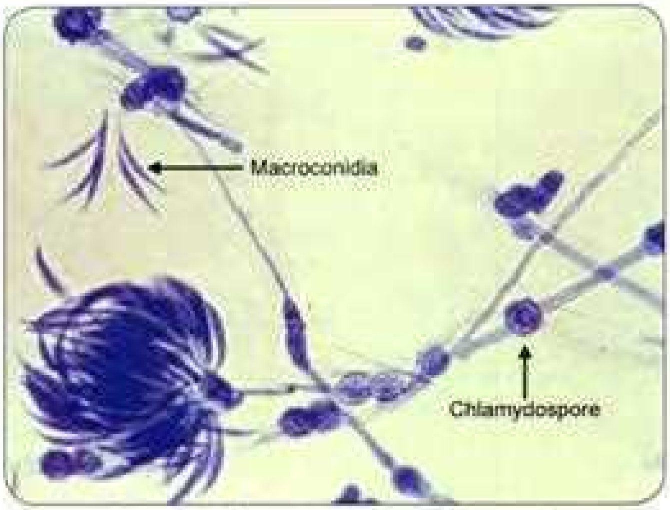


Fig. 52.33: *Fusarium* species (LPCB mount)

Source: Public Health Image Library, ID#: 17970/Centers for Disease Control and Prevention (CDC), Atlanta (*with permission*).

MYCOTOXICOSES

Mycotic poisoning can be classified into two varieties:

1. **Mycotoxicosis:** Refers to the disease produced following consumption of food contaminated by toxins liberated by certain fungi (see Table 52.12).
2. **Mycetism:** Refers to the toxic effects produced by eating poisonous fleshy fungi; usually different types of mushrooms (Table 52.13).

Table 52.13: Features of common mycetism

Mushroom poisoning	Produced by fungal species	Source	Clinical condition
Ergot alkaloid	*Claviceps purpurea*	Rye flour	St. Anthony's fire
Coprine poisoning	*Coprine atrementarius*	Butter	Antabuse like reaction
Muscarine	*Inocybe fastigiata*	Food	Cholinergic effect
Ibotenic acid, muscimol	*Amanita pantherina*	Edible mushroom	Abdominal pain, vomiting, diarrhea
Cyclopeptide	*Amanita phalloides*	Edible mushroom	Hepatocellular failure, green death cap

EXPECTED QUESTIONS

I. Essay:

1. A 29-year-old HIV-infected male presents to the clinic with a 5-day history of a mild burning sensation in his mouth that is accentuated when eating acidic or spicy foods. The oral examination showed creamy white patches on the oral mucosa. Scraping obtained from the patches revealed gram-positive oval budding yeast cells with pseudohyphae.
   a. What is the clinical diagnosis and the likely etiological agent?
   b. Name the risk factors predisposing for this condition.
   c. What are the other clinical manifestations caused by this organism?
   d. Describe the laboratory diagnosis of this clinical condition in detail.
2. A 21-year-old male had developed characteristic well-demarcated annular or ring-shaped pruritic scaly skin lesions with central clearing and raised edges. Culture of the skin scraping done on Sabouraud's dextrose

agar reveals velvety colonies with red pigment on the reverse. Microscopy of the culture isolate reveals plenty of tear drop-shaped microconidia and few, long, pencil-shaped macroconidia.
  a. What is the clinical diagnosis and what is the most likely etiological agent?
  b. Describe the various clinical manifestations produced by this organism.
  c. Add a note on the laboratory diagnosis of this condition.
3. Classify various types fungal diseases. Describe the etiology, clinical manifestations and laboratory diagnosis of mycetoma.

II. Write short notes on:
1. Cryptococcosis.
2. Histoplasmosis.
3. Dimorphic fungi.
4. Mucormycosis.
5. *Pneumocystis* pneumonia (PcP).
6. Aspergillosis.

III. Multiple Choice Questions (MCQs):

1. All are yeast or yeast like fungi except:
a. *Candida* b. *Trichosporon*
c. *Cryptococcus* d. *Trichophyton*

2. Fungi which do not have sexual stage:
a. Zygomycota b. Ascomycota
c. Basidiomycota d. Fungi imperfecti

3. Organism that does not affect nail:
a. *Trichophyton* b. *Epidermophyton*
c. *Microsporum* d. *Candida albicans*

4. A patient coming from Himachal Pradesh, presents with multiple skin lesions. Microscopy reveals cigar-shaped yeast cells and asteroid bodies. Microscopy of culture shows 'flower like' pattern. Identify the agent?
a. *Candida* b. *Sporothrix schenckii*
c. *Epidermophyton* d. *Rhizopus*

5. Germ tube test is diagnostic for:
a. *Candida glabrata* b. *Candida albicans*
c. *Cryptococcus* d. *Coccidioides immitis*

6. Example for fungus having branching, aseptate hyphae are all except:
a. *Rhizopus* b. *Absidia*
c. *Penicillium* d. *Mucor*

7. Most common fungus causing orbital cellulitis in a patient with diabetic ketoacidosis is:
a. *Mucor* b. *Aspergillus*
c. *Candida* d. *Cryptococcus*

8. Asteroid bodies is observed in:
a. Sporotrichosis b. Histoplasmosis
c. Candidiasis d. Chromoblastomycosis

9. Chromoblastomycosis is caused by:
a. *Epidermophyton* b. *Histoplasma*
c. *Exophiala* d. *Penicillium*

10. Which of the following organism is not been isolated in artificial culture media?
a. *Cryptococcus neoformans*
b. *Rhinosporidium seeberi*
c. *Histoplasma capsulatum*
d. *Penicillium marneffei*

11. Fluorescent dye used for detection of fungi in tissue specimen is:
a. Methenamine silver stain
b. Calcofluor white
c. Hematoxylin and Eosin staining
d. India ink

12. Which of the following is added in Sabouraud's Dextrose Agar to suppress the growth of contaminating (saprophytic) fungi?
a. Cycloheximide b. Chloramphenicol
c. Gentamicin d. Amphotericin B

13. Tinea versicolor is caused by:
a. *Candida albicans* b. *Trichophyton rubrum*
c. *Trichophyton violaceum*
d. *Malassezia furfur*

14. Broad-based budding is seen in:
a. *Histoplasma* b. *Blastomyces*
c. *Cryptococcus* d. *Penicillium*

15. Aflatoxin is produced by:
a. *Aspergillus flavus* b. *Histoplasma*
c. *Sporothrix schenckii* d. *Penicillium marneffei*

16. Spherules are seen in:
a. Chromoblastomycosis
b. Rhinosporidiosis
c. Mucormycosis d. Aspergillosis

17. Barrel-shaped arthroconidia are seen in:
a. Histoplasmosis b. Cryptococcosis
c. Coccidioidomycosis
d. Paracoccidioidomycosis

18. Sclerotic bodies are seen in:
a. Mucormycosis b. Aspergillosis
c. Rhinosporidiosis d. Chromoblastomycosis

19. Spaghetti and meatball appearance is seen in:
a. *Hortaea werneckii* b. *Trichosporon beigelii*
c. *Piedraia hortae* d. *Malassezia furfur*

20. The drug of choice for *Pneumocystis* pneumonia is:
a. Amphotericin B b. Flucytosine
c. Cotrimoxazole d. Voriconazole

21. Sickle-shaped large macroconidia is seen in:
a. *Microsporum* b. *Epidermophyton*
c. *Fusarium* species d. *Pneumocystis jirovecii*

22. The cysts resemble crushed ping-pong balls is seen with:
a. *Microsporum* b. *Pneumocystis jirovecii*
c. *Epidermophyton* d. *Fusarium* species

23. Azole active against mucormycosis is:
a. Voriconazole b. Fluconazole
c. Itraconazole d. Posaconazole

24. *Candida* species resistant to azoles is:
a. *C. albicans* b. *C. krusei*
c. *C. tropicalis* d. *C. dubliniensis*

25. β-1-3-D-glucan is detected for all, *except*:
a. *Candida* species b. *Cryptococcus* species
c. *Aspergillus* species d. *Pneumocystis jirovecii*

Answers

1. d **2.** d **3.** c **4.** b **5.** b **6.** c **7.** a **8.** a **9.** c **10.** b **11.** b **12.** a **13.** d **14.** b **15.** a **16.** b
17. c **18.** d **19.** d **20.** c **21.** c **22.** b **23.** d **24.** b **25.** b

Hospital Infection Control

SECTION 6

Section Outline

Hospital-Acquired Infections

53
CHAPTER

Chapter Preview

- Hospital-acquired infections
- Major HAI types
- Prevention of HAIs
- Hospital infection control committee

INTRODUCTION

Definition

Hospital-acquired infections (HAIs) or nosocomial infections or healthcare-associated infections (HAIs) can be defined as the infections acquired in the hospital by a patient:

- Who was admitted for a reason other than that infection.
- In whom the infection was not present or incubating at the time of admission
- Symptoms should appear at least after 48 hours after admission
- This include infections acquired in the hospital but appearing after discharge, and also occupational infections among staff of the hospital care facility.

CDC (Centers for Disease Control and Prevention, Atlanta) has established the National Healthcare Safety Network (NHSN) to monitor the incidence of nosocomial infections.

It is estimated that 5-10% of patients admitted to acute care hospitals develop HAIs. Treatment of these HAIs adds a huge economic burden to the hospital.

Factors Affecting HAIs

The principal factors that determine the likelihood that a given patient would acquire a nosocomial infection are:

- **Immune status:** Most admitted patients have impaired immunity either as a part of their preexisting disease or in some instances, due to the treatment they have received in the hospital
- **Hospital environment**: The hospital environment harbors a greater magnitude of microorganisms than that of community. Transmission of these organisms to the patients can cause nosocomial outbreaks of infection
- **Hospital organisms:** Most of the organisms present in the hospital environment are multidrug resistant. This is because of the increased antibiotic usage in the hospital. The minor population of resistant organisms present initially flourish in presence of constant antibiotic pressure and slowly replace the susceptible strains in the hospital
- **Diagnostic or therapeutic interventions** such as insertion of intravenous or urinary catheters, or endotracheal tube, may introduce infection in susceptible patients; most of which are due to the patient's endogenous flora
- **Transfusion:** Blood, blood products and intravenous fluids used for transfusion, if not properly screened, can transmit many blood-borne infections (BBI) such as HIV, hepatitis B and C viruses
- **Poor hospital administration:** Strong administrative support is essential to control the HAIs; failing of which promote the spread of HAIs.

Sources of Infection

Endogenous Source

The majority of nosocomial infections are endogenous in origin, i.e. they involve patient's own microbial flora which may invade the patient's body during some surgical or instrumental manipulations.

Exogenous Source

Exogenous sources are from hospital environment, staff, or patients.

- **Environmental sources** include inanimate objects, air, water and food in the hospital. Inanimate objects in the hospital are medical equipment (endoscopes, catheters, etc.), bed pans, surfaces contaminated by patients' excretions, blood and body fluids
- **Healthcare workers** may be potential carriers, harboring many organisms; which may be multidrug resistant, e.g.

nasal carriers of Methicillin-resistant *Staphylococcus aureus* (MRSA)
- **Other patients** of the hospital may also be the source of infection.

Microorganisms Implicated in HAIs

Hospital-acquired infections can be caused by almost any microorganism, but those that survive in the hospital environment for long periods and develop resistance to antimicrobials and disinfectants are particularly important.
The ESKAPE pathogens: They are responsible for a substantial percentage of nosocomial infections in the modern era and represent the vast majority of multidrug resistant isolates present in a hospital.
- ***E**nterococcus faecium*
- ***S**taphylococcus aureus*
- ***K**lebsiella pneumoniae*
- ***A**cinetobacter baumannii*
- ***P**seudomonas aeruginosa*
- ***E**nterobacter* species.

Other infections that can spread in hospitals include:
- *Escherichia coli*
- Nosocomially-acquired *Mycobacterium tuberculosis*
- *Legionella pneumophila*
- *Candida albicans*
- *Clostridium difficile* diarrhea.
- Blood-borne infections transmitted through contaminated needle prick injury or mucocutaneous exposure of blood includes HIV, hepatitis B and C viral infections.

Modes of Transmission

Microorganisms spread in the hospital through several modes (Table 53.1).

MAJOR HAI TYPES

Though several types of HAIs exist, there are four most common types (listed below) which are often monitored to estimate the burden of HAI in a hospital. Out of these, the first three are together called as device associated infections (DAIs).
1. Catheter-associated urinary tract infection (CAUTI, 33%)
2. Central line-associated blood stream infection (CLABSI, 13%)
3. Ventilator-associated pneumonia (VAP, 15%)
4. Surgical site infection (SSI, 31%).

The surveillance diagnosis and prevention of these major HAI types have been described in detail in Chapter 54.

Catheter-associated Urinary Tract Infection

UTI accounts for the majority of HAIs. It can be: catheter associated (CAUTI) and non-catheter associated; the former being more common type:

Table 53.1: Modes of transmission of hospital-acquired pathogens.

Route	Description
Contact transmission	
Direct contact	Skin-to-skin contact and thereby physical transfer of microorganisms between a susceptible host and an infected or colonized person (usually healthcare workers, rarely other patients) **This is the most important and frequent mode of transmission**
Indirect contact	This involves contact of a susceptible host with contaminated inanimate objects such as: • Dressings, or gloves, instruments (e.g. stethoscope) • Parenteral transmission through: Needle or sharp prick injury, splashes of blood or body fluids or excretions, contaminated saline flush, syringes, vials and bags
Inhalational mode	
Droplet transmission	Droplets of >5 µm size can travel for shorter distance (<3 feet) • Droplets generated from the infected person while coughing, sneezing and talking are propelled for a short distance through air and deposited on the host's body • This is an important mode of transmission of agents causing bacterial meningitis, diphtheria, and RSV, etc.
Airborne transmission	This refers to the airborne droplet nuclei (≤5 µm size) or dust particles that remain suspended in the air for long time and can travel longer distance: • This is more efficient mode than droplet transmission • Microorganisms transmitted by airborne transmission include *Legionella, Mycobacterium tuberculosis*, measles and varicella-zoster viruses
Vector-borne transmission	
	• Via vectors such as mosquitoes, flies, etc. carrying the microorganisms • This is a rare mode of transmission in hospital
Common vehicle transmission	
	Such as food, water, devices and equipment

- **Risk factors** that predispose patients to acquire a nosocomial UTI include: i) advanced age, ii) female gender, iii) severe underlying disease, iv) placement of a urinary catheter for >2 days
- **Organisms:** Gram-negative rods cause the majority of hospital-acquired UTIs and *E. coli* is the most common organism implicated. Gram-positive bacteria such as *S. aureus*, enterococci can occasionally cause CAUTI.

Central Line Associated Blood Stream Infection

Central line associated blood stream infection (CLABSI) is the fourth common cause of HAIs.

- **Organisms:** Coagulase negative staphylococci, and *S. aureus* are increasingly reported to cause CLABSI recently, followed by gram-negative rods and *Candida*
- **Risk factors** that predispose to acquire a CLABSI include:
  - *Patient related:*
    - Age (<1 year and >60 years)
    - Malnutrition
    - Low immunity
    - Severe underlying disease
    - Loss of skin integrity (burn or bed sore)
    - Prolonged stay in ICUs.
  - *Device related:* Presence of central line
  - *HCW related:* Poor infection control practices such as hand hygiene.

Ventilator-associated Pneumonia

Ventilator-associated pneumonia (VAP) are the second common cause of HAIs next to UTI.

- **Risk factors** of developing hospital-acquired pneumonia include:
  - *Device related:* Endotracheal intubation
  - *Patient related:* (i) prolonged ICU stay leading to increased risk of colonization of hospital MDROs, (iii) aspiration of oropharyngeal flora due to various reasons such as semiconscious state, supine position, etc.
  - *HCW related:* Poor infection control practices such as poor hand hygiene.
- **Organisms:** Gram-negative rods such as *Acinetobacter* species and *Pseudomonas* species account for majority of VAP.

Surgical Site Infection (SSI)

Surgical site infection is defined as infection that develops at the surgical site within 30 days of surgery (within 90 days for breast, cardiac and joint surgeries).

- Though SSI is a major threat in the hospitals, it is often under reported because 50% of SSIs develop after the discharge
- **Organisms:** Surgical site wounds are classified as clean, clean-contaminated, contaminated or dirty
  - *For clean wound:* The skin flora of the surgery team or the environmental organisms are the major pathogens; most common being *S. aureus*
  - *For other types:* The patient's endogenous flora (anaerobes and gram-negative rods) are the common agents.
- **Risk factors** for nosocomial wound infection include:
  - Advanced age, obesity, malnutrition, diabetes
  - Infection at a remote site that spread through blood stream
  - Preoperative shaving of the site
  - Inappropriate timing of prophylactic antimicrobial agent.

  Note: The antimicrobial prophylaxis is usually given to the patient to prevent the seeding of organisms on the surgical site. It is given 1 hour prior to the incision, usually along with the induction of anesthesia.

PREVENTION OF HAIs

The preventive measures for HAIs can be broadly categorized into (i) standard precautions and (ii) transmission-based or specific precautions.

Standard (Routine) Precautions

Standard precautions are a set of infection control practices (see box below) used to prevent transmission of diseases that can be acquired by contact with blood, body fluids, non-intact skin (including rashes), and mucous membranes. These measures should be followed when providing care to:

- All individuals, whether they appear infectious/symptomatic or not
- All specimens (blood or body fluids) whether they appear infectious or not
- All needles and sharps whether they appear infectious or not.

Note: Universal precautions was a term used in the past to refer to the infection control practices to avoid contact with patients' body fluids, by means of wearing the nonporous articles such as medical gloves, goggles, and face shields. Now it is replaced by the word "standard precaution" which in addition include the measures to prevent contact (i.e. skin and mucosal) transmission.

Standard Precautions

They are indicated while handling all patients, specimens and sharps. Components of standard precautions include:

- **Hand hygiene:** (details explained later)
  - Wash hands promptly after contact with infective material
  - Use no touch technique wherever possible.
- **Personal protective equipment (PPE):** described later

Contd...

Contd...

- **Biomedical waste including sharp handling:** All biomedical waste including sharp should be segregated and disposed according biomedical waste rule 2016 (Chapter 55)
- **Spillage cleaning:** Clean up spills of infective material promptly (see Table 53.3)
- **Disinfection:** Ensure that patient-care equipment, supplies and linen contaminated with infective material are either discarded, or sterilized between each patient use
- **Respiratory hygiene and cough etiquette** (refer Table 53.4).

Hand Hygiene

Hands are the main source of transmission of infections during healthcare. Hand hygiene is therefore the most important measure to avoid the transmission of harmful microbes and prevent healthcare-associated infections.

Any healthcare worker involved in direct or indirect patient care needs to be concerned about hand hygiene and should be able to perform it correctly and at the right time.

Types of Hand Hygiene Methods

Hand Rub

Alcohol based (70–80% ethyl alcohol) and chlorhexidine (2–4%) based hand rubs are available. The duration of contact has to be at least for 20–30 seconds.

- **Advantage:** After a period of contact, it gets evaporated of its own hence drying of hands is not required separately
- **Indications:** Hand rub is indicated during routine rounds in the wards or ICUs; in all the moments or situations requiring hand hygiene, except when the hands are visibly dirty or soiled, when it will be ineffective.

Hand Wash

Antimicrobial soaps (liquid, gel or bars) are available. If facilities are not available, then even ordinary soap and water can also be used. The duration of contact has to be at least for 40–60 seconds. Hand washing is indicated in the following situations:

- When the hands are visibly soiled with blood, excreta, pus, etc.
- Before and after eating
- After going to toilet
- Before and after shift of the duty.

Five Moments for Hand Hygiene

The World Health Organization (WHO) has published standard guidelines describing the situations or opportunities when hand hygiene is indicated in healthcare sectors (Fig. 53.1).

Fig. 53.1: Five moments for hand hygiene

Source: World Health Organization (WHO) (with permission).

Steps of Hand Rubbing and Hand Washing

WHO has also laid down the guidelines describing the appropriate steps involved for an effective hand rubbing and hand washing (Fig. 53.2).

Personal Protective Equipment (PPE)

Personal protective equipment are used to protect the skin and mucous membranes of HCWs from exposure to

Table 53.2: Personal protective equipment (PPE)

Gloves (non-sterile)	Used when there is a risk of infection to HCWs (e.g. while touching blood, body fluids, secretions, excretions of patients, items/equipment or environment)
Gloves (sterile)	Used when there is a risk of infection to HCWs as well as to the patients (during surgeries /invasive procedures)
Plastic apron	Used during surgeries
Gown	Used during surgeries and when soiling is likely to be expected
Surgical mask	Used during surgeries and while handling patients on droplet precautions
N95 mask	Used while handling patients on airborne precaution (tuberculosis)
Cap, face shield, goggles	Used when spillage of blood is suspected, e.g. during major cardiac surgeries, etc.
Surgical shoes	Used mainly in ICUs and operation theaters to protect HCWs and environment from transmission of organisms

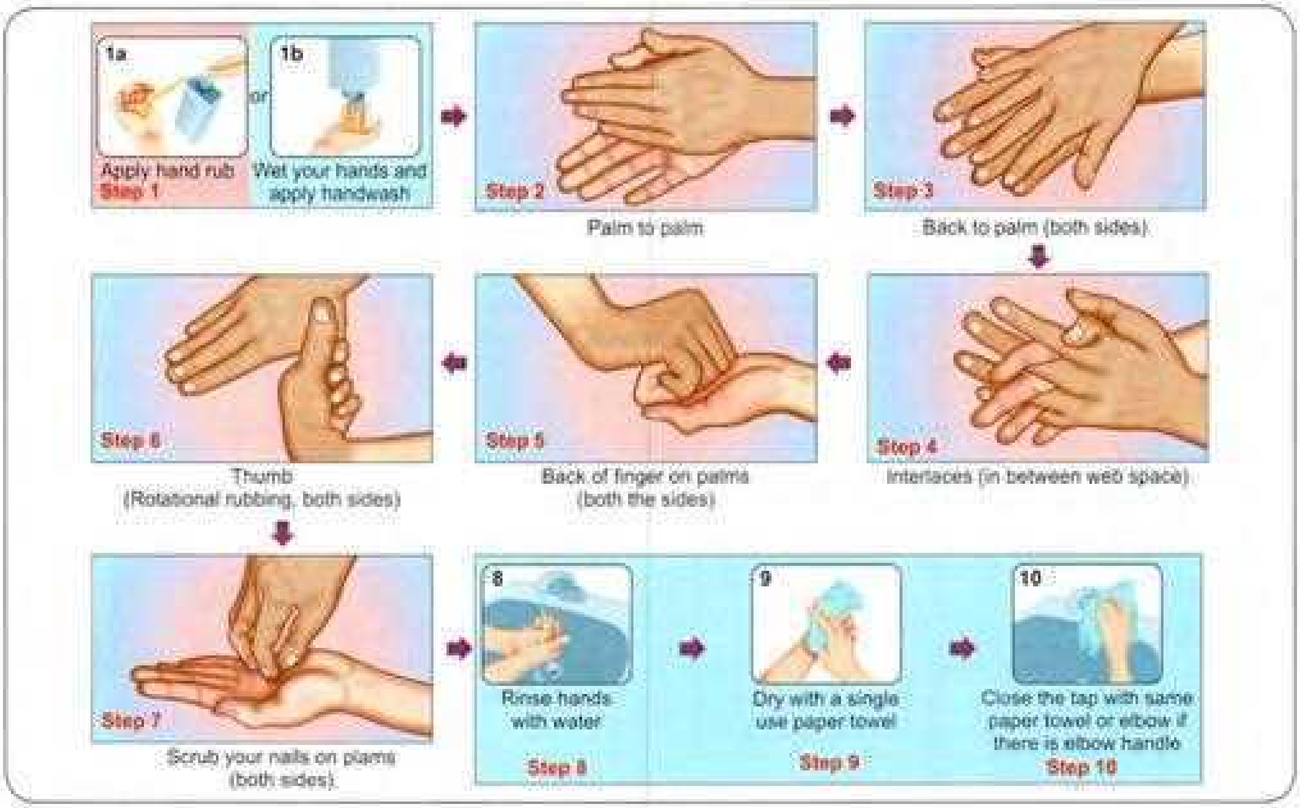


Fig. 53.2: Steps of hand rubbing and hand washing (WHO): Hand rub step 1 to 7 (20–40 seconds); Hand wash step 1 to 10 (40–60 seconds)

Figs 53.3A to I: Personal protective equipment (PPE): **A.** Gloves; **B.** Plastic apron; **C.** Gown; **D.** Surgical mask; **E.** N95 mask; **F.** Cap; **G.** Face shield; **H.** Goggles; **I.** Surgical shoes

blood and/or body fluids and from the HCW's hands to the patient during sterile and invasive procedures.

- The various PPE used in healthcare settings are described in Table 53.2 and Figure 53.3
- Selection of appropriate PPE is based on:
  - The level of risk associated with contamination of skin, mucous membranes, and clothing by blood and body fluids during a specific patient care activity or intervention (as a part of standard precaution)
  - Route of transmission of suspected organisms—contact, droplet and inhalation (as a part of transmission-based precaution).
- Though most PPE are used as a part of transmission-based precautions; there are some indications where some PPE are used as a part of standard precaution
- The PPE must be removed immediately following the indication for which it was used
- **Donning and doffing:** In order to minimize the risk of transmission of infection, donning (wearing) and doffing (removing) of PPE must be performed in a particular sequence
  - Donning (wearing): Gown first → Mask or respirator → Goggles or face shield → Gloves
  - Doffing (removing): Gloves → Face shield or goggles → Gown → Mask or respirator.

Spill management and respiratory hygiene and cough etiquette have been described in Tables 53.3 and 53.4 respectively.

Table 53.3: Spill management for blood and body fluids

Spill management of blood and body fluids: Bring the spill kit to the site of spillage, wear appropriate PPE (gloves and gown); put 'no entry' sign board near the spill area.

If spillage is small (<10 mL):
- Wipe up spill immediately with absorbent material and discard into appropriate bin
- Wipe the area with 10% sodium hypochlorite and allow to dry
- Remove PPE and perform hand hygiene

If spillage is large (>10 mL):
- Place disposable paper towels over spill to absorb the spillage and then pour 10% sodium hypochlorite on top of absorbent paper towels and leave for 15 minutes.
- Remove the absorbent papers; put fresh disposable paper towels to clean the area and then discard these into appropriate waste bin.

Table 53.4: Respiratory hygiene and cough etiquette

Anyone with signs and symptoms of a respiratory infection, regardless of the cause, should follow or be instructed to follow respiratory hygiene and cough etiquette:
- Cover the nose/mouth with single-use tissue paper when coughing, sneezing, wiping and blowing noses
- If no tissues are available, cough or sneeze into the inner elbow rather than the hand
- Follow hand hygiene after contact with respiratory secretions and contaminated objects/materials
- Keep contaminated hands away from the mucous membranes of the eyes and nose

In high-risk areas of airborne transmission such as pulmonary medicine OPD:
- Give mask to the patients with cough and make separate queue away from the general queue
- Sputum collection should be done in an open space or in a well-ventilated room

Transmission-based precautions (Specific precautions)

These are the additional precautions taken over and above the standard precautions when a disease of a specific transmission is suspected and where standard precautions may not be sufficient enough to prevent the transmission of infection. Based on the specific modes of transmission as discussed earlier (see Table 53.1); there are three types of transmission-based precautions (Table 53.5).

Contact Precautions

It is required when disease with contact transmission is suspected; for example,
- Patients with enteric infections and diarrhea which cannot be controlled
- Highly contagious skin lesions
- *Clostridium difficile* infection
- Multidrug resistant organisms (MDROs) isolated in a hospital setting, for example,
  - MRSA (Methicillin resistant *S. aureus*)
  - VRE (Vancomycin resistant enterococci)
  - CRE (Carbapenem resistant Enterobacteriaceae).

The components of contact precaution include:
- **Isolation:** Individual room for the patient (optional); otherwise cohorting of patients (desirable). Cohorting involves placing the patients infected with the same pathogen together in the same room or in the same cubicle of a ward
- **Patient movement:** Patient should be confined to the room. If transfer is required, then hand hygiene and appropriate PPE must be used and patient's colonized area must be covered
- **PPE:** Staff must wear gloves and gowns on entering the room
- **Patient dedicated equipment:** Equipment such as blood pressure cuffs, nebulizers, stethoscope must be used for single patient and must be disinfected before re-use
- **Hand washing** should be done before and after contact with the patient, and on leaving the room
- **Environmental cleaning** (floor, clothes, toilet) and equipment cleaning, with appropriate disinfectant is essential.

Droplet Precautions

It is required when disease with droplet transmission is suspected; for example, respiratory syncytial virus, *Mycoplasma*, parainfluenza, pertussis, plague, meningococcus, *C. diphtheriae*.

The components of droplet precaution include:
- **Isolation:** Individual room for the patient (essential), negative pressure room (desirable).
- **Patient movement:** Restricted movement of the patient; patient wears a surgical mask while leaving the room.
- **PPE:** Surgical mask for healthcare workers while entering the room.

Airborne Precautions

It is required when disease with airborne transmission is suspected; for example, pulmonary TB, chickenpox, measles and SARS (severe acute respiratory syndrome).

The components of airborne precaution include:
- **Isolation:** Individual room should be provided with adequate ventilation with negative pressure facility (essential).
- **Patient movement:** Patient should be confined to the room all the time.
- **PPE:** Staff should wear high-efficiency masks (N95 mask) while entering the room. Patients may wear surgical mask all the time.

HOSPITAL INFECTION CONTROL COMMITTEE

The hospital infection control program is organized and run by the Medical Superintendent (MS), for which

Table 53.5: Measures be followed during standard and transmission-based precautions

Type	Indication	Isolation[1]	Gloves	Gown	Mask	Eye protection	Handling of equipment	Visitors
Standard	Should be followed for: • All patients • All blood/body fluids • All sharps Irrespective of their infection status	Not required	As required*	If soiling likely	Wear surgical mask during procedures likely to generate splash from blood and body fluids	As required**	Reprocess before reuse on next patient	No additional precaution
Contact	MDROs, *C. difficile* Diarrheal pathogens Highly contagious skin infections	Essential	Essential	Essential	Surgical mask—Required if infectious agent is also transmitted by droplet	As required**	Single use or reprocess before reuse on next patient	Same precautions as for staff
Droplet	Respiratory syncytial virus *Mycoplasma* Parainfluenza Pertussis Plague Meningococcus	Essential	As required*	If soiling likely	Surgical mask is essential	As required**	Same as contact	Restrict visitor numbers and precautions same as for staff
Airborne	Pulmonary TB Chickenpox Measles SARS	Essential (negative pressure)	As required*	If soiling likely	N95 respirator is essential	As required**	Same as contact	Restrict visitor numbers and precautions same as for staff

*Gloves are used when likely to touch blood, body fluids and contaminated items; must be removed after single use.
**Eye protection is required during procedures likely to generate contamination with aerosols.
[1]Single room or cohorting.

he/she constitutes the Hospital Infection Control Committee (HICC).

The HICC provides a forum for multidisciplinary input and cooperation, and information sharing, required for hospital infection control and prevention. The HICC is advisory to the MS and makes its recommendations to the MS.

HICC Constitution

The hospital infection control committee (HICC) should include wide representations from relevant departments/health sectors as follows:

- Chairperson, usually the Medical Superintendent
- Secretary, usually the head of department of microbiology
- Hospital Infection Control Officer (HICO), usually a representative from the department of microbiology
- Hospital Infection Control Nurses (HICN)
- Head of all the clinical (all medical and all surgical) departments
- Nursing Superintendent
- Head of the staff clinic
- Operation Room Supervisor
- In-charge of Central Sterile Supplies Department (CSSD)
- In-charge of pharmacy
- In-charge of hospital linen
- In-charge of hospital laundry
- In-charge of hospital kitchen
- Epidemiologist
- In-charge of engineering department of hospital.

Functions of HICC

The HICC supervises the implementation of the hospital infection control program. The various functions of the committee include:

- **HAI surveillance:** Maintains surveillance of hospital-acquired infections. The four key parameters used for HAI surveillance are as follows (refer chapter 54):
  1. CA-UTI (Catheter-associated urinary tract infection).
  2. CLABSI (Central line-associated bloodstream infection).
  3. VAP (Ventilator-associated pneumonia).
  4. SSI (Surgical site infection).
- **Develops a system** for identifying, reporting, analyzing, investigating and controlling hospital-acquired infections.
- **Antimicrobial stewardship program (AMSP):** Develops antibiotic policies, monitors the antibiotic usage, advises the MS on matters related to the proper use of antibiotics, and also recommends remedial measures when antibiotic resistant strains are detected (Refer Chapter 57 for detail).
- **Policies:** Reviews and updates the hospital infection control policies and guidelines from time to time.
- **Education:** Conducts teaching sessions for healthcare workers regarding matters related to HAIs.

- **Staff health:** Monitors employee health activities regarding matters related to HAIs such as needle stick injury prevention, hepatitis B vaccination, etc.
- **Outbreak management:** Develops strategies to identify infectious outbreaks, their source and implements preventive and corrective measures
- **Other departments:** Communicates and cooperates with other departments of the hospital with common interests such as:
  - Pharmacy
  - Central Sterile Supplies department (CSSD)
  - Linen and laundry department(s)
  - Antimicrobial Usage Committee
  - Biomedical Safety Committee
  - Blood Transfusion Committee.
- **Reviews** risks associated with new technologies, and monitor infectious risks of new devices and products, prior to their approval for use
- **HICC Meetings:** HICC shall meet regularly not less than once a month and as often as required. However, in an emergency (such as an outbreak), this committee must be able to meet promptly.

EXPECTED QUESTIONS

I. Write short notes on:

1. Modes of transmission of hospital-acquired pathogens.
2. Prevention of healthcare associated infections.
3. Hospital infection control committee (HICC).
4. Hand hygiene.
5. Standard (routine) precautions.

II. Multiple Choice Questions (MCQs):

1. Hand rub should not be used in which condition?
   a. Before touching patient
   b. After touching patient
   c. After touching patient's surrounding
   d. Hands are visibly soiled

2. How many moments of hand hygiene have been laid down by WHO?
   a. 5 b. 6
   c. 7 d. 8

3. Hand rub should be performed for minimum of how much duration?
   a. 20 seconds b. 40 seconds
   c. 60 seconds d. 2 minutes

4. Hand wash should be performed for minimum of how much duration?
   a. 20 seconds b. 40 seconds
   c. 60 seconds d. 2 minutes

5. Which is not a component of WHO's 5 moments of hand hygiene?
   a. Before touching the patient
   b. After touching the patient
   c. Before touching patient's surrounding
   d. After touching patient's surrounding

6. What is the correct sequence of donning of PPEs?
   a. Gloves → Gown → Mask → Goggles
   b. Gown → Mask → Goggles → Gloves
   c. Goggles → Gloves → Mask → Gown
   d. Mask → Gown → Goggles → Gloves

7. Large blood spillage should be disinfected with:
   a. Phenol b. Soap and water
   c. Lysol d. Hypochlorite

8. Negative pressure room is required for?
   a. Standard precaution
   b. Contact precaution
   c. Droplet precaution
   d. Airborne precaution

9. Which transmission-based precaution has to be followed while handling patients with chickenpox?
   a. Standard precaution
   b. Contact precaution
   c. Droplet precaution
   d. Airborne precaution

10. Airborne precaution is followed for all of the following, *except*:
   a. Pulmonary TB b. Chickenpox
   c. Measles d. MRSA

11. Which is not an ESKAPE pathogen?
   a. *Enterococcus faecium*
   b. *Staphylococcus aureus*
   c. *Klebsiella pneumoniae*
   d. *Proteus mirabilis*

Answers

1. d **2.** a **3.** a **4.** b **5.** c **6.** b **7.** d **8.** d **9.** d **10.** d **11.** d

Major Hospital-acquired Infection Types: Surveillance and Prevention

54
CHAPTER

Chapter Preview

- Hospital-acquired Infection Surveillance
- Prevention of Major Types of HAIs

HOSPITAL-ACQUIRED INFECTION SURVEILLANCE

Hospital-acquired infections or healthcare-associated infections (HAIs) surveillance is a system that monitors the HAIs in a hospital. Main objectives of HAI surveillance include:

- Provides endemic or baseline HAI rate and information on type of HAIs in the hospital
- Helps in comparing HAI rates within and between hospitals
- Identifies the problem area; based on which root cause analysis can be conducted to find out the breakdowns in infection control measures and then the appropriate corrective measures are implemented
- Provides timely feedback to the clinicians; thus, reinforces them to adopt best practices.

Targeted Surveillance

National Healthcare Safety Network (NHSN) division of CDC (centers for disease control and prevention) provides guidelines for the surveillance of HAIs.

- **Where to conduct:** HAI surveillance should be conducted only for high-risk locations such as intensive care units (ICUs)
- **What type of HAIs to be monitored:** As technically difficult, only the major type of HAI to be monitored such as:
  - Catheter-associated urinary tract infection (CAUTI)
  - Central line-associated blood stream infection (CLABSI)
  - Ventilator-associated event (VAE)
  - Surgical site infection (SSI).
- **Who will conduct:** The infection control nurses (ICNs) under the supervision of the officer in-charge of HICC conduct HAI surveillance
- **HAI surveillance diagnostic criteria:** The NHSN has provided the diagnostic criteria for four major types of HAIs (Tables 54.1 to 54.4). These criteria are made very objective so as to maintain the uniformity of data collection between hospitals which helps in accurate comparison of HAI rates between hospitals of same and different nations.

Method of Conducting HAI Surveillance

The HAI surveillance cycle consists of data collection → data analysis → data interpretation → data dissemination.

- **Data Collection:** The infection control nurses (ICNs) visit daily to the high-risk areas (ICUs) and collect the clinical data of patients on devices (urinary catheter, central line, ventilator) and also patients admitted following surgeries. They also prospectively check the laboratory investigations to confirm a diagnosis
- **Data Analysis:**
  - The four types of HAIs are diagnosed according to HAI surveillance criteria of NHSN/CDC (Tables 54.1 to 54.4)
  - Then the HAI rates are calculated as per the formulae given in Table 54.5
  - Then the monthly report of location wise HAI rates of the hospital is generated.

Table 54.1: NHSN surveillance diagnostic criteria for catheter associated urinary tract infection (CAUTI)

Device criteria	Presence of a urinary catheter for >2 days
Clinical criteria	Presence of any one symptom of UTI such as fever, suprapubic tenderness, urgency, frequency or dysuria
Culture criteria	Isolation of significant count ($\geq 10^5$/mL) of a UTI pathogen from urine

Table 54.2: NHSN Surveillance diagnostic criteria for CLABSI (Central line-associated blood stream infection)

	Age	Blood culture criteria		Clinical criteria
		Organism isolated	No. of cultures positive	
LCBI-1	Any age	LCBI pathogen[1]	1	Symptoms not required
LCBI-2	<1 year	LCBI commensal[2]	2	Any one symptom[3]
LCBI-3	<1 year	LCBI commensal[2]	2	Any one symptom[4]
Device criteria = catheter present for > two calendar days				
LCBI *plus* catheter criteria met = called as CLABSI LCBI *without* catheter criteria met= called as non-CLABSI				

LCBI- laboratory confirmed blood stream infection
[1]LCBI pathogen, e.g. common hospital-acquired pathogens
[2]LCBI commensal, e.g. Coagulase negative staphylococci
[3]LCBI-2 symptoms—fever, chills, hypotension
[4]LCBI-3 symptoms—fever, hypothermia, bradycardia, apnea

Table 54.3: NHSN surveillance diagnostic criteria for VAE (Ventilator associated events)

Stage-1: VAC (ventilator-associated condition)	
Device criteria	Presence of a mechanical ventilator at least for two calendar days
Worsening oxygenation criteria	• Baseline period during which the daily minimum FiO_2 (fraction of inspired oxygen) and PEEP (positive end-expiratory pressure) values are stable or decreasing for 2 days followed by • Period of worsening of oxygenation—increased FiO_2 (by ≥20%) or PEEP (≥3 cm water) for at least 2 days
Stage-2: IVAC (infection-related ventilator associated complications): VAC plus the following criteria	
Clinical criteria	Any one out of four: Fever or hypothermia or Leukocytosis or leukopenia
Antibiotic criteria	New antimicrobial agent started and continued for ≥4 days
Stage-3: PVAP (Possible ventilator-associated pneumonia): IVAC plus the culture criteria	
Culture criteria	Isolation of significant count of a pneumonia pathogen from respiratory specimens such as tracheal aspirate, bronchoalveolar lavage, etc.

- **Data Interpretation:** HAI rates are compared:
  - For the same location across different time frames
  - Between different locations of same or different hospital during same time frame.
- **Data Dissemination:** The monthly HAI surveillance report should be shared with all clinical departments and administrators. It is also presented during HICC meetings. Accordingly, the appropriate corrective actions are planned in the problem areas.

Table 54.4: NHSN surveillance diagnostic criteria for surgical site infection (SSI)

Definition and types of SSIs	
Definition: Surgical site infections (SSI) are defined as infections that develop at the surgical site within 30 days of surgery (within 90 days for breast, cardiac and joint surgeries) **SSIs are classified based on the level where infection is developed:** • *Superficial SSI:* develops at the level of superficial incisional site (skin and subcutaneous level) within 30 days regardless of type of surgery • *Deep SSI:* develops at the level of deep incisional site (muscle and fascial level) within 30 days for all surgeries except breast, cardiac and implant surgeries (90 days) • *Organ space SSI:* develops at the level of organ space site within 30 days for all surgeries except breast, cardiac and implant surgeries (90 days)	
One among the following must be met:	
Clinical criteria	i. Presence of purulent pus from the corresponding level of surgical site or ii. Presence of local signs of infections (pain/tenderness, swelling, erythema, heat, etc.)
Culture criteria	Positive culture from the discharge collected at the corresponding level of surgical site
Other evidence	i. For superficial SSI—Surgeon's diagnosis is taken as diagnostic criteria ii. For deep or organ space SSI—histopathological, imaging or gross anatomical evidence of abscess should be present

Table 54.5: Formulae of HAI infection rates

HAI infection rates	Formulae
CA-UTI Rate	No. of CA-UTI cases/ total no. of urinary catheter days × 1000
CLABSI Rate	No. of CLABSI cases/ total no. of central line days × 1000
VAE Rate	No. of VAE cases/ total no. of ventilator days × 1000
SSI Rate	No. of SSI/No. of surgeries done × 100

PREVENTION OF MAJOR TYPES OF HAIs

Prevention of Device-associated Infections (DAIs)

The majority of device-associated infections (DAIs) encountered in hospital are CAUTI, CLABSI and VAP.

- Presence of device itself is a major risk factor for developing such infection. This is because of various reasons: (i) risk of introduction of patients own flora, (ii) risk of introduction of HCW's hand flora due to improper handling during insertion or daily maintenance of the device; (iii) ability of the invading organism to produce biofilm over the device; (iv) persistence of organisms as majority of them are MDROs (multi-drug resistant organisms)

- Strict aseptic techniques must be followed while insertion and daily maintenance of the devices
- The preventive measures for each of the DAIs are grouped as bundle care approach (described below).

Bundle Care Approach

Most hospitals follow bundle care approach for the prevention of DAIs.

- Bundle care comprises of 3 to 5 evidence-based elements with strong clinician agreement; each of the component must be followed during the insertion or maintenance of the device
- Compliance to the bundle care is calculated as all-or-none way, i.e. failure of compliance to any of the component leads to non-compliance to the whole bundle
- The components of bundle care approach for prevention of DAIs have been described in Table 54.6.

Prevention of Surgical site infection (SSI)

Preventive measures of SSI can be categorized into preoperative, perioperative and postoperative measures. Both WHO and CDC recently published the guidelines for prevention of SSI which has been summarized in Table 54.7.

Table 54.6: Bundle care approach for prevention of device-associated infections (DAIs)

Bundle care for urinary catheter	
Insertion bundle	**Maintenance bundle**
1. Catheter should be inserted only when appropriate indication is present (e.g. acute urinary retention) 2. Only the sterile items are used for insertion of catheter 3. Catheter is inserted by non-touch technique with strict asepsis 4. Closed drainage system must be used 5. Catheter of appropriate size must be used 6. Catheter must be properly secured after placement (by plaster-tube-plaster technique)	1. Daily catheter care (vaginal or meatal care) must be given regularly and by strict aseptic measures such as hand hygiene and single use gloves 2. Catheter is properly secured all the time 3. Drainage bag must be always above the floor and below the bladder level 4. Closed drainage system is used all the time 5. While collection of urine from bag, the following steps must be followed —Hand hygiene, change of gloves between patients; use of separate jug for each bag, use of alcohol swabs for disinfection of outlet 6. Daily assessment of readiness for removal of catheter must be documented
Bundle care for central line	
Insertion bundle	**Maintenance bundle**
1. Hand hygiene prior to insertion of central line 2. Use maximum sterile PPE: gloves, gown, drapes, cap and mask 3. Site of insertion—Subclavian preferred, avoid femoral 4. Skin preparation—by antiseptics such as chlorhexidine 5. Skin must be completely dry after use of antiseptics 6. Use semi-permeable dressing 7. Hand wash after procedure must be performed 8. Document data and time of insertion	1. *Daily aseptic central line care during handling:* ➢ Hand hygiene must be performed ➢ Hub decontamination by alcohol 2. Daily documentation of local signs of infection 3. Change of dressing with 2% chlorhexidine 4. Daily assessment of readiness for removal of central line must be documented
Maintenance bundle for mechanical ventilator	
1. Adherence to hand hygiene 2. Elevation of the head of the bed to 30–45°—this is to prevent oropharyngeal aspiration to respiratory tract 3. Daily oral care with chlorhexidine 2% solution 4. Need of PUD (peptic ulcer disease) prophylaxis should be assessed daily; if needed only sucralfate should be used 5. DVT (deep vein thrombosis) prophylaxis should be provided if needed 6. Daily assessment of readiness to remove mechanical ventilator must be documented	

Table 54.7: Prevention of surgical site infection (SSI)

Preoperative measures
1. **Preoperative bathing:** It should be performed using a plain soap or an antimicrobial soap to reduce the bacterial load, especially at the site of incision 2. **For MRSA carriers:** Decolonization with mupirocin ointment must be done for patients undergoing surgery who are nasal carriers of MRSA 3. **Hair removal:** For patients undergoing any surgical procedure, hair removal should not be done or, if absolutely necessary, it should be removed only with a clipper. Shaving is strongly discouraged at all times

Contd...

Contd...

Intraoperative measures
1. **SAP:** Surgical antimicrobial prophylaxis (SAP) must be provided for all except clean surgeries. ➢ *Timing*—SAP must be administered within 60–120 minutes before incision, which usually coincides with induction of anesthesia ➢ *Choice*—It depends upon local antibiotic policy. Cefazolin or cefuroxime are usually preferred ➢ *Frequency*—SAP is usually given as a single dose. Repeat dose may be required only for: ♦ Duration of surgery exceeds >4 hours ♦ Cardiac surgeries ♦ Drugs with lower half-lives (redosing required if duration of surgery exceeds 2 half-lives) ♦ Extensive blood loss during surgery ➢ *For ESBL prevalent area*—No added or modified agent for SAP should be added. ESBL screening for patients is not routinely recommended. 2. **Surgical hand disinfection-** Scrubbing with either antimicrobial soap (chlorhexidine) or with alcohol-based hand rub must be performed before donning sterile gloves; before surgery and in between surgeries 3. **Surgical site preparation** should be performed with alcohol-based chlorhexidine antiseptic solution before the commencement of surgery 4. **Perioperative maintenance** of oxygenation (target FiO_2 80%), temperature (normothermia), blood glucose level (target level of <200 mg/dL), adequate circulating volume (normovolemia) and nutritional support are necessary during surgery and immediate 4–6 hours postoperative period
Postoperative measures
1. **Wound dressing**—Daily dressing of surgical site and removal of any discharge present at the site must be performed. Perform hand hygiene and use gloves before dressing 2. **OT disinfection**—Thorough postoperative disinfection of operation theater must be performed with a high level disinfectant in between cases and after the last case (terminal disinfection) 3. **Periodic monitoring of the air quality of operation theater** for various parameters must be performed such as no. of air exchanges, temperature, humidity, pressure and microbial contamination 4. **SAP prolongation is not recommended** in any situations (e.g. presence of a wound drain) for the purpose of preventing SSI as it promotes development of antimicrobial resistance

Adapted from WHO's Global guidelines on the prevention of surgical site infection, 2016 and CDC's Guideline for the prevention of surgical site infection, 2017
Abbreviation: ESBL, extended spectrum beta lactamase.

EXPECTED QUESTIONS

I. Write short notes on:
1. HAI surveillance.
2. Bundle care approach for prevention of device-associated infections.
3. Prevention of surgical site infection.

II. Multiple Choice Questions (MCQs):

1. **Which parameter is not included in HAI surveillance?**
   a. CA-UTI (catheter-associated urinary tract infection)
   b. CLABSI (central line-associated bloodstream infection)
   c. VAP (ventilator-associated pneumonia)
   d. Open wound infections
2. **For device-associated infection, the device should be present in place at least for how many calendar days?**
   a. 1 b. 2
   c. 3 d. 4
3. **Among the following ventilator associated events, which requires to meet the worsening oxygenation criteria?**
   a. VAC (ventilator-associated condition)
   b. IVAC (infection-related ventilator-associated complication)
   c. PVAP (possible ventilator-associated pneumonia)
   d. All of the above
4. **Among the following ventilator-associated events, which requires culture to be positive?**
   a. VAC (ventilator-associated condition)
   b. IVAC (infection-related ventilator associated complications)
   c. PVAP (possible ventilator-associated pneumonia)
   d. All of the above
5. **A patient is admitted since 5 days. He develops fever after placement of a urinary catheter for >2 days. Urine culture revealed *Escherichia coli* (≥10^5/mL). What is the surveillance diagnosis?**
   a. Hospital-acquired CAUTI
   b. Hospital-acquired non-CAUTI
   c. Community-acquired CAUTI
   d. Community-acquired non-CAUTI

Answers

1. d **2.** b **3.** d **4.** c **5.** a

Biomedical Waste Management

55
CHAPTER

Chapter Preview

- Biomedical Waste Rule 2016
- Treatment and disposal methods
- Waste segregation in hospitals

The waste generated from the healthcare facilities carries a higher potential for infections and injuries. Therefore, it is essential to have safe and reliable methods of segregation and disposal of hospital waste. The Ministry of Environment and Forests (MoEF) has formulated biomedical waste rule in 1998; which was later revised in 2016 with an amendment added in 2018 (Table 55.1).

BIOMEDICAL WASTE RULE 2016

Definition

According to Biomedical Waste Rule, 2016 of India, Biomedical wastes are defined as wastes that are generated during the laboratory diagnosis, treatment or immunization of human beings or animals, or in research activities pertaining thereto, or in the production of biologicals (Table 55.1).

Waste Generated in Hospitals

It is estimated that quantity of solid waste generated in hospitals varies from 1/2 to 2 kg/bed in government hospitals, private hospitals and nursing homes. However, biomedical waste accounts for a minor proportion of total waste generated in hospitals.

In developing countries, the waste generated in hospitals falls into the following categories:

- **General waste (80%):** Vast majority of waste falls in the general waste category, which may be disposed with the usual domestic and urban waste management system. They do not cause any harm to humans
- **Pathological and infectious waste (15%):** This is the component of hospital waste that produces maximum hazards. Pathogens in the infectious waste may infect health care workers by entering through ingestion, inhalation or direct skin-to-skin contact
- **Chemical and pharmaceutical waste (3%):** Most of the chemicals (e.g. disinfectants) and pharmaceutical wastes are toxic, genotoxic (affect genetic system), corrosive, flammable, explosive or shock sensitive
- **Sharp waste (1%):** Needle sticks and other sharps are of great concern as they are capable of transmitting blood-borne pathogens such as HIV, hepatitis B and C viruses, etc.
- Less than 1% accounts for special waste such as cytotoxic drug, radioactive waste, broken thermometers and used batteries.

Situation in India

According to the Ministry of Environment and Forests (MoEF), gross generation of biomedical waste in India is about 4,05,702 kg/day, of which only 2,91,983 kg/day is properly disposed, which means that almost 28% of the wastes is left untreated and not disposed, finding its way in dumps or water bodies and re-enters our system. Karnataka tops the chart among all the states in generation of biomedical waste.

TREATMENT AND DISPOSAL METHODS

There are several methods of disposal of biomedical waste. Though incineration is widely used, the recently developed alternative methods are becoming increasingly popular.

Incineration

It has been the method of choice of disposal of biomedical waste.

- Incineration is a high temperature dry oxidation process that reduces organic and combustible waste into nonorganic incombustible matter, resulting in a very significant reduction of waste volume and weight

Table 55.1: Biomedical Waste Management Rule, India, 2016 (including the amendment added in 2018)

Category	Type of waste	Type of Bag/container	Treatment/disposal options
Yellow 	Human anatomical waste Animal anatomical waste	Yellow colored non-chlorinated plastic bags	Incineration/plasma pyrolysis/deep burial
	Soiled waste		Incineration/plasma pyrolysis/ deep burial/autoclaving or hydroclaving + shredding/mutilation
	Expired/discarded medicines—pharmaceutical waste, cytotoxic drugs	Yellow colored containers/non-chlorinated plastic bags	Incineration (cytotoxic drugs at temperature >1200°C)
	Chemical solid waste	Yellow colored containers/non-chlorinated plastic bags	Incineration or plasma pyrolysis or encapsulation
	Chemical liquid waste	Separate collection system leading to effluent treatment system	Pre-treated before mixing with other waste water
	Discarded linen contaminated with blood/body fluids	Non-chlorinated yellow plastic bags/suitable packing material	Non-chlorinated chemical disinfection followed by incineration/plasma pyrolysis
	Microbiology, other clinical laboratory waste, blood bags, live/attenuated vaccines	Autoclave safe plastic bag/ container	Pre-treat to sterilize with non-chlorinated chemicals on-site as per NACO/ WHO guidelines (Blue book 2014) + incineration
Red 	**Infectious plastic waste** Disposable items such as tubing, bottles, intravenous tubes and sets, catheters, urine bags, syringes (without needles and fixed needle syringes) and vacutainer with their needles cut) and gloves	Red colored non-chlorinated plastic bags or containers	• Autoclaving/microwaving/ hydroclaving + shredding • Mutilation/sterilization+ shredding Treated waste sent to authorized recyclers or for energy recovery or plastics to diesel or fuel oil or for road making, whichever is possible. Plastic waste should not be sent to landfill sites
White (Translucent) 	**Waste sharps including metal sharp** Needles, syringes with fixed needles, needles from needle tip cutter or burner, scalpels, blades, or any other contaminated sharp	Puncture-proof, leak-proof, tamper-proof containers	• Autoclaving/dry heat sterilization+ shredding/ mutilation • Encapsulation in metal container or cement concrete • Sanitary landfill/designated concrete waste sharp pit
Blue 	a. **Glasswares:** Broken or discarded and contaminated glass including medicine vials and ampoules except those contaminated with cytotoxic wastes b. **Metallic body implants**	Puncture proof and leak-proof container	Disinfection (by soaking the washed glass waste after cleaning with detergent and sodium hypochlorite treatment) or through autoclaving/microwaving/ hydroclaving and then sent for recycling

Note:
- Biomedical waste rule does not specify any specific color coded bag for general waste segregation in hospital. Depending up on local policy, hospitals choose any color coded bag for general waste (for e.g. JIPMER uses black bag for general waste)
- Hypochlorite should be used at 1–2% concentration.
- In-situ pretreatment of waste can be done as per WHO Blue Book 2014.
- The chlorinated plastic bags (except blood bags) and gloves should be phased out by non-chlorinated bags by 27th March 2019.
- Every health care facility should have their own STP (sewage treatment plant); and those with less than 10 beds have to establish STP by 31st December 2019.
- Barcoding system should be introduced to monitor the segregation compliance.

Abbreviations: NACO, National AIDS Control Organization; WHO, World Health Organization.

- Incineration is usually done for those wastes that cannot be reused, recycled or disposed off in a landfill site, for example, human and animal anatomical waste, microbiological waste, solid non-plastic infectious waste
- **Incineration should not be done for:**
  - Pressurized gas containers
  - Reactive chemical waste
  - Halogenated plastics such as PVC (polyvinyl chloride)
  - Waste with heavy metals such as mercury, silver salts, radiographic waste, broken thermometers.

Autoclave

Autoclaving is a thermal process where steam is brought into direct contact with waste in a controlled manner and for sufficient duration to sterilize the wastes. For ease and safety in operation, the system should be horizontal type and exclusively designed for the treatment of biomedical waste. For optimum results, prevacuum-based system is preferred against the gravity type system. It shall have tamper-proof control panel with efficient display and recording devices for critical parameters such as time, temperature, pressure, date and batch number, etc.

Chemical Disinfection

Chemicals such as sodium hypochlorite (1-2%) are added to waste to kill or inactivate the pathogens within it. It results in disinfection rather than sterilization.

- It is more suitable for liquid waste such as blood, urine, stool and hospital sewage
- However, solid waste, such as microbiological cultures and sharps, etc. may be disinfected chemically with certain limitations.

Effluent Treatment Plant

The liquid effluent generated during the process of washing containers, vehicles, floors, etc. is first subjected to chemical treatment and then disposed in effluent treatment plant.

Microwaving

In microwaving, microbial inactivation occurs as a result of the thermal effect of electromagnetic radiation spectrum lying between the frequencies 300 and 300,000 MHz.

- It is an intermolecular heating process
- The heating occurs inside the waste material in the presence of steam
- The efficacy of microwave disinfection should be monitored regularly.

Shredder

Shredding is a process by which waste are deshaped or cut into smaller pieces so as to make the wastes unrecognizable. It helps in prevention of reuse of biomedical waste and also acts as identifier that the waste has been disinfected and is safe to dispose off.

Sanitary Landfill

It is a small deep burial pit of 2 meters depth. It should be half filled with waste, then covered with lime within 50 cm of the surface, before filling the rest of the pit with soil. It is especially designed for disposal of hospital waste.

- For health and safety, a landfill site should be constructed away from residency, forests and coastal waters
- If the facilities are not available to treat the waste before disposal, landfills are regarded as an acceptable route of disposal. However, medical waste should not be disposed in open dump.

Encapsulation

Encapsulation method involves filling containers with waste, adding immobilizing material and sealing the containers, to prevent the access to unscrupulous activities. The process uses cubic boxes made up of metallic drums which are three quarters filled with sharps or chemicals or pharmaceutical wastes and then filled with a medium such as plastic foam, cement mortar or clay material.

Inertization

The process of inertization involves mixing waste with cement and other substances before disposal to minimize the risk of toxic substances contained in the waste migrating into surface or ground water.

Plasma Pyrolysis

Plasma pyrolysis makes use of an ionized gas in the plasma state to convert electrical energy to temperatures of several thousand degrees using plasma arc torches or electrodes. The system provides high temperatures combined with high UV radiation flux which destroys pathogens completely.

WASTE SEGREGATION IN HOSPITALS

Waste segregation refers to the basic separation of different categories of waste generated at source in the hospital and thereby reducing the risks as well as cost of handling and disposal. Segregation is the most crucial step in biomedical waste management.

According to Biomedical Waste Rule (2016), segregation should be done by using containers of four different colors, each is designated for segregation of a particular waste category (see Table 55.1). The differences between

Biomedical Waste Rule 1998 and 2016 has been depicted in Table 55.2.

- Yellow bag—for infectious non-plastic waste
- Red bag—for infectious plastic waste
- White or translucent sharp container (puncture-proof box)—for metal sharp
- Blue container (puncture-proof box)—for broken glass items and metal implants
- Plastic bags must be labeled with biohazard logos (Fig. 55.1) and should be non-inflammable, autoclave stable and non-chlorinated
- All segregated waste must be sent for final disposal within 48 hours of collection.

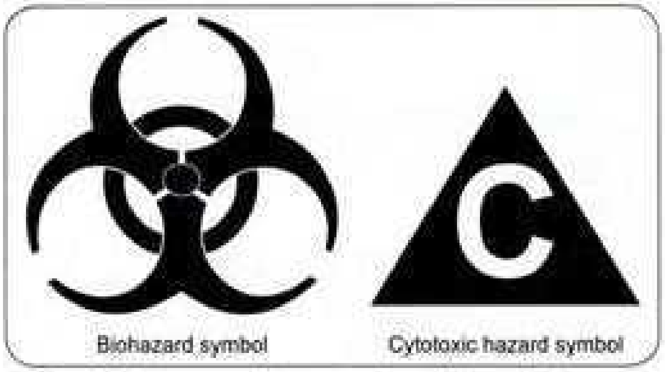


Fig. 55.1: Logos used for segregation of biomedical waste

Table 55.2: Differences between Biomedical Waste Rule 1998 and 2016

Properties	Biomedical Waste Rule 1998	Biomedical Waste Rule 2016
No. of categories	Ten	Four
Overlapping of category	Yes, many categories are segregated into >1 color bags	No, there is no overlapping between categories and color coded bags at all
Maximum limit for the release of furans*	Not specified	Specified
Incinerator	May have only one chamber	Upgraded to have secondary chamber
Chlorinated bags	Were used	To be phased out by non-chlorinated bags in two years
Cytotoxic drugs	Black color bag	Yellow color bag
Use of bar code	Not there	To be introduced within two years
Methods of disposal recommended	Incineration, shredding, autoclave, microwaving, sharp pits, etc.	Newer methods are introduced such as plasma pyrolysis, inertization and encapsulation
Disposal facility	Occupier must have disposal facility	Occupier need not have disposal facility if outsourcing facility is available within 75 Km
Giving treated biomedical solid waste to the municipal solid waste (incineration ash)	Allowed	Not allowed
Majority of idea	Discarding the biomedical wastes	For recycling of the biomedical wastes

*Toxic gases like dioxins, furans, mercury and polychlorinated biphenyls are released from incinerators into the atmosphere due to burning of plastics.

EXPECTED QUESTIONS

I. Write short notes on:

1. Categories of biomedical waste.
2. Disposal methods available for biomedical waste.
3. Type of containers used for disposal of biomedical waste.

II. Multiple Choice Questions (MCQs):

1. **Anatomical waste should be segregated in which color bags?**
   a. Yellow b. Red
   c. Blue d. Black
2. **Microbiological waste should be segregated in which color bags?**
   a. Yellow b. Red
   c. Blue d. Black
3. **Sharps should be segregated in which color box?**
   a. Yellow b. Red
   c. Blue d. White
4. **Solid waste (items contaminated with blood and body fluids including cotton, dressings belong to which category of biomedical waste?**
   a. Yellow b. Red
   c. Blue d. White
5. **Plastic items should be segregated in which color bag?**
   a. Yellow b. Red
   c. Blue d. White
6. **Before segregation of microbiological waste, pre-treatment with what concentration of hypochlorite is recommended?**
   a. 1-2% b. 5%
   c. 10% d. 15%

Answers

1. a **2.** a **3.** d **4.** a **5.** b **6.** a

Needle Stick Injury Prevention and Management

56
CHAPTER

Chapter Preview

- Prevention of needle stick injury
- Post-exposure management

INTRODUCTION

An occupational exposure is defined as:

- Percutaneous injury, e.g. needle stick injury (NSI) or other sharp injury.
- Splash injury:
  - Contact with the mucous membrane (e.g. eye or mouth);
  - Contact with non-intact skin (abraded skin or afflicted with dermatitis);
  - Contact with the intact skin when the duration is prolonged (e.g. several minutes or more).

Occupational injury is often loosely termed as needle stick injury though it includes injury through needle or other sharps and splashes.

Agents transmitted:

Hepatitis B virus (HBV), Hepatitis C virus (HCV) and HIV are three major blood-borne viruses (BBVs) that are transmitted through NSI. The risk of transmission is highest for HBV (30%) followed by HCV (3%) and HIV (0.3%).

Infectious specimens for NSI:

- *Potentially infectious body fluids* include blood, genital secretions (semen, vaginal secretions) and all body fluids (CSF, synovial fluid, pleural fluid, peritoneal fluid, pericardial fluid, amniotic fluid)
- *The following are not considered potentially infectious,* unless visibly contaminated with blood: Feces, nasal secretions, saliva, sputum, sweat, tears, urine and vomitus.

Factors that influence risk of contracting infection following NSI:

The risk of infection following exposure depends on the following factors:

- Type of needle (hollow bore needle has a higher risk than solid needle)
- Device visibly contaminated with blood
- Depth of injury (higher is the depth, more is the risk)
- Volume of blood involved in the exposure
- Viral load present in the blood at the time of exposure
- Timely performing first aid
- Timely start of appropriate post-exposure prophylaxis (PEP) for HBV and HIV.

PREVENTION OF NEEDLE STICK INJURY

Precautions During Handling Needles

The following measures should be taken during handling needles to prevent occupational exposures:

- Standard infection control precautions must be followed such as hand hygiene and appropriate use of personal protective equipment (PPE) (e.g. gloves, gowns, masks, and goggles) while handling blood or body fluids
- Work surfaces must be disinfected with 1% sodium hypochlorite solution
- Health care workers (HCWs) must be immunized against HBV
- Spillage of blood and other body fluids must be promptly cleaned and surface disinfected with 10% sodium hypochlorite solution
- Disposable needles should be used. Needles should never be reused
- **Never recap needles:** If unavoidable, single hand-scoop technique may be followed
- **Disposal after use:** Needles must be disposed into the sharp box immediately after use. Needles/sharps should not be left on trolleys and bed side tables

- **Engineering control measures:** Various devices are specially designed with safety features to prevent NSI such as retractable lancets, safety lock syringe with a protective sheath and needleless IV systems.

Precautions During Surgical Procedures

Confine and contain approach should be implemented for every surgical procedure.

- *Passing of sharp instruments* during surgery must be according to the plan decided by surgeon and his assistant nurse. Sharp instruments should always be passed by non-touch approach, not directly by hands
- *Suturing:* Needles must never be picked up with the fingers while suturing. Forceps or a needle holder is ideal for holding needle. Where practical, blunt needles should be used to close the abdomen
- Preoperative testing of a patient for BBVs is not mandatory; should be performed if clinical indication present
- **Patient known to have BBV infections may require the following additional precautions for surgical operation:**
  - The lead surgeon should ensure that all members of the team know about infection hazards and appropriate measures should be followed, such as use of *double gloves*
  - The surgical team must be limited to essential members of *trained staff* only
  - It may help theater decontamination if *such cases posted last in the list*, but this is not essential.

POST-EXPOSURE MANAGEMENT

Steps of Post-exposure Management

The following are the sequential steps to be followed following an occupational exposure (Table 56.1):

1. **First aid:** First aid has to be started as early as possible (Table 56.2).
2. **Report to the designated nodal center:** Every hospital must have a nodal center for management of NSI. In most hospitals, HICC office acts as nodal center, other hospitals may designate staff clinic or casualty for the purpose. Nodal centers perform the following functions as mentioned below (step 3–9).
3. **Take first dose of PEP for HIV:**
   - The first dose of PEP for HIV should be taken as early as possible. Effect is maximum if taken <2 hours and effect is nil if taken after 72 hours of exposure
   - NACO recommends the PEP as single tablet of TLE (Tenofovir 300 mg plus Lamivudine 300 mg plus Efavirenz 600 mg)
   - If the HIV negative status of the source is documented in patient case record or at hospital information system, then the first dose of PEP is not required
   - If test report is not available, then do not immediately perform the test or check the report from laboratory registers as it delays the institution of PEP.
4. **Testing for BBVs:** The following tests are done for both source and HCW. The test format should be a rapid method (immunochromatographic test or flow through assay) and result should be available within 1-2 hours.
   - Anti-HIV antibody detection
   - HBsAg detection
   - Anti-HCV antibody detection
   - Anti-HBs antibody (done for HCW if previously vaccinated for HBV and titer not tested).

   HCW's baseline status is determined because later it may be difficult to attribute whether the infection was acquired due to this occupational exposure or any other prior exposure. This may guide while taking decision, when the HCW claims for compensation from the health authorities.
5. **Decision on post-exposure prophylaxis** (PEP) for HIV and HBV is taken based on standard guidelines (NACO for HIV and CDC for HBV) as described in Tables 56.3 and 56.4 respectively.
6. **Informed consent and counseling:** Almost every person feels anxious after exposure. They should be counseled and provided with psychological support.

Table 56.1: Steps of post-exposure management

1. First aid
2. Report to designated nodal center
3. Take first dose of PEP for HIV
4. Testing for BBVs
5. Decision on PEP for HIV and HBV
6. Documentation and recording of exposure
7. Informed consent and counseling
8. Follow-up testing of HCWs
9. Precautions during the follow up period

Abbreviations: PEP, Post-exposure prophylaxis; HCW, Health care worker; BBV, Blood-borne virus.

Table 56.2: First Aid: Management of exposed site

Do's	Don'ts
• Earlier the first aid, lesser is the chance of transmission of BBVs • For splash injury: Irrigate thoroughly the site (e.g. eyes or mouth or other exposed area) vigorously with water at least for 5 minutes • Spit fluid out immediately if gone into mouth and rinse the mouth several times • If wearing contact lenses, leave them in place while irrigating. Once the eye is cleaned, remove the contact lens and clean them in a normal manner	• Do not panic • Do not place the pricked finger into the mouth reflexively • Do not squeeze blood from wound • Do not use antiseptics and detergents

- They should be informed about the risks and benefits of PEP medications
- PEP is not mandatory. Exposed persons should however be made to understand the risk of acquiring transmission if PEP is not taken.

7. **Documentation and recording of exposure:**
   - A ***structured proforma*** should be used to collect the detail information related to exposure such as date, time, and place of exposure, type of procedure done, type of exposure, duration of exposure, source status and volume and type of specimen involved
   - ***Consent form:*** For prophylactic treatment, the exposed person must sign a consent form. If the individual refuses to initiate PEP, it should be documented. The designated officer for PEP should keep this document.
8. **Follow-up testing** of HCWs for BBVs should be done if the source status is positive/unknown.
   - ***HIV testing follow-up is done:*** At 6 weeks, 3 months and 6 months after exposure

Table 56.3: Revised NACO Guidelines for post-exposure prophylaxis (PEP), 2015

Exposure code (EC)	HIV source code (SC)	PEP Recommendation
1, 2 or 3	Negative	Not warranted
1	1	Not warranted
1	2	PEP is recommended Duration of PEP: 28 days Regimen (TLE): Single daily dose of • Tenofovir 300 mg plus • Lamivudine 300 mg plus • Efavirenz 600 mg
2	1	
2	2	
3	1 or 2	
2 or 3	Unknown (in area with high prevalence)	

Source material: Blood, body fluids or other potentially infectious material (CSF, synovial, pleural, pericardial and amniotic fluid, and pus) or an instrument contaminated with any of these substances

Exposure code:
1. EC-1 (Mild exposure): Mucous membrane/non-intact skin exposure with small volumes, or less duration
2. EC-2 (Moderate exposure):
   - Mucous membrane/non-intact skin with large volumes/splashes for several minutes or more duration OR
   - Percutaneous superficial exposure with solid needle or superficial scratch
3. EC-3 (Severe exposure): Percutaneous exposure with:
   - Large volume transfer
   - By hollow needle, wide bore needle or deep puncture
   - Visible blood on device
   - Needle used in patient's artery or vein

Source HIV Status Code (SC):
1. SC-1: HIV positive, asymptomatic or low viral load (<400 copies/mL)
2. SC-2: HIV positive, symptomatic (advanced AIDS or primary HIV infection), high viral load
3. SC Unknown: Status of the patient is unknown and neither the patient nor his/her blood is available for testing
4. HIV negative: Tested negative according to NACO strategy

The first dose of PEP
Should be started within 2 hours (for greater impact) and definitely within 72 hours. No need to provide PEP if exposure occurred >72 hours

PEP not required in the following situations:
1. If exposure occurred >72 hours before
2. If exposed person is HIV positive: Exposed individuals who are known or discovered to be HIV positive should not receive PEP. They should be referred to ART clinic for counseling and initiation of ART
3. If the skin is intact
4. If source is HIV negative
5. Exposure with low-risk specimens like tear, saliva, urine, stool, vomitus, nasal secretion, sweat, etc.
6. For exposures with EC-1 and SC-1
7. Source unknown if HIV prevalence is low

Side effects and compliance to PEP:
- Common side effects are:
  - At the beginning: Nausea, diarrhea, muscular pain, headache or fatigue
  - Later during the course: Anemia, leukopenia or thrombocytopenia
- Compliance of >95% to the PEP schedule is required to maximize the efficacy of PEP. Hence, the person should be counseled to continue the PEP and to take medication to minimize the side effects of PEP

*Regimen for exposure in pregnant women is essentially same as that of non-pregnant persons.
Abbreviations: NACO, National AIDS Control Organization; ART, antiretroviral therapy.

Table 56.4: Post-exposure prophylaxis (PEP) for hepatitis B

HCW status	If source is positive or unknown for HBsAg	If source is negative for HBsAg
If the exposed person is vaccinated and the antibody titer is protective (≥10 mIU/mL)	No further treatment is required: • Regardless of the HBV status of the source* • Regardless if the titer falls down later*	
If the exposed person is vaccinated and the titer is not protective (<10 mIU/mL)	HBIG-1 dose should be started immediately, given maximum within 7 days Vaccine: Start the second series (3 doses)	Vaccine: Start the second series (3 doses)
If the exposed person is not vaccinated or partially vaccinated	HBIG-1 dose should be started immediately maximum up to 7 days Vaccine: Complete the vaccine series from the last dose given (do not restart)	Vaccine: Complete the series of 3 doses from the last dose given (do not restart)
Nonresponders (If the exposed person is vaccinated for 2 series, i.e. 6 doses and the titer is not protective)	HBIG-2 doses at 1 month apart (0.06 mL/kg or 10–12 IU/kg)	Nothing is required

Note:
HCWs who are vaccinated but anti-HBs <10 mIU/mL, or who are unvaccinated or incompletely vaccinated must be checked for their HBsAg status at baseline and follow-up testing 6 months later.
HBIG and HBV vaccine can be administered simultaneously but at different sites.
HBIG provides a temporary protection for 3–6 months.
Anti-HBs antibody titer should be checked only after 2 months of last dose of vaccine and 6 months after HBIG administration; otherwise, it will give erratic results.
Previous report of Anti-HBs titer is acceptable only if it is documented. Verbal reports should not be considered.
* In a previously protected person, the memory B cells will start producing antibodies soon after the antigenic challenge, hence revaccination by booster doses is not recommended even if the titer falls down later.
Adapted from CDC guideline, 2013.
Abbreviations: HBIG, hepatitis B immunoglobulin; HCW, health care worker; HBsAg, hepatitis B surface antigen.

- HBV and HCV follow-up testing is done at 6 months after exposure.

9. **Precautions during the follow-up period:** If the source status is positive/unknown, then the following precautions should be adopted by the HCW during the follow-up period, especially the first 6–12 weeks.
   - Refraining from blood, semen, organ donation
   - Abstinence from sexual intercourse or use of latex condom
   - Women should not breastfeed their infants
   - The exposed person is advised to seek medical evaluation for any febrile illness that occurs within 12 weeks of exposure.

EXPECTED QUESTIONS

I. **Write short notes on:**
1. Sequential steps to be followed after a needle stick injury.
2. Post-exposure prophylaxis for HIV.
3. Post-exposure prophylaxis for Hepatitis B.

II. **Multiple Choice Questions (MCQs):**
1. **The decreasing order of risk of transmission following occupational exposure:**
   a. HIV>HBV>HCV
   b. HBV>HIV>HCV
   c. HBV>HCV>HIV
   d. HCV>HBV>HIV
2. **All are potentially highly infectious specimen for occupational injury; *except*:**
   a. Blood b. Semen
   c. CSF d. Saliva
3. **The sequence of steps to be followed after accidental exposure to blood/fluid:**
   a. First aid → Reach to nodal center and try to get report of source status → Take first dose of PEP for HIV → Testing of source and HCW status for BBVs → Prophylactic treatment for HIV and HBV
   b. Reach to nodal center and try to get report of source status → First aid → Take first dose of PEP for HIV → Testing of source and HCW status for BBVs → Prophylactic treatment for HIV and HBV
   c. Take first dose of PEP for HIV → Testing of source and HCW status for BBVs → First aid → Reach to nodal center and try to get report of source status → Prophylactic treatment for HIV and HBV
   d. Prophylactic treatment for HIV and HBV → Take first dose of PEP for HIV → Testing of source and HCW status for BBVs → First aid → Reach to nodal center and try to get report of source status

Answers
1. c **2.** d **3.** a

Antimicrobial Stewardship

57
CHAPTER

Chapter Preview

- Implementation of Antimicrobial Stewardship Program
- Monitoring the Compliance to Antimicrobial Stewardship Program

INTRODUCTION

Antimicrobial stewardship program (AMSP) provides strategies for rationalizing the use of antimicrobials in the hospital.

Definition

Centers for disease control and prevention (CDC) has defined antimicrobial stewardship as use of the right antimicrobial agent, for the right patient, at the right time, with the right dose, route and frequency, causing the least harm to the patient and future patients.

Why AMSP is Needed

Antimicrobial stewardship program in a hospital is required for the following reasons:

Antimicrobial Resistance (AMR)

AMR is a rising threat across the globe. The multidrug resistance organisms (MDROs) are prevalent in every country though the extent and the severity of the problem varies. Extensive use of antimicrobials is the single most important factor for the bacteria to undergo mutation to become resistant and then the resistant strain flourish exponentially in presence of selective pressure of antimicrobials.

Misuse and Over-use of Antimicrobials

Though last seven decades since the discovery of penicillin, witnessed that the antimicrobials were highly effective and have saved millions of lives, at the same time, this has also led to their misuse through various ways such as use without a prescription and overuse for self-limiting infections, non-bacterial infections and treatment of colonizer/contaminant.

Widespread Use of Antimicrobials in Other Sectors

World's largest antimicrobial use occurs for animal non-therapeutic purpose (70%), followed by animal therapeutic purpose (15%). Human use accounts only 15% of total antimicrobial consumption, out of which only 9% is being used for human therapeutic purpose. This data explains that just bringing in stewardship program in health care facility would not bring down antimicrobial use dramatically. A robust plan should also be in place for control of antimicrobial use in animals.

Poor Antimicrobial Research

Research in development of new antimicrobial is a huge investment for the pharmaceutical industry. More so, soon after the discovery of an antimicrobial, the bacteria develop resistance mechanisms to tackle the antimicrobial. As a result, the investment goes waste. It is also hypothesized that there could be a return to the pre-antibiotic era, where many people could suffer or die from untreatable bacterial infections.

IMPLEMENTATION OF ANTIMICROBIAL STEWARDSHIP PROGRAM

The key steps of implementation of AMSP in a hospital is as follows.

Administrative Support (Leadership)

The most important prerequisite for implementing AMSP is a strong administrative support. They should be publicly committed to the program and provide necessary funding and infrastructure support.

Set up AMS Team

Antimicrobial stewardship team (AMS team) is a multidisciplinary committee which is responsible for framing, implementing and monitoring the compliance to antimicrobial policy of the hospital. AMS team is led by the antimicrobial steward who may be an infectious disease physician or infection control officer or clinical microbiologist. **Antimicrobial steward** is the central driving force behind this program. A larger hospital may require more than one antimicrobial steward. Other members of AMS team include stewardship nurses, clinical pharmacists and officer in-charge of pharmacy.

Infrastructure Support

- **Support from microbiology laboratory**
  - *Automations:* Facility for automated culture (e.g. BACTEC or BacT/ALERT), identification (MALDI-TOF) and sensitivity (e.g. VITEK) should be available. This reduces the turn-around time to 24–48 hours; compared to conventional cultures which takes 3-5 days
  - *Biomarkers:* Facility for testing biomarkers such as procalcitonin and C-reactive protein (CRP) must be available
  - *Molecular tests:* Facility to perform rapid molecular tests must be available; e.g. Biofire FilmArray multiplex PCR
  - *Emergency laboratory:* Emergency lab running round the clock is a marker of a quality microbiology laboratory.
- **Hospital information system (HIS):** Fully functional HIS including laboratory information system will augment the stewardship program by many folds
- Supporting manpower availability.

Framing Antimicrobial Policy

Every hospital should frame their own hospital antimicrobial policy which is usually a pocket hand book with system/syndrome wise indications for antimicrobial choice and their dosage.

- It should be prepared by AMS team after discussing with all the clinicians, microbiologists and administrators
- The policy must be compliant to the standard national and international antimicrobial guidelines and local antibiogram pattern
- Common consensus between all the clinicians must be arrived, before framing the policy; then only the clinicians will adhere to the policy later.

Implementing AMS Strategies

Two types of strategy are available for implanting AMSP.

- Front end strategies (formulary restriction)
- Back end strategies (prospective audit and feedback).

Front End Strategy (Formulary Restriction)

This involves classifying antimicrobial agents into restricted, semi-restricted and non-restricted antimicrobials with indications for their use combined with an approval system by AMS team (Table 57.1).

- This strategy sounds more attractive, impact is immediate and appears to be the most ideal way to achieve antimicrobial stewardship, but practically implementing formulary restrictions is not that easy
- It creates lot of confusion as it directly impacts the clinician's freedom to choose antimicrobials
- More so availability of the AMSP consultants to give approval all the time further complicates the problem especially in emergency situations.

Back End Strategy

This is done by **prospective audit and feedback.** Though difficult to perform, but it is the most effective strategy to implement AMSP.

- The AMS team goes for stewardship round during which they discuss with the clinical team in detail about the compliance to the antimicrobial policy in terms of appropriateness of the antimicrobials used, dosage with renal adjustment, compliance to susceptibility report, etc. The clinical team gives justification about the non-compliance
- The prospective audit and feedback is mutually agreed upon constructive discussion between AMS team and clinical team on the cases with daily follow up
- Although back end strategy is more labor-intensive, it has several advantages:
  - It is more widely practiced
  - It is more easily accepted by clinicians
  - It provides a higher opportunity for educating and training the health care professionals
  - Impact is delayed but sustainable improving the overall quality of antimicrobial prescribing practice.

Table 57.1: Proposed formulary restriction

Restricted antimicrobials	Semi-restricted antimicrobials	Unrestricted antimicrobials
Pharmacy supply of >1 days requires prior approval by AMS team	Pharmacy supply of >3 days requires prior approval by AMS team	Pharmacy supply does not require AMS team approval
Colistin Carbapenem Tigecycline	Teicoplanin, Vancomycin, Daptomycin, Linezolid, Third and fourth generation cephalosporins	First and second generation cephalosporins, Cotrimoxazole, Azithromycin, Clarithromycin, Fluoroquinolones

Educate and Train

Similar to any other health care program, AMSP also needs continuous education, training, motivation and assessment of the health care providers. Developing antimicrobial stewardship is a behavioral change within the person. Hence, adequate motivational education is a must to bring in such change.

MONITORING THE COMPLIANCE TO ANTIMICROBIAL STEWARDSHIP PROGRAM

It is said that "If you cannot measure it, you cannot improve it". Measurement of the compliance to AMSP is achieved by looking at both process and outcome indicators.

1. **Policy adherence indicator** (process indicator): This is achieved by conducting antimicrobial stewardship audit as described under backward strategy. Both prescription errors and administrative errors can be calculated.
2. **Antimicrobial usage outcome indicator**: This is calculated by:
   - DDD (Defined daily dosage)
   - DOT (days of therapy).
3. **AMR outcome indicator:** The change in AMR pattern is analyzed by conducting periodic AMR surveillance.
4. **Clinical outcome indicators** such as morbidity (e.g. length of stay) and mortality (e.g. infection-related deaths) indicators.
5. **Financial outcome indicators** such as antimicrobial cost per patient day or per year or per admission.

EXPECTED QUESTIONS

I. Write short notes on:

1. Strategies of antimicrobial stewardship program.
2. Monitoring of antimicrobial stewardship program.

II. Multiple Choice Questions (MCQs):

1. **Antimicrobial stewardship program in a hospital is required for the following reasons, *except*:**
   a. Rapid development of antimicrobial resistance
   b. Misuse and over-use of antimicrobials
   c. Widespread use of antimicrobials in humans compared to animal industry
   d. Poor antimicrobial research
2. **Who can act as antimicrobial steward?**
   a. Infectious disease physician
   b. Clinical microbiologists
   c. Medicine consultant
   d. Any of the above
3. **Which is not a Back End Strategy of Antimicrobial stewardship program?**
   a. Prospective audit and feedback is an example
   b. Formulary restriction is an example
   c. It is labor intensive than front-end strategy
   d. Sustainable than front-end strategy
4. **Which is the correct method of framing antimicrobial policy by Antimicrobial stewardship (AMS) team?**
   a. AMS team discuss with each other and frame the policy based on standard guideline
   b. AMS team discuss with each other and frame the policy based on standard guideline as well as local AMR pattern
   c. AMS team discuss with each other and frame the policy based on standard guideline as well as local AMR pattern, then discuss with each clinician for their suggestions
   d. AMS team just copy the guideline from any other renound institute of India
5. **Maximum consumption of antibiotics occurs for:**
   a. Human therapeutic use
   b. Human non-therapeutic use
   c. Animal therapeutic use
   d. Animal non-therapeutic use

Answer

1. c **2.** d **3.** b **4.** c **5.** d

Environmental Surveillance (Bacteriology of Water, Air, Surface, Milk, and Food)

58
CHAPTER

Chapter Preview

- Water surveillance
- Air surveillance
- Surface surveillance
- Milk surveillance
- Food surveillance

WATER SURVEILLANCE

"**Wholesome water**" refers to the water that is fit to use for drinking, cooking, food preparation or washing without any potential danger to human health. Drinking water should have the following three properties to be labeled as 'wholesome water'.

- **Biological quality:** It should be free from potentially pathogenic microorganisms
- **Chemical quality:** It should be free from harmful chemical substances such as metals, solvents, pesticides and hydrocarbons
- **Physical quality:** It should have pleasant taste, color and odor.

As a part of public health program, drinking water supplies should be regularly tested for microbiological, chemical and physical quality. Discussion in this chapter is confined to the assessment of bacteriological quality of water.

Bacterial Flora in Water

Bacterial flora present in water may be of various types (Table 58.1).

Naturally occurring water bacteria, soil bacteria and sewage bacteria usually arise from decomposing organic matter and are not pathogenic to humans. Their presence in water does not warrant threat. It is the sewage intestinal bacteria which are of major concern to humans and their presence in water supplies has to be monitored regularly.

Supplies of drinking water contaminated with sewage and other excreted products may cause life-threatening water-borne diseases such as typhoid, cholera, polio, viral hepatitis A and E, etc. (Table 58.2). Hence, drinking water supplies should be regularly tested to confirm that they are free from such contamination.

- However, it is impracticable to attempt directly to detect the presence of all the different kinds of water-borne pathogens because they are usually present in minute quantity and intermittently
- Instead, it is wise to test the water supplies for those microorganisms which indicate that fecal contamination has taken place. These organisms are called **indicator organisms** (Table 58.3).

Indicator Organisms

Indicator organisms are usually the commensal bacteria of intestine which satisfy two properties:

1. They should be present in excess number than any pathogen so that they can be detected easily; at the same

Table 58.1: Bacterial flora in water

Natural water bacteria	
Includes those organisms that are commonly found in water, free from gross pollution	• *Micrococcus* • *Pseudomonas* • *Serratia* • *Flavobacterium* • *Alcaligenes* • *Acinetobacter*
Soil bacteria	
These organisms are frequently washed into the water during heavy rains and are not normal inhabitants of water	• *Bacillus subtilis* • *Bacillus megaterium* • *Enterobacter aerogenes* • *Enterobacter cloacae*
Sewage bacteria	
These include: • Majority are the normal inhabitants of the intestine of man and animals • Other sewage bacteria are the bacteria that live mainly on decomposing organic matter	**Intestinal bacteria** (through sewage): *Escherichia coli, Enterococcus faecalis, Clostridium perfringens, Salmonella, Vibrio cholerae* **Other sewage bacteria:** *Proteus,* *Clostridium sporogenes* and others

Table 58.2: Water-borne pathogens

Microorganisms	Water-borne pathogens
Bacterial	*Vibrio cholerae* *Salmonella* Typhi, *S.* Paratyphi A and B *Shigella* species *Yersinia enterocolitica* *Campylobacter jejuni* Diarrheagenic *Escherichia coli*
Viral	Hepatitis A virus Hepatitis E virus Poliovirus Rotavirus
Protozoal	*Entamoeba histolytica, Giardia lamblia, Balantidium coli, Cryptosporidium, Cystoisospora*
Helminthic	*Ascaris lumbricoides* *Enterobius vermicularis,* *Trichuris trichiura*
Helminths transmitted through aquatic hosts	*Dracunculus medinensis* *Diphyllobothrium latum* Schistosomes

Table 58.3: Indicator organisms of fecal contamination of water

Indicator organisms	Interpretation (Presence in water indicates)
Coliform (other than *E. coli*)	Remote contamination with either fecal (presumptive) or soil and vegetation
Fecal (thermotolerant) *E. coli*	• Confirms recent fecal contamination of water • Most sensitive indicator
Fecal streptococci	Confirms remote fecal pollution
Clostridium perfringens	Remote contamination
Pseudomonas aeruginosa	• Least reliable indicator • Useful in hospitals and food establishments
Bacteriophages	• Phage specific for *E. coli*—indicates fecal pollution of water • Indirectly indicates viral pollution of water

time, they should not be able to proliferate in water to any extent.

2. They should be more resistant than the pathogens to the stresses of aquatic environment and disinfection processes.

Indicator organisms themselves are not pathogens. But their presence in water supplies indicates that there is a contamination of sewage and the water supplies needs to be disinfected. However, it is also to be kept in mind that mere presence of these indicator organisms does not assure the presence of water-borne pathogens.

There are a number of intestinal commensals used as indicator organisms, described below.

Coliform (Presumptive Coliform)

They are the members of Enterobacteriaceae that are present in large numbers in sewage.

- Among them, only *Escherichia coli* is a reliable indicator as it is not found in other sources
- However other coliforms such as *Klebsiella, Citrobacter, Enterobacter*, etc. are much less abundant in feces than *E. coli*, and survive for longer time than *E. coli*.
  - They are also found in the environment as saprophytes
  - Therefore, their presence in water may indicate either remote fecal pollution of water (long enough to have allowed *E. coli* to die out) or contamination from soil and vegetations.

Fecal Escherichia coli

It is regarded as the essential indicator of pollution of water with feces of human or animal origin.

- It is defined as the thermotolerant coliform organism that—(1) ferments lactose at 44°C with production of acid and gas and (2) gives a positive indole test at 44°C
- Fecal *E. coli* is the most numerous coliform in humans and animal intestine and is derived almost exclusively from these sources
- It does not survive in water for long time, and therefore is the **best indicator** of recent human or animal fecal pollution of water
- Its presence in water indicates a potentially dangerous fecal pollution of water.

Fecal Streptococci

These are the commensals of intestine belonging to Group D streptococci.

- They are less abundant in feces than *E. coli*, can survive longer in water than *E. coli*. Hence their presence along with coliforms, despite absence of *E. coli* confirms fecal pollution of water
- Fecal streptococci can be either enterococci or non-enterococcal streptococci. Most of the species are abundant in animal feces, except *Enterococcus faecalis* which is abundant in human feces.

Sulfite Reducing Clostridia (C. perfringens)

These reduce sulfite to sulfide.

- Though *Clostridium perfringens* is less numerous in human feces (least sensitive indicator), their spores can survive for longer time in the environment
- Their presence indicates remote or intermittent fecal pollution of water.

Pseudomonas aeruginosa

Though it can survive in the environment for long time, it can multiply in various aquatic habitats. Hence, its presence in water is not necessarily good indicator of fecal contamination. However, it is frequently encountered in hospital environment and a cause of food spoilage.

Bacteriophages

The phages that infect *E. coli* can be used as indicator of fecal pollution of water. They may also be used as indicator of viral pollution as their occurrence and resistance in the environment and to chlorine is similar to that of enteroviruses.

Bacteriological Examination of Water

Collection and Transport of Water Sample

Heat sterilized screw capped bottles (at least 200 mL holding capacity) should be used for collection of water. Extreme care should be taken to avoid contamination of the bottle with the bacteria present in the surrounding environment or hands of the collecting person. At least 150 mL of water should be collected.

- Sodium thiosulfate is added to neutralize the bactericidal effect of residual chlorine present in water if any
- **Water from tap:** Water should be collected only after running it from the tap for 2-3 minutes
- **Water from streams or lakes:** The bottle should be opened only after immersed at a depth of 30 cm with its mouth facing the current
- **Water from well:** It is collected by bottles tied with heavy weight (stones)
- **Transport:** The bottle should be properly labeled and sent to the laboratory as quickly as possible at least within 6 hours. If delay is anticipated, the bottles should be kept in an ice box and protected from light.

Presumptive Coliform Count (Multiple Tube Method)

Presumptive coliform count (multiple tube method) is the standard test employed for bacteriological analysis of water. This test is followed by differential coliform count to confirm the presence of fecal *E. coli*. In multiple tube method, the presumptive coliform count is determined, which is expressed as the most probable number (MPN) of coliform organisms in 100 mL of water.

- **Medium:** MacConkey purple broth (double strength and single strength) in bottles or tubes is the standard medium of choice
  - Durham's tube is used to detect gas production
  - Bromocresol purple is used as indicator.
- **Procedure:** Measured amount of water samples are added to tubes containing MacConkey purple broth by sterile graduated pipettes as under:
  - 50 mL of water—added to one bottle of 50 mL double strength medium
  - 10 mL of water each—added to 5 tubes of 10 mL double strength medium
  - 1 mL of water each—added to 5 tubes of 5 mL single strength medium.
- **Result:** The inoculated tubes are incubated at 37°C for 48 hours. Positive test is indicated by (1) a color change in the medium from purple to yellow (due to lactose fermentation) and (2) gas collected in the Durham's tube
- **Interpretation:** The interpretation of presumptive coliform count is as follows:
  - **Presumptive coliform count** (Most probable number): An estimate of coliform count per 100 mL of water is calculated from the tubes showing acid and gas production using the McCrady's probability table (Table 58.4)
  - **Quality of water supply** is determined by the presumptive coliform count. The most probable numbers of 0, 1-3, 4-9 and ≥10 per 100 mL are interpreted as excellent, satisfactory, intermediate and unsatisfactory respectively (Table 58.5)
  - Detection of coliform bacteria does not always indicate fecal contamination; as some of them may be found in environment. Hence, it is further tested by differential coliform count to detect the fecal *E. coli*.

Differential Coliform Count (Eijkman Test)

The Eijkman test is done to confirm that the coliform bacilli detected in the presumptive test are fecal *E. coli*. This is done by:

- Subculturing the positive tubes (of the previously done presumptive coliform test) on lactose containing medium such as brilliant green bile broth for detection of lactose fermentation with production of acid and gas at 44°C and
- Demonstrating positive indole test at 44°C.

Other Methods of Water Analysis

Membrane Filtration Method

Principle: This method is based on the filtration of a known volume of water through a cellulose membrane with pore diameter of 0.45 or 0.2 µm. The bacteria are retained on the surface of the membrane filter. When such membrane is transferred on to a petri dish containing selective differential culture medium (e.g. membrane lauryl sulfate lactose broth with added agar) at an appropriate temperature (e.g. 37 and 44°C) and incubated; characteristic colonies of coliforms/thermotolerant coliforms develop, which can be counted directly.

- **Advantage:** In contrast to the multiple-tube method, the membrane-filtration method:
  - Gives direct count of total coliforms and thermotolerant coliforms present in water
  - More useful in testing dialysis water
  - Requires lesser media and consumables
  - Less labor intensive.

Table 58.4: McCrady's probability table for calculating most probable number (MPN)/100 mL of water

Number of tubes giving a positive reaction out of			
1 tube of 50 mL	5 tubes of 10 mL each	5 tubes of 1 mL each	MPN/100 mL
0	0	0	<1
0	0	1	1
0	0	2	2
0	1	0	1
0	1	1	2
0	1	2	3
0	2	0	2
0	2	1	3
0	2	2	4
0	3	0	3
0	3	1	5
0	4	0	5
1	0	0	1
1	0	1	3
1	0	2	4
1	0	3	6
1	1	0	3
1	1	1	5
1	1	2	7
1	1	3	9
1	2	0	5
1	2	1	7
1	2	2	10
1	2	3	12
1	3	0	8
1	3	1	11
1	3	2	14
1	3	3	18
1	3	4	21
1	4	0	13
1	4	1	17
1	4	2	22
1	4	3	28
1	4	4	35
1	4	5	43
1	5	0	24
1	5	1	35
1	5	2	54
1	5	3	92
1	5	4	161
1	5	5	>180

Table 58.5: Classification of quality of drinking water supply according to bacteriological tests

Quality of drinking water supply	Most probable number (MPN)/100 mL of water	
	Coliform count/100 mL	Thermotolerant *E. coli* count/100 mL
Excellent	0	0
Satisfactory	1–3	0
Intermediate	4–9	0
Unsatisfactory	≥10	≥1

- **Disadvantage:** Turbid water cannot be tested by this method. Consumables are more expensive than the former method.

Method Used for Streptococci and Clostridia

For isolation of fecal streptococci, medium containing sodium azide is used and for *Clostridium perfringens*, differential reinforced *Clostridium medium* can be used.

Plate Count (Pour Plate)

Water sample is added to molten agar and poured on to petri dishes and incubated at differential temperatures. It expresses the number of colony forming bacteria per mL of water. It is more cumbersome; not routinely followed.

Presence—Absence Method

This method detects just the presence or absence of organism in water. It is more useful in outbreak situations where there is a need for urgent reporting. Various commercial kits are available such as *Manjas method*, where H_2S coated strips are used for *Salmonella*. It is a presumptive test; has to be confirmed by other methods.

Water Testing in Dialysis Settings

The microbiological contamination of dialysate fluid poses a major problem. Therefore, its purity has to be ensured periodically (e.g. monthly).

- **Membrane filtration** is the method of choice for testing dialysis water because it permits testing of large volume of water
- **Endotoxin detection:** Apart from bacteriological testing, the dialysis water is also tested for presence of endotoxin
  - *Methods:* Two methods are available: gel clot assay (Limulus amebocyte lysate assay) and kinetic test method (e.g. colorimetric method)
  - *Permissive level:* Water used to prepare dialysate and to reprocess hemodialyzers should contain a total microbial count <100 CFU/mL and endotoxin unit <0.25 EU/mL [AAMI (Association for the Advancement of Medical Instrumentation) guideline].

Examination for Specific Water-borne Pathogens

Specific water-borne pathogens may be isolated from water by employing enrichment and selective media.

- **For isolation of *Salmonella* Typhi:** Equal volume of water is added to the double strength selenite F broth followed by incubation and then subcultured onto selective medium
- **For *Vibrio cholerae*:** Water sample is mixed with alkaline peptone water in 1:9 ratio, incubated and subcultured onto selective medium
- Isolated organisms are identified by biochemical tests and serotyping
- Pathogenic organisms may also be isolated by membrane filtration method as described earlier.

AIR SURVEILLANCE

Air is an important vehicle of transmission of many pathogenic organisms. Therefore, the examination of air to detect the number of bacteria carrying particles is important particularly in critical areas such as operation theaters (OTs), bone marrow transplant units, etc.

Indication (CDC Recommendation)

Routine air sampling (i.e. random, undirected sampling) is not recommended because (i) HAI rates are not related with levels of general microbial contamination of air or environmental surfaces, (ii) there is no standard guideline mentioning for permissible levels of microbial contamination of environmental surfaces or air.

CDC recommends targeted air surveillance, which should be carried out for the following indications:

- Investigation of an outbreak
- For research purpose
- After reconstruction or newly constructed buildings
- After fumigation or fogging (to monitor the quality)
- For short-term evaluation of a change in infection-control practice.

Evaluation of the Quality of Air in OT

Microbiological Parameters

Evaluation of the quality of air in OTs can be performed routinely by two broad methods:

1. **Microbiological sampling method:** which can be done in two ways.
   a. Methods that measure bacteria carrying particles (chiefly larger particles) settle down by gravity from air on to the exposed surface, e.g. settle plate method.
   b. Methods that count the number of bacteria carrying particles in a given volume of air, e.g. the slit sampler method and air centrifuge method.
2. **Particle count method:** Here, the airborne particle concentrations in OT is measured by means of a laser light scattering instrument (particle counter).
   - The principle followed here is microorganisms are transferred by particles in the air. If the number of particles in the theater is reduced, then microbiological load in OT is also automatically reduced, thus lowering the risk of infection at the surgical site
   - The particle count of an OT is considered acceptable only when it falls in the acceptable range according to the international standards system ISO 14644-1.

Settle Plate Method

Petri dishes containing an agar medium of known surface area are left open for 30 minutes to 1 hour. Then, the plates are incubated at 37°C for 24 hours (Fig. 58.1A).

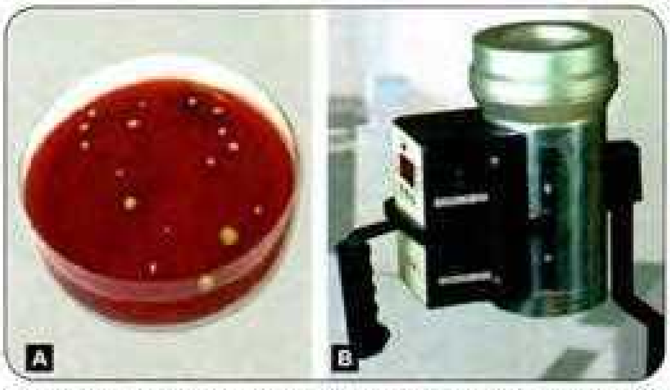
- **Colony count:** Large bacteria carrying dust particles settle onto the medium. The number of colonies formed on the plate indicates the number of settled particles containing bacteria
- Blood agar is the preferred medium for an overall count of pathogenic, saprophytic and commensal bacteria
- Malt extract agar may be used for molds.

Slit Sampler Method

This is the most efficient and convenient method for counting the number of bacteria carrying particles suspended in a unit volume of air.

Procedure: A special equipment called "slit sampler" (Fig. 58.1B) is available which has three parts—(1) an area to hold a petri dish, (2) suction pump and slit, (3) outer surface has a slit of 0.33 mm width and 27.5 mm length and 3 mm depth.

- Air is sucked through the equipment at a rate of one cubic foot (28.3 liter) per minute for 10 minutes and



Figs 58.1A and B: A. Settle plate method showing blood agar with settled particles containing bacterial colonies; **B.** Air sampler (HiMedia)

Source: Department of Microbiology, JIPMER, Puducherry (with permission).

directed onto a plate containing culture medium through the slit
- The plate is rotated mechanically so as to allow the organisms to spread out evenly on the medium
- The culture media are incubated and the colonies are counted. The number of colonies gives the number of bacteria carrying particles present in the air.

Non-microbiological Parameters

The number of bacteria in air at any given point of time depends upon various non-microbiological parameters such as: air changes per hour, air velocity, positive pressure environment, temperature and relative humidity inside OT, well maintained air handling units, well designed wall, floor and ceiling and number of persons present (Table 58.6). Therefore, there should be a periodic monitoring of these parameters.

SURFACE SURVEILLANCE

Environmental surface sampling has been used to determine a) reservoirs of potential environmental pathogens, b) survival of microorganisms on surfaces, and c) the sources of the environmental contamination.
- **Locations:** It is required for high-risk locations such as operation theaters and ICU settings
- **Sites for sampling (high touch areas):** Surface sampling is taken from sites where there is high risk of contaminations
- **Indications (CDC recommendation):**
  - Surface sampling is currently indicated for research, as a part of an epidemiologic investigation, or during an outbreak investigation
  - Routine periodic surface surveillance is not recommended.
- **Method:** Moistened sterile swabs (soaked in sterile saline) are used to take the samples from high-risk areas as mentioned above and then inoculated on to blood agar for the recovery of aerobic bacteria
- **Reporting:** Only pathogenic organisms isolated are reported. A semi-quantitative report (as heavy, moderate or light growth) is desirable. Contaminants such as aerobic spore bearers are not reported
- **Newer technique** such as luminometer (expresses bacterial contamination as CFU/mL) and glow gel techniques are available which are easy to perform though expensive.

MILK SURVEILLANCE

Milk can occasionally contain bacteria which are derived from three sources:
1. From animals (fecal contamination or through infected udder, teat canals and skin).

Table 58.6: Quality of air in OT (non-microbiological parameters)

Properties	Recommendations
Air changes per hour	Minimum 20 numbers of air changes per hour; out of which four should be fresh air
Air velocity	25–35 FPM (feet per minute) It is checked by anemometer
Positive pressure (PP)	• Difference in positive pressure between OT and adjoining areas is required to prevent outside air entry into OT • PP should be maintained in OT at all times (operational and non-operational hours) • Minimum PP of 2.5 Pascal is recommended
Temperature	21 +/– 3°C (orthopedic theaters, 18°C +/– 2°C)
Relative humidity	20 to 60% (ideal 55%)
Air handling in the OT	There should be dedicated AHU (air handling unit) for each OT and should not be linked to air conditioning of any other area/OT • Window and split AC should never be used in OT as they are pure recirculating units and have pockets for microbial growth • Air is supplied through terminal HEPA filters in the ceiling • The minimum size of the filtration area should extend one feet on each side of the OT table to cover the entire OT table and surgical team • Validation of HEPA filters is done biannually by DOP (Dispersed Oil Particulate) test
Wall, floor and ceiling	• Should be anti-static and made up of non-porous, smooth, seamless materials • Paints used should have antibacterial, anti-fungal property
Occupancy	Maximum occupancy of 5–8 persons at any time inside the OT is allowed

Abbreviation: HEPA, High efficiency particulate arrestance.

2. From hands of the milk handlers.
3. From the environment.

The milk-borne pathogens (Table 58.7) pose a threat to the community.

Methods used for Disinfecting/Sterilizing Milk

In general, there are four methods by which milk is sterilized or disinfected.
1. **Thermized milk:** It is raw milk that has been heated for 15 seconds at 57–68°C. The efficacy of this process is tested by methylene blue reduction test.
2. **Pasteurization:** Milk is heated to high temperature for short time (72° C for 15 seconds). Efficacy of pasteurization is tested by the phosphatase test.
3. **Ultra-heat treated milk:** The milk is exposed to very high temperature of 135°C for 1 second so that all microorganisms with their spores are destroyed.

Table 58.7: Milk-borne diseases (By Joint FAO/WHO Expert committee on Milk Hygiene, 1970)

Diseases primarily of animal origin, transmitted to man by milk	
Primary Importance	**Lesser Importance**
• Tuberculosis (*M. bovis*) • Brucellosis • Salmonellosis • *Coxiella burnetii* (Q fever) • Staphylococcal food poisoning • Streptococcal infections	• Cowpox • Hand-foot-and-mouth disease • Anthrax • Leptospirosis • *Campylobacter jejuni* infection • Tick-borne encephalitis viruses
Diseases primarily of human origin, transmitted by milk	
Primary Importance	**Lesser Importance**
• Typhoid and paratyphoid fever • Shigellosis • Cholera • Enterohemorrhagic *E. coli* (EHEC) infection	Non-diarrheal diseases: • Streptococcal infection • Staphylococcal food poisoning • Diphtheria • Tuberculosis • Enteroviral infections • Viral hepatitis

4. **Sterilized milk:** The milk is heated at 100°C for long periods such that it can pass the turbidity test.

Methods for Bacteriological Examination of Milk

This is carried out by several methods:

- Colony count methods such as viable count and coliform count methods
- **Chemical tests:**
  - Methylene blue reduction test
  - Phosphatase test (it is based on the principle that if the pasteurization is effective, it should inactivate the enzyme alkaline phosphatase which is normally present in the milk)
  - Turbidity test.
- Detection of specific pathogens such as tubercle bacilli, *Brucella* (refer Chapter 35 for detail), etc.

FOOD SURVEILLANCE

There are a number of food-borne pathogens, transmitted by various sources of food (Table 58.8). They pose a significant public health problem causing morbidity and mortality.

Table 58.8: Agents of food poisoning and their common food source

Agents of food poisoning	Food source
Staphylococcus aureus	Ham, poultry, potato or egg salad, mayonnaise, cream pastries
Bacillus cereus	Fried rice
Clostridium perfringens	Beef, poultry, legumes, gravies
B. cereus	Meat, vegetables, dried beans, cereals
Vibrio cholerae	Shellfish, water
Enterotoxigenic *Escherichia coli*	Salads, cheese, meat, water
Enterohemorrhagic *E. coli*	Ground beef, roast beef, salami, raw milk, raw vegetables, apple juice
Salmonella species	Beef, poultry, eggs, dairy products
Campylobacter jejuni	Poultry, raw milk
Shigella species	Potato or egg salad, lettuce, raw vegetables
Vibrio parahaemolyticus	Mollusks, crustaceans

Therefore, examination of food materials is essential to determine the number and types of bacteria present in order to control the standards of hygiene practices followed and also to investigate outbreaks of food poisoning.

Viable Plate Count

Viable plate count (or standard plate count) is the standard method followed for bacteriological examination of food.

- **Food sampling:** (1) 10 g of food material is taken in a sterile container and is homogenized in 90 mL of sterile diluent, e.g. Ringer's solution. (2) for the food contaminated only on its surface, such as intact vegetable or fruit, 100 g of food is taken in a sterile container containing 100 mL of sterile water and then shaken well so that all bacteria present on its surface will come out and are dissolved in water
- **Food processing:** Serial dilutions of homogenate or diluent is made, and then plated onto appropriate medium. The coliform count on MacConkey broth and differential count detecting thermotolerant *E. coli* can be made by the methods as described for water analysis.

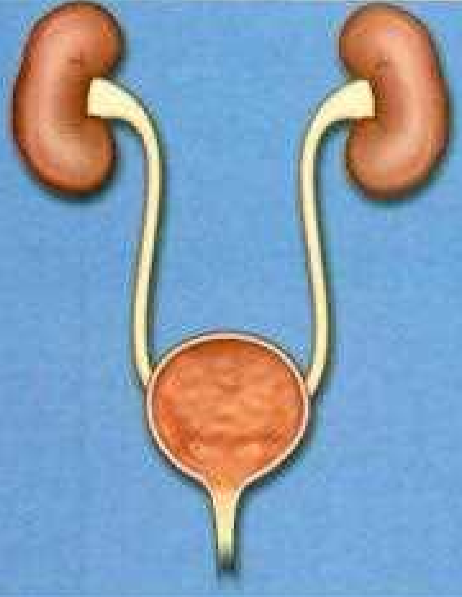
EXPECTED QUESTIONS

I. **Write short notes on:**
   1. Indicator organisms for fecal contamination of water.
   2. Eijkman test.
   3. Settle plate method.

II. **Multiple Choice Questions (MCQs):**
   1. **Which is not used as indicator organism of fecal contamination of water?**
      a. Fecal *E. coli* b. Fecal streptococci
      c. *Pseudomonas* d. *Vibrio cholerae*
   2. **Evaluation of the quality of air in OTs can be performed by all the following methods, *except*:**
      a. Settle plate b. Slit Sampler
      c. Particle count d. Presence- absence

Answer

1. d **2.** d



Clinical Microbiology
(Infective Syndromes)

SECTION 7

Section Outline

Normal Microbial Flora of Human Body

59
CHAPTER

Chapter Preview

- Microbiology of normal flora
- Role of normal flora
  - Beneficial effects
  - Harmful effects
- Probiotics
- Prebiotics

INTRODUCTION

Normal flora (also called "indigenous microbiota") refers to the diverse group of microbial population that every human being harbors on his/her skin and mucous membranes.

- In humans, the normal flora is located in various sites such as gastrointestinal tract (GIT), respiratory tract, genitourinary tract and skin
- Although there are many species of normal flora, these microbes typically fall into one of the two categories: resident flora and transient flora.

Resident Flora

These organisms are life-long members of the body's normal microbial community.

- They are very closely associated with a particular area. When disturbed, they again re-establish themselves. For example, *Escherichia coli* is a resident flora of the intestine
- They do not cause harm; rather they have beneficiary effect on the host (described later).

Transient Flora

The transient flora consists of microorganisms that inhabit the body surface or mucous membranes temporarily for a short interval.

- Many of the transient flora are potential pathogens which may cause disease under certain conditions, e.g. pneumococcus and meningococcus in nasopharynx
- In hospitals, patients may acquire many resistant organisms as transient flora from the healthcare workers and hospital environment. For examples, MRSA (Methicillin-resistant *Staphylococcus aureus*) in nose and skin, multidrug resistant gram-negative organisms such as *Klebsiella, Escherichia coli, Pseudomonas, Acinetobacter* in respiratory tract
- In contrast to resident flora, they can be easily eliminated from the body surface by following proper hand hygiene and other infection control practices.

MICROBIOLOGY OF NORMAL FLORA

The resident microbial flora is more or less constant for a given area of the body at a given age.

- Humans acquire the normal flora soon after the birth and then continue to harbor until death
- Although life is possible without normal flora (e.g. germ free experimental animals), but they certainly have a definite role in maintaining health and normal functions of their host
- The presence of the normal microbial flora in a given body site depends upon various local factors:
  - Local temperature, moisture, pH (acidic or alkaline)
  - Presence of certain nutrients and inhibitory substances
  - Environmental flora (hospital or community)
  - Immune status of the individual
  - Anatomical site: Skin or mucosa (gastrointestinal, respiratory or urogenital).
- Most of the normal flora predominantly contain bacteria and to a less extent some fungi. The existence of viruses and parasites as normal flora is doubtful
- The total population of normal flora in humans is roughly about 10^{14} bacteria; which is more than total number of cells (10^{13}), present in human body
- Overall, anaerobic flora dominates over aerobes; the ratio of anaerobic/aerobic bacteria varies depending upon the body site (Table 59.1)

Table 59.1: Microbiology of normal flora

Anatomical site	Total bacteria/ g or mL	Anaerobic/ aerobic ratio	Anaerobic normal flora (common)	Aerobic normal flora	
Mouth				**Predominant**	**Less predominant**
Saliva	10^8–10^9	1:1	• Anaerobic cocci • *Actinomyces* • *Fusobacterium* • *Bifidobacterium* • *Prevotella* • Spirochetes	Viridans streptococci	
Tooth surface	10^{10}–10^{11}	1:1			
Gingiva	10^{11}–10^{12}	10^3:1			
Nasopharynx				**Predominant**	**Less predominant**
			• *Prevotella* species • Anaerobic cocci • *Fusobacterium*	• Streptococci (α and non-hemolytic) • *Neisseria* (non-pathogenic species) • Diphtheroids • *Staphylococcus epidermidis*	• *Haemophilus* • Meningococcus • Pneumococcus • *Staphylococcus aureus* • Gram-negative rods • Yeasts
Gastrointestinal tract				**Predominant**	**Less predominant**
Stomach	0–10^3	1:1	*Lactobacillus*	*Helicobacter pylori*	
Jejunum/ileum	10^5–10^7	1:1	• Anaerobic cocci • *Bacteroides fragilis* • *Fusobacterium* • *Bifidobacterium* • *Prevotella* • *Clostridium*	• Enterobacteriaceae and other gram-negative rods • Enterococci • Streptococci (α and non hemolytic), *S. agalactiae*	• Diphtheroids • *Candida albicans* and other yeasts • *Staphylococcus aureus*
Terminal ileum and colon	10^{11}–10^{12}	10^3:1			
Female genital tract				**Predominant**	**Less predominant**
Vagina	10^7–10^9	10:1	• Anaerobic cocci • *Lactobacillus* • *Prevotella* • *Bifidobacterium* • *Clostridium*	• *Corynebacterium* species • *Lactobacillus* species • Streptococci (α, non-hemolytic and *S. agalactiae*) • *Neisseria* (non-pathogenic species)	• Enterococci • Enterobacteriaceae and other gram-negative rods • *S. epidermidis* • *Candida albicans* and other yeasts
Skin				**Predominant**	**Less predominant**
	10^3–10^4	3:2	• *Propionibacterium* • Anaerobic cocci	• *Staphylococcus epidermidis* • Diphtheroids • *Micrococcus* species • *Neisseria* (Non-pathogenic species) • Streptococci (α and non hemolytic)	• *Staphylococcus aureus* • *Candida* species • *Acinetobacter* species

- GIT is the predominant site, where over 400 species of different bacteria have been counted till date
- The most common normal flora in humans is *Bacteroides fragilis*; however among aerobes, it is *Escherichia coli*; both are a part of intestinal flora.

The microbiological profile of the normal flora in various sites of human body is given in Table 59.1.

ROLE OF NORMAL FLORA

Various microorganisms present in the normal flora have different relationship with the host.

- They may have beneficiary effect on the host or;
- They may be harmful to the host (if enter into a wrong site, cause endogenous infection), or;
- They may exist as commensals (inhabiting the host for long periods without causing detectable harm or benefit).

Beneficial Effects

The normal microbial flora has several beneficial effects to the host (Fig. 59.1) which is proved experimentally by comparing between the conventional animals having normal flora with germ-free animals (animals lacking normal flora) and gnotobiotic animals (animals harboring

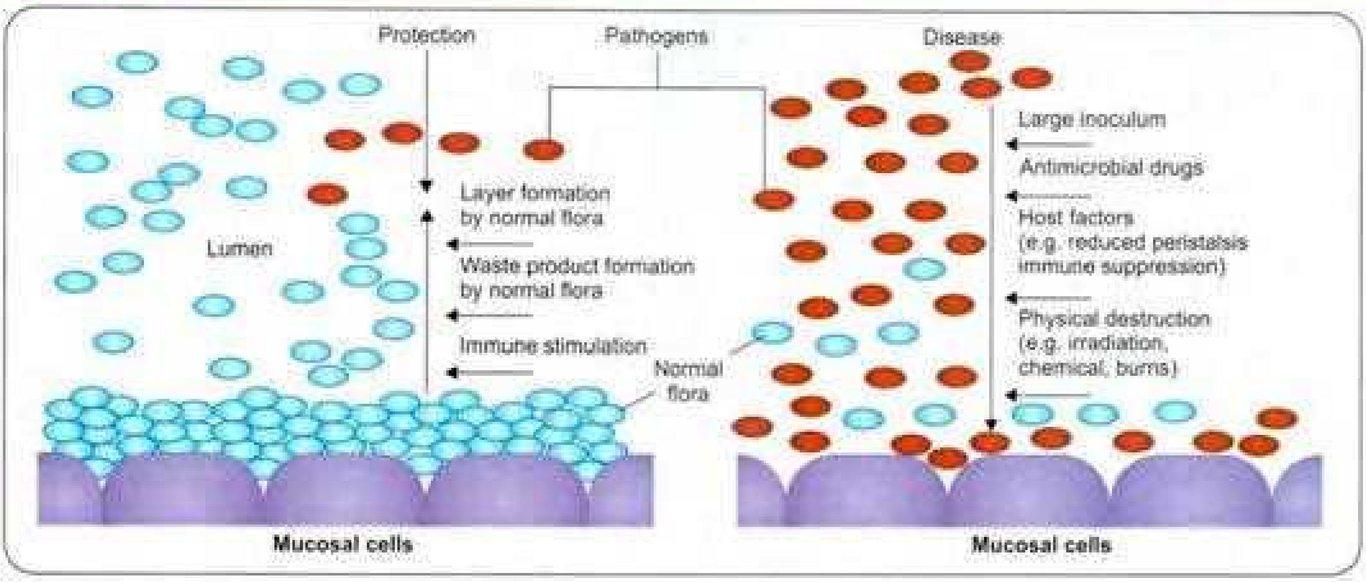


Fig. 59.1: Beneficial effect of normal flora in health and disease

certain few known microorganisms). The beneficial effects of normal flora are as follows:

- **Prevent colonization of pathogen:** The normal flora prevent colonization of pathogens by competing for attachment sites or for essential nutrients
- **Synthesize vitamin:** Human enteric bacteria secrete several vitamins such as Vitamin K and B complex (e.g. Vitamin B12) in excess; which can be absorbed by the host as nutrient
- **Waste produced antagonize other bacteria:** Normal flora may inhibit or kill other nonindigenous organisms by producing a variety of waste substances such as:
  - Fatty acids and peroxides
  - Lactic acid: Lactobacilli present as normal flora in vagina of adult females maintain the acidic pH by producing lactic acid, thereby prevent the growth of pathogenic bacteria
  - Bacteriocin: Some bacterial flora may produce bacteriocin or colicins which are antibiotic-like substances that can inhibit or kill other bacteria.
- **Immune stimulation:** Normal microbial flora being foreign to the host stimulates the host's immune system
  - **Development of lymphatic tissues:** Immune stimulation helps in development of lymphatic tissues in the local sites (e.g. Peyer's patches in intestine)
  - **Stimulate antibody production:** The antigens of the normal microbial flora stimulate the host immune system to produce antibodies that cross-react with pathogens having related or shared antigens and prevent their entry.
- **Prevent allergic diseases (Hygiene hypothesis):** It states that a lack of early childhood exposure to symbiotic microorganisms (e.g. gut flora or probiotics), infectious agents and parasites increases susceptibility of the individual to allergic diseases by suppressing the natural development of the immune system
- **Complement activation:** The endotoxins liberated by the gram-negative population of normal flora may help the defense mechanisms of the body by triggering the alternative complement pathway.

Disturbed Normal Flora Promote Infection

When the composition of normal flora is disturbed, it facilitates pathogenic organisms to enter and cause disease. Several mechanisms by which the normal flora is disturbed are as follows:

- **Injudicious use of broad spectrum antimicrobial agent:** It may completely suppress the normal flora thus permitting the pathogen (exogenous and endogenous) to take the upper hand and cause infection. For example, *Clostridioides difficile*, causing pseudomembranous colitis
- **Host factors** such as immune suppression, reduced peristalsis may promote the pathogen to grow
- **Physical destruction** of the normal flora: by irradiations, chemicals, burns, etc.
- If the **inoculum size** of the entering pathogen is high then it can dominate over the normal flora
- **Minor trauma** in mouth (e.g. by dental procedure, chewing or vigorous brushing) can promote passage of small numbers of bacteria (e.g. viridians streptococci) transiently into bloodstream, which can cause bacterial endocarditis.

Table 59.2: Diseases produced by normal flora

Diseases produced by normal flora	Anatomical site from which the flora is transferred
Urogenital infections including UTI	Intestinal flora such as *Escherichia coli*, *Klebsiella*, *Proteus*
Endocarditis	Oral flora (Viridans streptococci)
Dental caries and periodontal disease	Oral flora (*Streptococcus mutans*)
Peritonitis, abdominal infection	Intestinal flora
Pneumonia	Transient respiratory flora
Septicemia	From any site

Harmful Effects

Normal flora may produce the following harmful effects, out of which the first two are significant.

- **May be agents of disease:** Members of the normal flora may cause various endogenous disease (Table 59.2)
  - When the host immunity is lowered, the transient flora may invade and produce disease, e.g. gram-negative organisms (*E. coli*) colonizing the respiratory tract can cause pneumonia
  - If they enter a wrong site or tissue (e.g. blood, sterile body cavities)—then even the resident flora can produce disease. For example, *E. coli* which is a resident flora of intestine may cause urinary tract infection if enters into urinary tract.
- **Transfer to susceptible hosts:** Some pathogens of humans that are members of the normal flora for one host can produce disease if transferred to the other host. For example, the pathogens that colonize the upper respiratory tract (such as meningococcus, pneumococcus, etc.) can produce disease in susceptible hosts
- **Bacterial synergism:** Bacterial vitamins and growth factors produced by members of the normal flora may promote the growth of the potential pathogens
- **Contribute to the drug resistance of pathogens:** Some members of normal flora produce enzymes such as beta lactamases which destroy the beta lactam antibiotics; thus indirectly contribute to the drug resistance of pathogens that are otherwise susceptible to the drug
- **Competition for host nutrients:** Bacteria in GIT absorb some of the host's nutrients for their survival.

Probiotics

The term "Probiotics" is defined as the live microorganisms (part of normal flora) which, when administered in adequate amounts, confer a health benefit to the host.

- They are extremely useful in the conditions where the normal intestinal flora is suppressed
- Probiotics are commercially available in the form of capsule or sachet, consisting of mixture of some important beneficiary bacteria and yeast of human intestinal flora such as *Bacillus coagulans, Bifidobacterium longum, Lactobacillus acidophilus, Saccharomyces boulardii*, etc.

Probiotics are found to have beneficiary role in the following conditions/diseases:

- **To treat various forms of GIT conditions like:**
  - Gastroenteritis due to any cause
  - Antibiotic-associated diarrhea
  - Lactose intolerance
  - Irritable bowel syndrome and colitis
  - Necrotizing enterocolitis
  - *Helicobacter pylori* infection.
- Reducing serum cholesterol level by breaking down bile in the gut, thus inhibiting its reabsorption
- Reducing blood pressure (by producing ACE inhibitor-like peptides during fermentation)
- Immune function restoration and preventing infections
- Modulate inflammatory and hypersensitivity responses, hence can be given in allergic disorders, eczema and atopic dermatitis
- Bacterial vaginosis (restoring the acid pH of vagina by lactic acid producing bacteria).

The live organisms contained in probiotics must remain live to have their action on the large intestine. More so, they have to compete with the existing flora to get themselves established. They can exert their beneficiary effect only after that. Hence, nowadays, instead of probiotics, another related preparation called prebiotics is being increasingly used.

Prebiotics

In contrast to probiotics, prebiotics are the dietary non-digestible fibers which when administered, stimulate the growth and activity of commensal microorganisms and thereby exert beneficiary effect to the host indirectly.

EXPECTED QUESTIONS

I. **Write short notes on:**
1. Resident flora and transient flora.
2. Harmful effects of the normal flora.
3. Beneficial effects of the normal flora.
4. Probiotics.

Urinary Tract Infections

60
CHAPTER

CASE SCENARIOS

Case scenario-1 (Lower UTI): A 32-year-old female was admitted with dysuria (burning micturition) and increased frequency of micturition for the past 2 days.

Case scenario-2 (Upper UTI): A 28-year-old female was admitted with high grade fever, vomiting, flank pain with increased frequency of micturition for the past 3 days.

In both the case scenarios, urine specimens were collected in a sterile containers and sent for microscopy, culture and sensitivity testing.

Questions:

1. What is your clinical diagnosis in both the cases?
2. What are the risk factors associated with this clinical condition?
3. What are the etiological agents, pathogenesis and clinical manifestations of this disease?
4. What are the various methods of specimen collection?
5. Describe the laboratory diagnosis in detail?
6. What are the treatment modalities according to the etiological agents?

Explanation:

Both the cases are examples of urinary tract infections (UTI) (Table 60.1).

The first scenario is a case of lower UTI (burning micturition and increased frequency) and the second scenario is a case of upper UTI (high-grade fever, vomiting, and flank pain with increased frequency).

Explanation to all other questions has been described subsequently in the chapter.

URINARY TRACT INFECTION

Urinary tract infection is defined as a disease caused by microbial invasion of the urinary tract that extends from the renal cortex of the kidney to the urethral meatus.

- The presence of detectable bacteria in urine is named as bacteriuria
- Presence of pus cells in urine is referred to as pyuria.

Classification

- UTIs may be broadly classified into two types—lower UTI and upper UTI (Table 60.1) depending upon the anatomical sites involved
- Depending upon the source of infection, UTI can be of two types: hospital acquired and community acquired.

Epidemiology

Urinary tract infections (UTIs) are among the most common bacterial infections that need medical care; accounting for second most common infection after respiratory tract infections in the community. Whereas in hospitals, they are the most common HAIs (hospital acquired infections) accounting for 35% of total HAIs.

Predisposing Factors

- **Prevalence:** About 10% of humans develop UTI in some part of their life
- **Gender:** UTI is predominantly a disease of females. The higher prevalence in females is due to the anatomical structure of female urogenital system, (1) short urethra, and (2) close proximity of urethral meatus to anus; so that there is more chance of introduction of endogenous bacteria into the urinary tract
- **Age:** Incidence increases with age
  - During first year of life, the prevalence is around 2% in both females and males
  - After that, the incidence of UTI decreases in males until old age where they again show an increased

Table 60.1: Comparison between lower and upper UTIs

	Lower UTI	Upper UTI
Sites involved	Urethra, and bladder	Kidney and ureter
Symptoms	Local manifestations: dysuria, urgency, frequency	Local and systemic manifestations (fever, vomiting, abdominal pain)
Route of spread	Ascending route	Both ascending (common) and descending route
Occurrence	More common	Less common

prevalence because of the prostate enlargement which interferes with emptying of the bladder
- Whereas in females, the incidence keeps increasing after first year of life
  - During 5–17 years, the incidence of bacteriuria is about 1–3%
  - Thereafter in adult life, the incidence is around 10–20%
  - Reinfection is common in females (20–40 years of age), as many as 50% would suffer a reinfection within one year.
- **Pregnancy:** Anatomical and hormonal changes in pregnancy favor development of UTIs. Most females develop asymptomatic bacteriuria during pregnancy. In some, it can lead to serious infections in both mother and fetus
- **Structural and functional abnormality** of urinary tract may cause obstruction to the urine flow and urinary stasis; which predisposes to infection
  - Structural obstruction: For example, urethral stricture, renal and ureteric stones, prostate enlargement, tumors, renal transplants, etc.
  - Functional obstruction: For example, neurogenic bladder due to spinal cord injury or multiple sclerosis.
- **Bacterial virulence** such as expression of pili helps in bacterial adhesion to uroepithelium
- **Vesico-ureteric reflux:** If the normal valve-like mechanism at the vesico-ureteric junction is weakened, it allows urine from bladder up into ureters and sometimes into the renal pelvis
- **Genetic factors:** Genetically determined receptors present on uroepithelial cells may help in bacterial attachment.

UTI is the leading cause of gram-negative sepsis (urosepsis) especially in hospitalized patients and the urinary catheters are the origin of nearly 50% of nosocomial UTIs.

Etiology

Escherichia coli (uropathogenic *E. coli*) is by far the most common cause of all forms of UTIs (i.e. community acquired & nosocomial UTI and upper & lower UTI) accounting for 70% of total cases.
- The endogenous flora such as gram-negative bacilli (e.g. *E. coli, Klebsiella, Proteus,* etc.) and enterococci are the important agents
- In hospital acquired UTIs, the agents are often multidrug resistant. In addition to the members of Enterobacteriaceae, other organisms such as staphylococci, *Pseudomonas, Acinetobacter* are also increasingly reported
- In general, viruses and parasites are not considered as urinary pathogens except for few (Table 60.2). Among fungi, *Candida albicans* is a frequent cause of UTI.

Table 60.2: Common microorganisms causing UTIs

Bacterial agents	Other agents
Gram-negative bacilli: • ***Escherichia coli:*** Most common • *Klebsiella pneumoniae* • *Proteus mirabilis* • *Pseudomonas aeruginosa* • *Acinetobacter* species • *Enterobacter* species • *Serratia* species	**Fungus:** *Candida albicans* **Parasites:** • *Schistosoma hematobium* • *Trichomonas vaginalis*
Gram-positive cocci: • *Staphylococcus saprophyticus** • *Staphylococcus aureus* • *Staphylococcus epidermidis* • *Enterococcus* species	**Viruses:** • Herpes simplex virus • Adenovirus • JC and BK virus • Cytomegalovirus

Abbreviations: UTI, Urinary tract infection; JC, John Cunningham
*Common in sexually active females.

Pathogenesis

Bacteria invade the urinary tract mainly by two routes—ascending and descending routes (Fig. 60.1).

Ascending Route

It is the most common route; the enteric endogenous bacteria (*E. coli,* other gram-negative bacilli, enterococci) enter the urinary tract which is facilitated by sexual intercourse, or instrumentation (e.g. catheterization), etc.

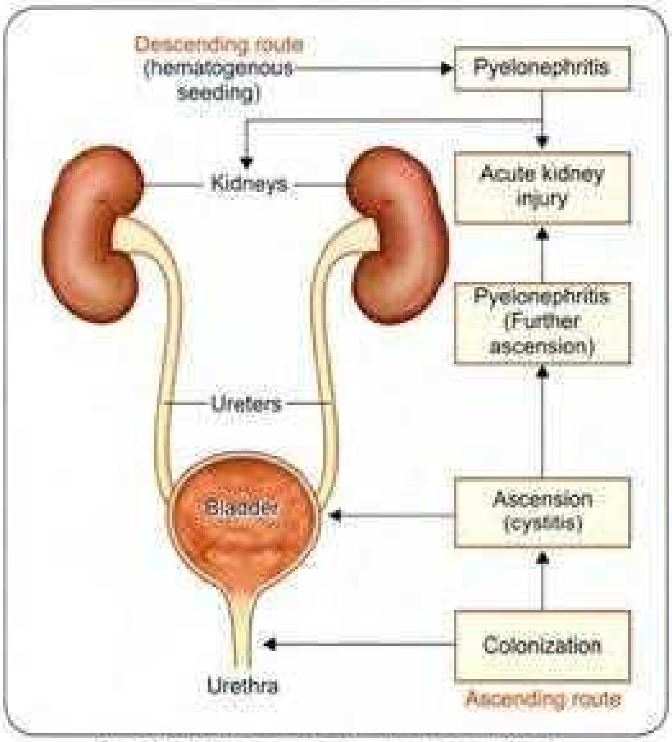


Fig. 60.1: Pathogenesis of urinary tract infection

- **Colonization:** Adhesion to urethral epithelium is the first and the most important step in pathogenesis. A number of virulence factors (e.g. P fimbriae, mannose resistant fimbria in *E. coli*) help in adhesion
- **Ascension:** Following colonization, pathogen ascends through urethra upwards towards bladder to cause cystitis. Bacterial toxins may facilitate ascension by inhibiting peristalsis (urinary stasis)
- **Further ascension** through ureter may occasionally occur if there is vesico-ureteric reflux leading to pyelonephritis (infection of renal parenchyma causing an acute inflammatory response)
- **Acute tubular injury:** If the inflammatory cascade continues, tubular obstruction and damage occurs which may lead to interstitial nephritis.

Descending Route

This refers to invasion of renal parenchyma through hematogenous seedling of the pathogen, which occurs as a consequence of bacteremia. This accounts for 5% of total UTIs. Although most infections affecting kidney are acquired by ascending route, certain organisms are particularly invasive and their association with pyelonephritis often indicates a descending route of origin; for example—*Staphylococcus aureus, Salmonella, Mycobacterium tuberculosis, Leptospira* and *Candida*.

Host Defense Mechanisms

Host defense mechanisms play an important role in prevention of UTI. They can be grouped into—(1) factors related to urine, (2) activation of host's mucosal immunity by the uropathogens (Table 60.3).

Clinical Manifestations

UTIs may be presented in various forms:

1. Asymptomatic bacteriuria.
2. Lower UTI: Cystitis, and acute urethral syndrome.
3. Upper UTI (pyelonephritis).

Table 60.3: Host defense mechanisms against UTIs

Urine factors	Mucosal immunity
Acidic urine: inhibits pathogens	Uroepithelial secretion of cytokines (induced by bacterial LPS)
High urine osmolality: inhibits pathogens	Mucosal IgA- prevent attachment of pathogen to uroepithelium
Urinary inhibition of bacterial adherence	Tamm Horsfall protein (uromucoid)—a glycoprotein secreted by epithelial cells of kidney, serves as anti-adherence factor by binding to type-I fimbria of *E. coli*
Mechanical flushing by urine flow	In men: (1) Zinc in prostatic secretion is bactericidal, (2) long urethra

Abbreviation: LPS, Lipopolysaccharide.

Asymptomatic Bacteriuria

It refers to isolation of specified quantitative count of bacteria in an appropriately collected urine specimen, obtained from a person without symptoms of UTI. It is more common in females and its incidence increases with age (1% among school girls to more than 20% in old age).

Clinical Significance

- Asymptomatic UTI is clinically significant in certain group of people such as pregnant women (as chances of complication in mother and fetus are more), people undergoing prostatic surgery or any urologic procedure where bleeding is anticipated. Therefore, in this group the routine screening and treatment for asymptomatic UTI is highly recommended
- In contrast, asymptomatic UTI is not clinically significant in non-pregnant, pre-menopausal women, old age, catheterized patient, or patients with spinal injury. In such cases, neither screening nor treatment of asymptomatic UTI is needed.

Cystitis (Infection of Bladder)

It is characterized by localized symptoms such as:

- Dysuria (pain while micturition), frequency, urgency, and suprapubic tenderness (over the bladder area)
- Urine becomes cloudy, with bad odor, and in some cases grossly bloody (hematuria)
- There is no associated systemic manifestation.

Acute Urethral Syndrome

This is another form of lower UTI seen in young sexually active females, characterized by:

- Presence of classical symptoms of lower UTI as described for cystitis
- Bacterial count is often low (10^2 to 10^4 CFU/mL)
- Pyuria is present
- **Agents:** Most are due to usual agents of UTI, few cases may be caused by gonococcus, *Chlamydia*, herpes simplex virus, etc.

Upper UTI or Pyelonephritis

Pyelonephritis refers to inflammation of kidney parenchyma, calyces and the renal pelvis, i.e. the part of ureter present inside the kidney.

- Associated with systemic manifestations such as—fever, flank pain, vomiting
- Lower tract symptoms such as frequency, urgency and dysuria may also be present.

Laboratory Diagnosis

- **Specimen collection:** Urine should be collected in a wide mouth screw capped sterile container by—(1) clean catch midstream urine, (2) suprapubic aspiration from bladder, (3) In catheterized patients—urine aspirated from the catheter tube after clamping distally and disinfecting, but never collected from urine bag

- **Transport:** Urine sample should be processed immediately. If delay is expected then it can be refrigerated or stored by adding boric acid
- **Direct examination:** Screening tests done are as follows:
  - Wet mount examination is done to demonstrate the pus cells in urine
  - Leukocyte esterase test—to detect the esterase enzyme liberated by leukocytes
  - Nitrate reduction test (Griess test)—to detect nitrate reducing bacteria
  - Catalase test—to detect catalase producing bacteria
  - Gram-staining of urine.
- **Culture:** Urine sample should be inoculated onto MacConkey agar and blood agar or CLED (cysteine lactose electrolyte deficient) agar
  - A count of $\geq 10^5$ **colony forming units (CFU)/mL** of urine is considered as significant—indicates infection (referred to as 'significant bacteriuria')
  - Colony count between 10^4 to 10^5 CFU/mL indicates doubtful significance; should be clinically correlated
  - **Low count of** $\leq 10^4$ CFU/mL is considered as insignificant—indicates presence of commensal bacteria (due to contamination during voiding)
  - **Quantitative culture** such as pour plate method is carried out to count the number of colonies.
- **Antibody coated bacteria test:** It is used to differentiate upper and lower UTI.

The laboratory diagnosis of UTI has been described in detail in Chapter 29. Culture identification features of common organisms causing UTI is described in Table 60.4.

TREATMENT **Urinary tract infections**

Treatment should be based on antimicrobial susceptibility testing report. Quinolones (e.g. norfloxacin), nitrofurantoin, cephalosporins, and aminoglycosides are among the preferred drugs.

Higher antibiotics such as carbapenem (e.g. meropenem), beta lactam-beta lactam inhibitor combinations (e.g. piperacillin-tazobactam) or fosfomycin are used for treatment of hopsital acquired UTIs due to multidrug resistant gram-negative bacilli.

Table 60.4: Culture identification features of common organisms causing UTI

	Culture	Culture smear and motility testing	Biochemical reactions
Escherichia coli (Fig. 29.1)	MAC or CLED: flat lactose fermenting colonies BA: gray moist colonies	Gram-negative bacilli Motile	Catalase positive, oxidase negative ICUT tests: I^+ C^- U^- TSI (acidic slant/acidic butt, gas$^+$, H_2S^-)
Klebsiella pneumoniae (Figs 29.2 and 3A)	MAC or CLED: mucoid lactose fermenting colonies BA: gray mucoid colonies	Gram-negative bacilli Non-motile	Catalase positive, oxidase negative ICUT tests: I^- C^+ U^+ TSI (acidic slant/acidic butt, gas$^+$, H_2S^-)
Proteus species (Fig. 29.3C)	MAC or CLED: lactose non-fermenting colonies BA: swarming type of growth	Gram-negative pleomorphic bacilli Motile	Catalase positive, oxidase negative ICUT tests: $I^{+/-}$ $C^{+/-}$ U^+ TSI (alkaline slant/acidic butt, gas$^{+/-}$, H_2S+)
Enterococcus (Fig. 22.7)	MAC: magenta pink colonies BA: translucent non-hemolytic colonies	Gram-positive cocci in pair, spectacle shaped Non-motile	Catalase negative Bile aesculin test positive
Staphylococcus aureus (Figs 21.2 to 21.3)	BA: golden yellow hemolytic colonies	Gram-positive cocci in clusters Non-motile	Catalase positive, coagulase positive
Staphylococcus saprophyticus	BA: white non-hemolytic colonies	Gram-positive cocci in clusters Non-motile	Catalase positive, coagulase negative, Resistant to novobiocin

Abbreviations: I, Indole test; C, Citrate test; U, Urease test; TSI, triple sugar iron agar test; +, positive; –, negative; MAC, MacConkey agar; BA, Blood agar; CLED, Cysteine lactose electrolyte-deficient agar.

EXPECTED QUESTIONS

I. Essay:

1. Describe the pathogenesis, clinical types, etiological agents and detail laboratory diagnosis of urinary tract infection.

II. Write short notes on:

1. Significant bacteriuria.
2. Difference between upper and lower UTI.
3. Asymptomatic bacteriuria.
4. Pathogenesis of UTI.

III. Multiple Choice Questions (MCQs):

1. **Which culture medium is preferred for processing of urine specimens?**
   a. TCBS agar b. CLED agar
   c. Chocolate agar d. XLD agar
2. **Which of the following is the most predominant normal flora of human intestine?**
   a. *Escherichia coli* b. *Bacteroides fragilis*
   c. *Fusobacterium* d. *Bifidobacterium*

Answers

1. b **2.** b

Diarrheal Diseases

61
CHAPTER

CASE SCENARIOS

Case scenario-1 (Watery Diarrhea): A 6-year-old boy developed severe watery diarrhea (12–15 times) and vomiting for 2 days. Stool collected has a rice water type of appearance. On enquiry, it was found that two other members of same family and few children of the same locality also suffered from similar presentation last week.

Case scenario-2 (Dysentery): In an outpatient department, a 2-year-old child presented with tenesmus, abdominal pain and passage of bloody diarrhea with mucus, eight times for the past 2 days.

Case scenario-3 (Food Poisoning): A group of patients presented to the emergency department with chief complaints of fever, vomiting and diarrhea. All of them had attended a birthday party 4–6 hours back where they had cream pastries.

In all these case scenarios, stool specimens were collected in sterile containers and sent for microscopy, culture and sensitivity testing.

Questions:

1. What is your probable clinical diagnosis in all these cases?
2. What are the etiological agents, pathogenesis and clinical manifestations?
3. Describe the laboratory diagnosis in detail.
4. What are the treatment modalities according to the etiological agents?

Explanation:

All these cases are examples of diarrheal diseases.

Case scenario-1: The first scenario is a case of watery diarrhea. Severe watery diarrhea (12–15 times) with rice water appearance stool is suggestive of 'cholera'. Similar cases in family and nearby places is pointing towards the possibility of an outbreak. The various causative agents of diarrheal diseases are listed in Table 61.1.

Case scenario-2: The history of tenesmus (recurrent inclination to pass stools, despite an empty colon) abdominal pain and frequent passage of bloody diarrhea with mucus is suggestive of dysentery. The various agents of dysentery are enumerated in Table 61.1.

Case scenario-3: Group of patients presenting with fever, vomiting and diarrhea with a common point source (consumption of cream pastries) indicates that it is a case of food poisoning. The various causative agents of food poisoning are enumerated in Table 61.2. Presentation after short period of intake of food (4–6 hours) indicates it may be a case of *S. aureus* or *Bacillus cereus* food poisoning.

Explanation to all other questions has been described subsequently in the chapter.

DIARRHEAL DISEASES

There are various clinical types of diarrheal diseases.

Diarrhea

Diarrhea is defined as passage of three or more loose or liquid stools per day, in excess than the usual habitat for that person (World Health Organization). It may be caused by microbial infections, or as a result of other gastrointestinal diseases such as inflammatory bowel diseases, coeliac disease, etc. Common microbial agents causing diarrhea and the mechanisms involved are summarized in Table 61.1.

Gastroenteritis

Gastroenteritis or infectious diarrhea may be defined as inflammation of the mucous membrane of stomach and intestine resulting in combination of diarrhea, vomiting and pain abdomen with or without mucus/blood/fever/dehydration.

Dysentery

Dysentery is characterized by diarrhea with increased blood and mucus, often associated with fever, abdominal pain, and tenesmus (a feeling of incomplete defecation).

Food Poisoning

Food poisoning refers to an illness acquired through consumption of food or drink contaminated either with microorganisms, or their toxins.

Table 61.1: Infectious agents of acute diarrhea and the underlying mechanism

Mechanism	Features	Examples of pathogens involved	
Non-inflammatory	**Location:** Proximal small bowel **Illness:** Watery diarrhea **Stool findings:** • No fecal leukocytes • Fecal lactoferrin—not increased	**Bacteria:** **(Mostly Enterotoxin mediated)** • *Vibrio cholerae* • *Escherichia coli:* ➢ Enteropathogenic ➢ Enterotoxigenic ➢ Enteroaggregative • *Clostridium perfringens* • *Bacillus cereus* • *Staphylococcus aureus* • *Aeromonas hydrophila* • *Plesiomonas shigelloides*	**Viruses:** • Rotavirus • Norovirus • Enteric adenoviruses **Parasites:** • *Giardia lamblia* • *Cryptosporidium* species • *Cyclospora* species • *Cystoisospora* species • Microsporidia
Inflammatory (invasion or cytotoxin)	**Location:** Colon or distal small bowel **Illness:** • Dysentery or • Inflammatory diarrhea **Stool findings:** • Fecal pus cells (polymorphonuclear leukocytes)—increased • Fecal lactoferrin—increased	**Predominantly dysentery:** • *Shigella* species • *Campylobacter jejuni* • Enterohemorrhagic *E. coli* • Enteroinvasive *E. coli* • *Vibrio parahaemolyticus* **Predominantly inflammatory diarrhea** • *Salmonella* species • *Yersinia enterocolitica* • *Listeria monocytogenes* • *Clostridium difficile* • *Aeromonas hydrophila* • *Plesiomonas shigelloides*	**Parasite** (predominantly dysentery) • *Entamoeba histolytica,* • *Balantidium coli*
Penetrating	**Location:** Distal small bowel **Illness:** Enteric fever **Stool findings:** Fecal mononuclear leukocytes (↑)	*Salmonella* Typhi, (enteric fever) *Yersinia enterocolitica* (typhoid like illness)	

- There are several non-bacterial agents that can cause food poisoning such as capsaicin (found in hot peppers), variety of toxins found in fish and shellfish, poisonous mushrooms and some chemical poisons
- The microbial causes of food poisoning have been listed in Table 61.2.

Traveler's Diarrhea

Traveler's diarrhea is the most common travel-related infectious illness.

- Occurs in about 20–50% of people traveling from temperate industrialized countries to tropical regions of Asia, Africa, and Central and South America
- Characterized by a sudden onset of abdominal cramps, anorexia, and watery diarrhea
- Microbial agents causing traveler's diarrhea are listed in Table 61.3.

EPIDEMIOLOGY

Worldwide, about 1.7 to 5 billion cases of diarrhea occur per year, with 1.26 million deaths; accounting for the second leading cause of death globally. It is more common in developing countries, where young children get diarrhea on an average three times a year.

ETIOLOGICAL AGENTS

The microbial agents of diarrheal diseases (gastroenteritis), dysentery and food poisoning are summarized in Tables 61.1 to 61.3 respectively.

PATHOGENIC MECHANISMS

Enteric pathogens have developed a variety of strategies to overcome host defenses (Table 61.4).

Inoculum Size

Enteric pathogens differ from each other in their infective dose (minimum dose required to initiate infection) as follows:

- *Shigella*, enterohemorrhagic *E. coli*, *Giardia*, or *Entamoeba*: 10–100 bacteria or cysts
- *Vibrio cholerae*: 10^5–10^8 bacilli
- *Salmonella*: 10^3–10^5 bacilli.

Table 61.2: Infectious agents of food poisoning

Incubation period, Organism	Symptoms	Common food sources
1–6 h		
Staphylococcus aureus	Nausea, vomiting, diarrhea	Ham, poultry, potato or egg salad, mayonnaise, pastries
Bacillus cereus	Nausea, vomiting, diarrhea	Fried rice
Clostridium botulinum	Nausea, vomiting, diarrhea	Canned food
8–16 h		
Clostridium perfringens	Abdominal cramps, diarrhea (vomiting rare)	Beef, poultry, legumes, gravies
B. cereus	Abdominal cramps, diarrhea (vomiting rare)	Meats, vegetables, dried beans, cereals
>16 h		
Vibrio cholerae	Watery diarrhea	Shellfish, water
Enterotoxigenic *E. coli*	Watery diarrhea	Salads, cheese, meat, water
Enterohemorrhagic *E. coli*	Bloody diarrhea	Ground beef, salami, raw milk, raw vegetables, apple juice
Salmonella species	Inflammatory diarrhea	Beef, poultry, eggs, dairy products
Campylobacter jejuni	Inflammatory diarrhea	Poultry, raw milk
Shigella species	Dysentery	Potato or egg salad, lettuce, raw vegetables
Vibrio parahaemolyticus	Dysentery	Mollusks, crustaceans

Table 61.3: Agents causing traveler's diarrhea

Etiologic agent	Comments
Bacteria (50–75%)	
Enterotoxigenic *E. coli* (10–45%)	Single most important agent
Enteroaggregative *E. coli* (5–35%)	Emerging enteric pathogen with worldwide distribution
Campylobacter jejuni (5–25%)	More common in Asia
Shigella	Major cause of dysentery
Salmonella	Common agent in India
Others	Including *Aeromonas*, *Plesiomonas*, and *Vibrio cholerae*
Viruses (<20%)	
Norovirus (<10%)	Associated with cruise ships
Rotavirus (<5%)	Common among children
Parasites (0–10%)	*Giardia lamblia*, *Cryptosporidium*, *Entamoeba histolytica*, *Cyclospora*

Adherence

Adherence to intestinal mucosa helps the organism to compete with the normal bowel flora and thereby colonizing the intestinal mucosa. This is crucial for the pathogenesis of many diarrheal agents such as Enteropathogenic *E. coli*, enterohemorrhagic *E. coli*, enterotoxigenic *E. coli* and *V. cholerae*.

Toxin Production

Enteric organisms can produce variety of toxins, which are implicated in pathogenesis of diarrhea (Table 61.4). These include:

Table 61.4: Pathogenic mechanisms of diarrheal agents

Toxins production		
Enterotoxins	**Cytotoxins**	**Neurotoxins**
Cholera toxin *Vibrio parahaemolyticus* *E. coli* • LT and ST of ETEC • EAST of EAEC • VT of EHEC *Clostridioides difficile* (toxin A) *Aeromonas* Rotavirus (NSP4) *Campylobacter jejuni*	*Shigella dysenteriae* type 1 Enterohemorrhagic *E. coli* *Clostridioides difficile* (toxin B)	*Staphylococcus aureus* enterotoxin *Bacillus cereus* toxin *Clostridium botulinum* toxin
Attachment within or close to mucosal cells	**Invasion of intestinal epithelium**	
E. coli • Enteropathogenic • Enterohemorrhagic *Cryptosporidium* species *Cyclospora* species *Cystoisospora* species Rotavirus Norovirus	*Shigella* species Enteroinvasive *E. coli* *Campylobacter jejuni* *Yersinia enterocolitica* *Plesiomonas shigelloides* *Entamoeba histolytica* *Balantidium coli*	

Abbreviations: LT, heat labile toxin; ST, heat stable toxin; ETEC, Enterotoxigenic *E. coli*; EAST, Enteroaggregative *E. coli* heat-stable enterotoxin; EAEC, Enteroaggregative *E. coli*; VT, verocytotoxin; EHEC, Enterohaemorrhagic *E. coli*; NSP4, non-structural protein-4.

- **Enterotoxins:** Cause watery diarrhea by acting directly on secretory mechanisms in the intestinal mucosa
- **Cytotoxins:** Cause destruction of mucosal cells, leading to inflammatory diarrhea
- **Neurotoxins:** Act directly on the central nervous system producing vomiting.

Invasion

In addition to producing cytotoxins, bacterial invasion is another mechanism by which destruction of intestinal mucosal cells takes place resulting in dysentery (Table 61.4).

Host Factors

Alterations of the host defense mechanisms can promote the diarrheal diseases.

- Suppression of the normal flora
- Neutralization of gastric acidity: Promote the acid labile pathogens (e.g. *V. cholerae*)
- Inhibition of intestinal motility: Interfere with the clearance of bacteria from small intestine
- Impaired host immunity
- **Genetic determinants:** Host genetic variation influences susceptibility to diarrheal diseases. People with blood group O show increased susceptibility to disease due *to V. cholerae, Shigella, E. coli* O157, and Norovirus.

LABORATORY DIAGNOSIS

Specimen Collection

- Fecal specimen (containing mucus flakes) is collected in a sterile screw capped wide mouthed container. In carriers, a rectal swab may be collected
- In food poisoning outbreaks, vomitus, stool or the suspected food materials are the ideal specimens. The food material should be homogenized or washed thoroughly in sterile diluent, e.g. Ringer's solution.

Microscopy

- **Wet mount preparation** in saline or iodine is done for detection of pus cells, RBCs and detection of parasitic cysts, trophozoites, eggs or larvae
- **Hanging drop preparation:** It is done for liquid specimens to demonstrate darting motility of *Vibrio cholerae*; which can be further confirmed by inhibition of motility by adding H-antisera
- **Gram-stained smear:** Gram-staining is not routinely done because of presence of normal flora in feces. It is recommended only in special situations where the typical morphology would suggest preliminary clue for diagnosis:
  - Presence of comma-shaped bacilli: *Vibrio cholerae*
  - Budding oval yeast cells in immunocompromised host or infant—suggestive of *Candida* species.
- **Acid fast staining** can be carried out for detection of oocysts of *Cryptosporidium, Isospora* and *Cystoisospora*
- **Electron microscopy** for detection of morphology of specific viruses causing gastroenteritis
  - Rotaviruses appear as spokes grouped around the hub of a wheel
  - Astroviruses have star-like morphology
  - Coronaviruses have cup-like depressions on the capsid surface.

Bacterial Culture

- Fecal specimen should be inoculated onto the following media
  - Enrichment broth: Selenite F broth and alkaline peptone water
  - Mildly selective medium: MacConkey agar
  - Highly selective medium such as: DCA (deoxycholate citrate agar), XLD (xylose lysine deoxycholate) agar and TCBS (thiosulfate citrate bile salt sucrose) agar.
- **Identification:** Appropriate biochemical tests are carried out for identification of the enteric pathogen. Then serotyping is performed with specific group or type specific antisera (Table 61.5)
- **Antimicrobial susceptibility test:** It is done to choose appropriate drug for treatment.

Tissue Culture

This is carried out for detection of enteric viruses and also for some diarrheagenic *E. coli*. Enterotoxigenic *Escherichia coli* (ETEC) penetrates HeLa and HEp-2 cell line, whereas verocytotoxin of enterohemorrhagic *Escherichia coli* (EHEC) is detected by its cytotoxic effect on Vero cell line.

Antigen Detection

ELISA and rapid tests (e.g. latex agglutination) based antigen detection methods are available e.g. detection of antigens of rotavirus, *Entamoeba histolytica, Giardia* and *Cryptosporidium* in stool.

Molecular Methods

Polymerase chain reaction (PCR) assays can be carried out for detection specific genes of enteric pathogens.

Toxin Detection

- ELISA-based formats are available for detection of enterotoxins in stool
- PCR for detection of genes coding for enterotoxins.

Steps of microbiological analysis of stool or food specimens to detect the pathogen responsible for gastrointestinal infections are given in Figure 61.1.

TREATMENT **Diarrhea**

Treatment depends up on severity.

- Fluid therapy is the main stay of treatment
- Anti-motility agents and adsorbents may be considered in moderate-to-severe diarrhea
- Empiric antibiotic therapy is required only for severe diarrhea (Table 61.6).

Table 61.5: Identification features/ detection methods of common organisms causing acute diarrhea or dysentery

	Presentation	Identification features
Vibrio cholerae (Figs 31.3 and 31.4B)	Watery diarrhea	Darting motility Coma-shaped gram-negative bacilli in culture smear Catalase and oxidase positive TCBS agar: sucrose fermenting yellow colored colonies ICUT tests: I^+ C^+ U^- TSI (acidic slant/acidic butt, gas^-, H_2S^-) Agglutinates with *Vibrio cholerae* O1 antisera and ogawa antisera (this is the most common pattern; though other serotypes are also present)
Shigella	Dysentery	Gram-negative bacilli, motile Catalase positive, oxidase negative MAC or DCA: non-lactose fermenting translucent colonies XLD: red colonies without black center ICUT tests: I^- C^- U^- TSI (alkaline slant/acidic butt, gas^-, H_2S^-) Agglutinates with *Shigella* polyvalent antisera and specific monovalent antisera
Group B *Salmonella*	Inflammatory diarrhea	Gram-negative bacilli, motile Catalase positive, oxidase negative MAC: non-lactose fermenting translucent colonies DCA: non-lactose fermenting colonies with black center XLD: red colonies with black center ICUT tests: I^- C^+ U^- TSI (alkaline slant/acidic butt, gas^+, H_2S^+) Agglutinates with *Salmonella* poly-O antisera and serotype (O_4) specific antisera
Viral agents	Diarrhea	Agents: Rotavirus, Norovirus, Adenovirus 40, 41, etc. • Detection of viral particles in stool specimen by electron microscopy • Detection of viral antigen by ELISA or • Detection of nucleic acid (RNA or DNA) by PCR in stool specimen
Intestinal parasites		**Stool microscopy detects**
Entamoeba histolytica	Dysentery	• Trophozoites and/or quadrinucleated round cyst • Detection of specific antigen (e.g. lectin)/specific genes in stool
Giardia intestinalis	Fatty diarrhea	Trophozoites (tear drop-shaped binucleated) with four pairs of flagella and/or Tetra-nucleated oval cyst with a central axoneme
Trichuris	Dysentery	Barrel-shaped eggs with mucus plugs at both ends, bile stained
Enterobius vermicularis	Nocturnal anal pruritus	Plano-concave egg containing larva, nonbile stained
Ascaris lumbricoides	Malabsorption	Fertilized egg: round-oval, thick albumin coat, floats in saturated saline, bile stained Unfertilized egg: elongated, thin albumin coat, does not float in saturated saline, bile stained
Hookworm	Diarrhea, anemia	Egg: Oval, contains segmented four blastomeres, clear space between blastomeres and egg shell, nonbile stained
Strongyloides	Diarrhea	Detection of rhabditiform larva in stool microscopy

Abbreviations: I, Indole test; C, Citrate test; U, Urease test; TSI, Triple sugar iron agar test; +, positive; –, negative; H_2S, Hydrogen sulfide; TCBS, Thiosulfate citrate bile salts sucrose agar; MAC, MacConkey agar; DCA, Deoxycholate citrate agar; XLD, Xylose lysine deoxycholate agar; ELISA, Enzyme-linked immunosorbent assay; PCR, Polymerase chain reaction.

Table 61.6: Treatment of diarrheal diseases (adapted from Sanford guideline)

Type of diarrhea	Definition	Treatment
Mild diarrhea	1–2 unformed stools per day Minimal symptoms No interference with daily routine activities	Hydration: Fluids Lactose-free diet and avoid caffeine No antibiotics required
Moderate diarrhea	3–5 unformed stools per day With/without symptoms Interferes with daily routine activities	Fluids + Anti-motility agents (loperamide) + Adsorbents (bismuth subsalicylate) No antibiotics required
Severe diarrhea	≥6 unformed stools per day ± temperature ≥101°F, tenesmus, blood or fecal leukocytes	Empirical: Ciprofloxacin or levofloxacin for 3–5 days • Azithromycin (for 3 days) for *Campylobacter* • Metronidazole or vancomycin for 10–14 days (for *C. difficile*)

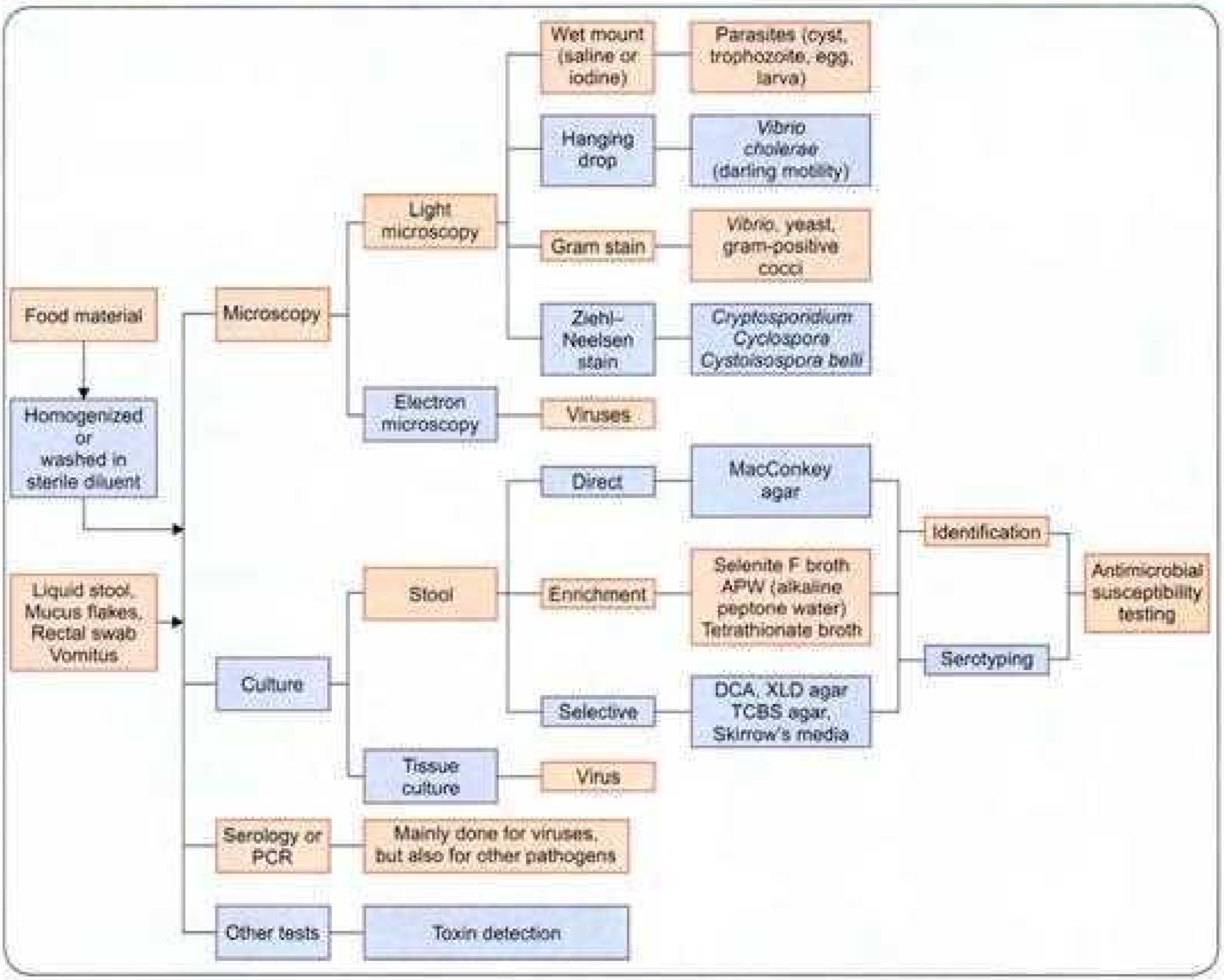


Fig. 61.1: Microbiological analysis of stool/food specimen to detect gastrointestinal pathogen(s)

Abbreviations: PCR, polymerase chain reaction; DCA, deoxycholate citrate agar; XLD, xylose lysine deoxycholate; TCBS, thiosulfate citrate bile salt sucrose.

EXPECTED QUESTIONS

I. Essay:

1. Describe in detail the clinical types, etiological agents and laboratory diagnosis of diarrheal diseases.

II. Write short notes on:

1. Food poisoning.
2. Dysentery.

III. Multiple Choice Questions (MCQs):

1. **All the following culture medium are used for processing of stool specimen, *except*:**
   a. TCBS agar
   b. CLED agar
   c. Deoxycholate citrate agar
   d. XLD agar
2. **Which is not an agent of dysentery?**
   a. *Shigella*
   b. *E. histolytica*
   c. *V. parahaemolyticus*
   d. *V. cholerae*
3. **Food poisoning within 4–6 hours of intake of food indicates presence of toxin of:**
   a. *S. aureus*
   b. *C. perfringens*
   c. *Salmonella*
   d. *V. cholerae*

Answers

1. b **2.** d **3.** a

Meningitis

62
CHAPTER

CASE SCENARIO (PYOGENIC MENINGITIS)

A 7-year-old girl was admitted to the hospital with complaints of high-grade fever, headache, vomiting, altered mental status, seizure and neck rigidity. CSF sample was collected by lumbar puncture in a sterile container and sent to laboratory for biochemical analysis, direct microscopic test, culture and sensitivity testing.

Questions:

1. What is your probable clinical diagnosis?
2. What are the etiological agents, pathogenesis and clinical manifestations of this disease?
3. Describe the laboratory diagnosis in detail?
4. What are the treatment modalities according to the etiological agents?

Explanation:

Case presenting with history of high-grade fever, headache, vomiting, altered mental status, seizure and neck rigidity is suggestive of meningitis.

Explanation to all other questions has been described subsequently in the chapter.

MENINGITIS

Definition

Meningitis is an inflammation of the meninges surrounding the brain and spinal cord. In true sense, it implies to infection of subarachnoid space or the leptomeninges (arachnoid and pia mater) but not dura mater.

Etiological Classification

Based on the changes in leukocytes in cerebrospinal fluid (CSF), meningitis can be grouped into:

- **Pyogenic meningitis:** It is characterized by elevated polymorphonuclear cells in CSF
  - It is exclusively caused by bacterial agents
  - According to the age, the agents involved may vary (Table 62.1)

Table 62.1: Causes of pyogenic meningitis

Age	Common causes
Neonates or Infants of 0–2 months	❑ *Escherichia coli* ❑ Group B *Streptococcus (S. agalactiae)* ❑ *Other gram-negative bacilli (like Klebsiella pneumoniae)* ❑ *Listeria monocytogenes*
2–20 years	❑ *Neisseria meningitidis*: Most common ❑ *Haemophilus influenzae* ❑ *Streptococcus pneumoniae*
>20 years (adults)	❑ *Streptococcus pneumoniae*: Most common agent ❑ *Haemophilus influenzae** ❑ *Neisseria meningitidis*
Elderly (>60 years)	*Listeria monocytogenes*
Overall	Most common agent is: *Streptococcus pneumoniae*

*Incidence of *H. influenzae* meningitis has been dramatically reduced after the start of Hib vaccination.

  - Overall *Streptococcus pneumoniae* is the most common cause of pyogenic meningitis
  - However for the neonatal meningitis, the common agents are Group B *Streptococcus*, *Escherichia coli*, and *Listeria*.
- **Aseptic meningitis:** It is characterized by elevated lymphocytes in CSF. The etiological agents include viral, tubercular, fungal and parasitic agents (Table 62.2). The term 'aseptic' is frequently a misnomer, implying a lack of infection. On the contrary, with the advent of PCR and other newer diagnostic methods, it is now possible to detect most of these agents.

Pathogenesis

Routes of Infection

Organisms may gain access to the meninges by several routes:

- **Hematogenous spread:** This is the most common route, where entry into the subarachnoid space is gained through the choroid plexus or through other blood vessels of the brain

Table 62.2: Causes of aseptic meningitis

Viruses	**Enteroviruses** (Polioviruses, echoviruses, Coxsackie viruses): the most common agents Herpes simplex virus 1 and 2 Other Herpes group: VZV, CMV, EBV Myxoviruses: Influenza A and B, parainfluenza virus, and mumps virus Arboviruses, and adenoviruses Rubella viruses and HIV
Bacteria	*M. tuberculosis, Treponema pallidum,* and *Leptospira*
Parasites	*Naegleria* species, *Acanthamoeba* species and *Toxoplasma gondii*
Fungi	*Cryptococcus neoformans*

Abbreviations: CMV, Cytomegalovirus; EBV, Epstein-Barr virus; HIV, Human immunodeficiency virus; VZV, Varicella-zoster virus.

- **Direct spread from an infected site** present close to meninges—otitis media, mastoiditis, sinusitis, etc.
- **Anatomical defect in central nervous system (CNS):** It may occur as a result of surgery, trauma, congenital defects which can allow organisms for ready and easy access to CNS
- **Direct intraneural spread along the nerve:** This is the least common route, occurs in cases of rabies virus or herpes simplex virus infection.

Predisposing Factors

Pathogenesis of meningitis depends upon various host and microbial factors.

- **Age:** Neonates have the highest prevalence of meningitis; probably due to, (1) their immature immune system, (2) acquiring the colonized organisms from mother's birth canal (e.g. *Listeria* or Group B *Streptococcus*), (3) ↑ permeability of blood brain barrier
- **Vaccination:** Widespread vaccination is shown to reduce the incidence of meningitis due to the particular agent, e.g. ↓ incidence of *H. influenzae* meningitis following implementation of Hib vaccination
- **Factors that promote infection at primary site:** Because respiratory tract is the primary portal of entry for many etiological agents of meningitis, the factors that predispose to respiratory infections can also increase the likelihood of development of meningitis. Such factors include alcoholism, diabetes, immunosuppression, splenectomy, etc.
- **Presence of CSF shunts** can also directly predispose to the pathogen entry
- **Breach in the blood brain barrier (BBB):** Organism can gain access through BBB by:
  - Loss of capillary integrity by disrupting the tight junctions of BBB
  - Transport within circulatory phagocytes
  - Crossing the endothelial cells by transport within the endothelial cell vacuoles.
- **Microbial virulence factors** may also contribute to the pathogenesis such as:
  - Capsular polysaccharide
  - Lipoteichoic acid
  - IgA proteases.

Clinical Manifestations

Patients with meningitis develop various manifestations such as:

- **Important symptoms:**
  - High-grade fever, vomiting
  - Intense headache
  - Photophobia (intolerance to bright light).
- **Important signs of meningism (meningeal irritation) such as:**
  - Neck rigidity
  - Kernig's sign (Severe stiffness of the hamstrings causes an inability to straighten the leg when the hip is flexed to 90 degrees)
  - Brudzinski's sign: Severe neck stiffness causes the patient's hips and knees to flex when the neck is flexed.

Laboratory Diagnosis

Specimen Collection and Transport

- CSF is the most ideal specimen. Other useful specimens are:
  - Blood (for culture)
  - Serum (for antibody or antigen detection)
  - Urine (for antigen detection).
- **CSF collection:** CSF is obtained by lumbar puncture under strict aseptic conditions. It is collected in three sterile containers, one each for cell count, biochemical analysis and bacteriological examination
- **CSF transport:** The CSF being the most precious specimen should be examined immediately
  - If delay is expected, it may be kept in an incubator at 37°C
  - When bacteriological examination is required, CSF should never be refrigerated as delicate pathogens such as *H. influenzae* may die.
- For virus isolation, it may be kept inside the freezer.

Biochemical Analysis

Biochemical analysis and cell count of CSF give a preliminary clue about the type of meningitis (Table 62.3). For example:

- In acute pyogenic meningitis, the CSF usually contains more than 1000 leukocytes/µL and predominantly neutrophils (90–95%)
  - The total protein content is elevated and the glucose level is markedly diminished or even absent
  - CSF pressure is highly elevated.

- **In Tuberculosis meningitis:** Cell count is slightly increased which is predominantly lymphocytic
  - Protein level in CSF is markedly increased; glucose level is slightly decreased
  - Cobweb coagulum formation occurs when CSF is kept for long time in a tube due to high fibrin content of CSF
  - CSF pressure is moderately elevated.
- **In viral meningitis:** Mild lymphocytosis is noticed in CSF. Protein content may be normal or slightly elevated; however glucose level is normal. CSF pressure is mildly elevated.

CSF Microscopy

- **Gram staining of CSF:** Microscopic examination of Gram stained smear may give a preliminary clue about the etiological agent of pyogenic meningitis based on the morphology of the bacteria (Table 62.3)
  - This helps in early initiation of appropriate antimicrobial agents (empirical therapy)
  - **Heaped smear:** As the bacterial load in CSF may be very low, so several drops of CSF should be placed at the same spot on the slide, each being allowed to air dry before the next is added
  - **Centrifugation:** Alternatively, CSF can be centrifuged (by cytospin) and the deposit is examined for Gram staining.
- **Ziehl-Neelsen staining** of CSF smear should be performed to detect *Mycobacterium tuberculosis*. If negative then PCR should be done to detect *M. tuberculosis* specific genes
- **India ink preparation** of CSF can be examined microscopically for detection of capsule of *Cryptococcus neoformans*, which appears as clear refractive unstained area surrounding the budding yeast cells
- **Wet mount preparation** can be examined for detection of trophozoites of parasites such as *Naegleria*.

Antigen Detection

- **From CSF:** After centrifugation of CSF, the supernatant can be used for antigen detection. Latex agglutination test

Table 62.3: Identification features/detection methods of common organisms causing meningitis

Agents of meningitis	Biochemical analysis	Direct demonstration	Culture identification
Streptococcus pneumoniae (Fig. 22.10)	**Pyogenic meningitis** ❑ *CSF pressure:* highly elevated (>180 mm of water) ❑ *Total leukocyte count:* Highly elevated, neutrophilic (100–10,000 per mm³) ❑ *Glucose:* Decreased to absent (<40 mg/dL) ❑ *Total proteins:* >45 mg/dL (usually markedly increased, >250 mg/dL)	Gram-positive cocci in pair, lanceolate shaped	Alfa hemolytic, draughtsman-shaped colony on blood agar Sensitive to optochin Bile soluble, ferments inulin
Streptococcus agalactiae (Fig. 22.6)		Gram-positive cocci in short chain (in neonate)	Beta hemolytic pin point colony on blood agar CAMP test positive, resistant to bacitracin
Neisseria meningitidis (Fig. 23.2)		Gram-negative cocci in pair, intracellular, inside the pus cells	Oxidase positive, growth on chocolate agar
Haemophilus influenzae (Fig. 33.2)		Pleomorphic gram-negative bacilli	Satellitism on blood agar with *S. aureus* streak line, growth surrounding disk containing combined X and V factors
Escherichia coli or other gram-negative bacilli (Fig. 29.1)		Gram-negative bacilli	Identification is based on colony grown on MacConkey agar and biochemical reactions
Cryptococcus neoformans (Fig. 52.20)	❑ *CSF pressure:* Slightly elevated/normal ❑ *Leukocyte count:* Slightly elevated and lymphocytic ❑ *Glucose:* Normal ❑ *Total proteins:* Normal or slightly elevated	India ink stain shows budding yeast cells with refractile capsule Latex agglutination test detecting capsular antigen in CSF	Blood agar or chocolate agar or SDA–showing mucoid white colonies
Viral meningitis		Detection of viral nucleic acid (DNA or RNA) in CSF by PCR or real time PCR	-
Tubercular meningitis (Figs 27.2 and 27.3)	❑ *CSF pressure:* Moderately elevated, cobweb coagulum seen ❑ *Leukocyte count:* Moderately elevated and lymphocytic ❑ *Glucose:* Slightly decreased ❑ *Total proteins:* Moderate to markedly increased	ZN stain of CSF showing acid fast bacilli Detection of specific genes in CSF by PCR or GeneXpert	Growth on Lowenstein Jensen medium (rough tough buff colonies) Growth on MGIT (mycobacterial growth indicator tube)

Abbreviations: SDA, Sabouraud Dextrose Agar; CAMP test, Christie, Atkins, and Munch-Peterson test; CSF, Cerebrospinal fluid; PCR, Polymerase chain reaction.

formats are available for detection of capsular antigens of common agents of meningitis such as:

- *Cryptococcus neoformans*
- *Streptococcus pneumoniae*
- *Streptococcus agalactiae*
- *Neisseria meningitidis*
- *Haemophilus influenzae.*

❖ **From urine:** Various test formats are available to detect capsular antigen in urine: e.g.
- *Streptococcus pneumoniae* (by immunochromatographic test)
- *Cryptococcus neoformans* (by latex agglutination).

Culture

❖ **Bacteriological culture:** Ideal media for bacteriological culture of CSF are the enriched media like chocolate agar and blood agar
- Brain Heart Infusion (BHI) broth: As the bacterial load is very low, a part of the CSF should be enriched by inoculating into brain heart infusion (BHI) broth
- Blood culture can be carried out by inoculating the blood directly into blood culture bottles
- Biochemical tests: Colonies grown on solid media should be subjected to biochemical tests for identification of the organism (Table 62.3)
- Antimicrobial susceptibility test should be done to initiate definitive therapy.

❖ **Fungal culture:** It is carried out by inoculating the CSF on SDA (Sabouraud's dextrose agar) or BHI agar

❖ **Viral culture:** Can done by inoculating the CSF onto appropriate cell lines.

Serological Test (Antibody Detection)

Antibody detection in serum may be useful for the diagnosis of underlying viral etiology; for example, detection of serum antibody against herpes simplex virus.

Bacterial Endotoxin Detection

Limulus lysate assay is widely used to detect endotoxins from gram-negative bacteria. The principle of this test—amebocytes from the horseshoe crab (*Limulus polyphemus*) undergoes coagulation when added with the patient's blood containing endotoxin.

Molecular Methods

❖ If tubercular meningitis is suspected—PCR should be done to detect *Mycobacterium tuberculosis* specific genes such as IS 6110 gene

❖ If pyogenic meningitis is suspected—multiplex PCR can be performed on CSF specimen by using multiple primers to detect the common etiological agents such as *S. pneumoniae*, meningococcus, *H. influenzae*, etc.

TREATMENT — Meningitis

The choice of antimicrobial agent is based on type of organism suspected and good CSF penetration ability of the agent.

If pyogenic meningitis is suspected

❑ *Empirical therapy* comprising of:
- ➢ Adult: IV cefotaxime or ceftriaxone and vancomycin is the recommended regimen. If *Listeria* is suspected, IV ampicillin can be added to the regimen
- ➢ For neonates: IV ampicillin plus gentamicin is the recommended regimen
- ➢ IV dexamethasone is added to the regimen to reduce intracranial pressure.

❑ *Definitive therapy:* After the culture report is available the definitive therapy is modified based on the organism isolated and its sensitivity pattern.

For tubercular meningitis: Antitubercular drug is started according to RNTCP guidelines.

For cryptococcal meningitis:

❑ Induction phase: Amphotericin B plus flucytosine for 2–4 weeks is recommended

❑ Maintenance phase: Fluconazole for 6–12 months is given.

For viral meningitis: Antiviral drugs such as IV acyclovir is given.

EXPECTED QUESTIONS

I. Essay:
1. Describe the clinical types, etiological agents and laboratory diagnosis of meningitis.

II. Write short notes on:
1. Aseptic meningitis.
2. Treatment of meningitis.

III. Multiple Choice Questions (MCQs):

1. **Which of the following is not a common cause of neonatal meningitis?**
   a. *E. coli* b. *S. agalactiae*
   c. *Listeria* d. *S. pneumoniae*

2. **53-year old woman presents with headache, vomiting, and neck rigidity. Indian ink staining of CSF reveals spherical budding yeast cells with clear halo surrounding. What is the etiological agent?**
   a. *Neisseria* b. *Haemophilus*
   c. *Cryptococcus* d. *Streptococcus*

3. **Biochemical analysis of pyogenic meningitis reveals all of the following, *except*:**
   a. CSF pressure: highly elevated
   b. Total leukocyte count: Highly elevated, neutrophilic
   c. Glucose: highly elevated
   d. Total proteins: markedly increased

Answers

1. d **2.** d **3.** c

Blood Stream Infections

63
CHAPTER

CASE SCENARIOS

Case scenario-1 (Infective Endocarditis):

A 75-year-old man was hospitalized with fever (101°F), severe back-pain and weakness in lower limbs. On examination, few non-tender, small erythematous nodular lesions on soles were seen. Echocardiogram showed valvular vegetations on mitral valve. He was diagnosed to have a cardiac valvular lesion 3 years back. Laboratory tests showed CRP 2.5 mg/dL, ESR 66 mm/h, leukocytes 15.6 × 10^9/L and creatinine 4.6 mg/dL. Two blood cultures sent were positive for viridans streptococci. The patient was immediately started on benzyl penicillin.

What is the probable clinical diagnosis? What are the typical etiological agents? Describe the diagnostic criteria used for this condition. How will you collect specimen when you suspect such clinical condition?

Explanation:

This is a case of infective endocarditis as it satisfies two major and three minor Duke Criteria (Table 63.2) which is used for clinical diagnosis of infective endocarditis.

- Past history of diagnosed cardiac condition (minor criteria-1 is met)
- Fever 101°F (minor criteria-2 is met)
- Severe back-pain and weakness in lower limbs and Janeway lesions (non-tender, small erythematous nodular lesions) seen (minor criteria-3 is met)
- Two blood cultures sent were positive for viridans streptococci. (major criteria-1 is met)
- Positive echocardiogram-valvular vegetations on mitral valve (major criteria-2 is met)
- Inflammatory markers raised (CRP 2.5 mg/dL, ESR 66 mm/hour, leukocytes 15.6 × 10^9/L and creatinine 4.6 mg/dL).

Explanation to all other questions has been described subsequently in the chapter.

Case scenario-2 (Sepsis):

A 42-year-old female presented with fever, chills and rigors, confusion, anxiety, difficulty in breathing, malaise and vomiting. On examination, the following signs were noticed: body temperature 102°F, heart rate 106 per minute and respiratory rate 22 per minute. Urine output was significantly decreased.

What is the probable clinical diagnosis? What scoring system is used to assess the severity of infection and extent of organ failure? How will you collect the specimen? Describe the laboratory diagnosis in detail?

Explanation:

This is a case of sepsis.

- Patient presented with fever and alteration in mental status, heart rate 106 per minute (increased) and respiratory rate 22 per minute (increased)-satisfies quick SOFA (sepsis related organ failure) criteria of sepsis (Table 63.5)
- SOFA scoring system is used to assess the severity of infection and extent of organ failure (Table 63.5)
- Laboratory diagnosis of blood stream infection including method of blood collection and blood culture has been explained subsequently in the chapter.

Case scenario-3 (CRBSI):

A 12-year-old male on central line presented with fever (>103°F), altered mental status, heart rate of 102 per minute and respiratory rate of 24 per minute. Blood was collected both from central line and peripheral line separately in BacT/ALERT bottles and sent for culture. Both central line and peripheral line bottles flagged positive for *E. coli* after 4 hours and 7 hours of incubation respectively.

What is the clinical condition. How will you diagnose this condition?

Explanation:

This is a case of CRBSI (catheter-related blood stream infection) as the following criteria are met (Table 63.3):

- Patient is on central line
- Presence of signs of sepsis: fever with altered mental status, increased heart rate, respiratory rate.
- Culture criteria (differential time to positivity >2 hours): Culture of both central line and peripheral line blood specimens revealed the same pathogen (*E.coli*);

with central line bottle flagged positive (at 4 hours of incubation) > 2 hours earlier than the peripheral line bottle (flagged at 7 hours of incubation).

Explanation to all other questions has been described subsequently in the chapter.

Case scenario-4 (FUO):

A 28-year-old male is presented with elevated temperature of (102°F) for >3 weeks. The patient is hospitalized for the past five days without elucidation of a cause.

What is your probable clinical diagnosis? List the various etiological agents? How will you approach for laboratory diagnosis in such case?

Explanation:

Fever (>101°F) for >3 weeks in a hospitalized patient for >3 days without elucidation of a cause is suggestive of 'Fever of unknown origin (FUO); classical type'. Explanation to all other questions has been described subsequently in the chapter.

BLOOD STREAM INFECTIONS

Blood stream infections (BSI) refer to presence of microorganisms in blood, which are a threat to every organ in the body.

- Microbial invasion of blood stream can have serious immediate consequences such as shock, multiple organ failure, and DIC (disseminated intravascular coagulopathies)
- Therefore, timely detection of the causative agent is one of the most important goals of microbiology laboratory
- All four categories of microbes (bacteria, viruses, fungi and parasites) can cause BSI; however as bacteria account for the majority of BSI, bacterial causes of BSI are addressed here.

Bacteremia and Septicemia

- **Bacteremia** refers to the presence of bacteria in blood without any multiplication
- **Septicemia** is a condition in which bacteria circulate and actively multiply in the bloodstream (and may produce their products, e.g. toxins) that cause harm to the host.

There are Three Types of Bacteremia

1. **Transient bacteremia:** It may occur spontaneously or with minor events such as brushing teeth or chewing food, instrumentation of contaminated mucosal site and surgery involving non-sterile site. These circumstances may also lead to septicemia.
2. **Continuous bacteremia:** Here, the organisms are released into the bloodstream at a fairly constant rate. It occurs in conditions such as:
   - Septic shock, endocarditis and other endovascular infections
   - During the early stage of certain infections including enteric fever, brucellosis, and leptospirosis.
3. **Intermittent bacteremia:** In most other infections, bacteria are released into blood intermittently.
   - Undrained abscess (bacteria are released approximately 45 minutes before a febrile episode)
   - Early course of meningitis, pneumonia, pyogenic arthritis and osteomyelitis.

Types of Bloodstream Infections (BSI)

There are two major categories of bloodstream infections: intravascular and extravascular.

Factors that contribute to the initiation of BSI are:

- Immunosuppression
- Use of broad spectrum antimicrobial agents can supress the normal flora; thus allowing the emergence of resistant strains of bacteria
- Invasive procedures or extensive surgeries that allow the bacteria to access the blood
- Prolonged survival of debilitated patients.

Intravascular Bloodstream Infections

Intravascular infections are those that originate within the cardiovascular system which include:

- Infective endocarditis
- Mycotic aneurysm
- Suppurative thrombophlebitis
- CRBSI (catheter-related blood stream infection).

These infections being present within the vascular system, lead to continuous bacteremia which result into serious and life-threatening events.

Infective Endocarditis

It is the infection of endocardium, characterized by presence of '*vegetation*' which is composed of mass of platelets, fibrin, microcolonies of organisms, and scanty inflammatory cells.

- Vegetations are most commonly present in heart valves followed by low-pressure side of a ventricular septal defect, and on the mural endocardium
- **Endocarditis can be classified** based on:
  - Onset-**acute** (rapidly damages the cardiac structures, and spread to extracardiac site, rapidly fatal) or **subacute** (slow evolution, metastasizes slowly)
  - Type of valve affected—can occur in native or in prosthetic valve
  - May be associated with intravenous drug abuse.
- The etiological agents of endocarditis depend on the type of endocarditis (Table 63.1).

Table 63.1: Agents of endocarditis
Agents of endocarditis
• *Staphylococcus aureus* • Coagulase-negative staphylococci (e.g. *Staphylococcus epidermidis*) • Streptococci (Viridans streptococci and others) • Enterococci • Pneumococci • Fastidious gram-negative coccobacilli (HACEK group) • Gram-negative bacilli • *Candida* species • Diphtheroids • Culture-negative endocarditis: Such as *Bartonella, Coxiella*
Most common agent in specific types of endocarditis
Native valve endocarditis: *Staphylococcus aureus* • Community acquired-Viridans streptococci • Health care acquired-*S. aureus* • Overall-*S. aureus*
Prosthetic valve endocarditis: It occurs following cardiac valve replacement • Early prosthetic valve endocarditis (occurs within 12 months of valve replacement)—*Staphylococcus epidermidis* is the most common agent • Late prosthetic valve endocarditis (occurs after 12 months of valve replacement)— Viridans streptococci is the commonest agent
Endocarditis in IV drug abusers: Young males are the most common victims. The skin is the commonest source of infection. • Right sided: Most common agent is *Staphylococcus aureus* • Left sided: Most common agent is *Enterococcus*, followed by *S. aureus* • Overall: Most common agent is *Staphylococcus aureus*
Most common cause of Subacute endocarditis: Viridans streptococci

❖ **Diagnosis (Duke Criteria):** This criteria is used for the clinical diagnosis of infective endocarditis (Table 63.2).

TREATMENT — Endocarditis

Empirical treatment consists of vancomycin and gentamicin/ceftriaxone. Rifampin may be added to the regimen if prosthetic valve endocarditis is suspected.

CRBSI (catheter-related blood stream infection)

Severely-ill patients in ICUs are often put on central line; which helps in administration of medication and parenteral nutrition. Central lines may get infected due to mishandling during insertion or during daily maintenance which leads to development of CRBSI. CRBSI can be diagnosed if clinical, microbiological and central line criteria are met as described in Table 63.3.

Extravascular Bloodstream Infections

Most cases of clinically significant bacteremia are of extravascular origin.

❖ The organisms multiply at the primary site such as lungs, and drained by lymphatics and reach the bloodstream

Table 63.2: Modified Duke criteria for the clinical diagnosis of infective endocarditis
Major Criteria
1. Positive blood culture: Any one of the following A. Typical IE organism isolated from two separate sets of blood cultures (Viridans streptococci, *Streptococcus gallolyticus*, HACEK group, *S. aureus or enterococci*) or B. Persistently positive blood culture with agents other than typical IE organisms: ➢ Blood culture sets drawn >12 h apart; or ➢ All of 3 or a majority of ≥4 separate sets of blood culture, with first and last drawn at least 1 h apart C. Single positive blood cultuer for *Coxiella burnetii* or phase I IgG antibody titer of >1:800
2. Evidence of endocardial involvement: Any one A. Positive echocardiogram ➢ Oscillating intracardiac mass on valve or ➢ Abscess, or ➢ New partial dehiscence of prosthetic valve B. New valvular regurgitation
Minor Criteria
1. **Predisposition:** Predisposing heart condition or IV drug use 2. **Fever** ≥ 38.0°C (≥100.4°F) 3. **Vascular phenomena:** Major arterial emboli, septic pulmonary infarcts, mycotic aneurysm, intracranial hemorrhage, conjunctival hemorrhages or Janeway lesions 4. **Immunologic phenomena:** Glomerulonephritis, Osler's nodes, Roth's spots or rheumatoid factor 5. **Microbiologic evidence:** Positive blood culture but not meeting major criterion as noted previously* or serologic evidence of active infection with organism consistent with infective endocarditis
Definite endocarditis if the followings are present: • Two major criteria or • One major criterion and three minor criteria or • Five minor criteria

*Excluding single positive cultures for coagulase-negative staphylococci and diphtheroids, which are common culture contaminants, and organisms that do not cause endocarditis frequently, such as gram-negative bacilli.
Abbreviation: IE, infective endocarditis.

Table 63.3: Diagnostic criteria of Catheter related blood stream infection (CRBSI)	
Catheter criteria	Presence of central line
Clinical criteria	Sign/symptoms of sepsis (fever, hypotension, tachypnea)
Culture criteria (Differential time to positivity)	Culture of both central line and peripheral line blood specimens must reveal the same pathogen; with central line bottle flagged positive at least 2 hours or more earlier, than the peripheral line bottle

❖ The organisms are either removed by the cells of the reticuloendothelial system or they multiply more widely and thereby causing septicemia

❖ **Portal of entry:** The most common portals of entry for bacteremia are the genitourinary tract (25%), followed

Table 63.4: Bacteria causing extravascular blood stream infection (BSIs) and their common sources

Organisms	Portal of entry/sources
E. coli and other gram-negative bacteria such as *Klebsiella, Proteus, Enterobacter, Pseudomonas*	Urinary tract (most common), Intestine (rarely)
Haemophilus influenzae-b	Meninges, epiglottis, lungs
Pneumococcus	Meninges, lungs
Brucella	Reticuloendothelial system
Salmonella Typhi	Small intestine, lymph nodes and reticuloendothelial system
Listeria	Intestine, meninges
Staphylococcus aureus and coagulase negative staphylococci	Surgical site infections

by respiratory tract (20%), abscesses (10%), surgical site wound infections (5%), and biliary tract (5%). In up to 25% of cases, the portal of entry remains uncertain

- **Agents:** The organisms invading the bloodstream depend upon the portal of entry and have been listed in Table 63.4.

Clinical Manifestations

Bloodstream infections have a bacteremia stage followed by septicemic stage. The clinical manifestations are evident only in the septicemic stage. In this stage, the bacteria multiply releasing their products (e.g. toxins) which travel to various organs affecting their functions. Based up on the severity and extent of organ failure; bloodstream infection can be divided into two stages: sepsis and septic shock (Table 63.4).

- **Sepsis:** The common signs and symptoms include:
  - Fever or hypothermia with/without chills and rigors
  - Hyperventilation leads to excess loss of CO_2 and subsequent respiratory alkalosis
  - Skin lesions, change of mental status and diarrhea.
- **Septic shock:** This is the gravest late stage complication of septicemia and is manifested as—hypotension, DIC and multiorgan failure (e.g. acute respiratory distress, renal failure, tissue destruction, etc.). The endotoxins of gram-negative bacteria have a direct effect on the pathogenesis of septic (or endotoxic) shock.

In sepsis, the severity and degree of organ failure can be determined by an assessment score called as SOFA (Sepsis-related Organ Failure Assessment) score (Table 63.5).

Laboratory Diagnosis

Diagnosis of bloodstream infection depends on isolation of the causative agent from blood.

Table 63.5: Definition of sepsis and assessment of severity and organ failure

Sepsis Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection
SOFA score Sepsis is diagnosed by SOFA (Sepsis-related organ failure assessment) score which in turn depends on six parameters. 1. Respiratory system—PaO_2/FiO_2 2. Coagulation system—Platelets count 3. Liver—Serum bilirubin 4. Cardiovascular—Mean arterial pressure (MAP) 5. Central nervous system—Glasgow coma scale score 6. Renal—Serum creatinine and urine output Organ dysfunction can be identified as an acute change in total SOFA score ≥2 points following the infection
qSOFA (Quick SOFA) Criteria Determination of SOFA score takes considerable time as it depends up on a number of laboratory parameters. However, before the result of SOFA score is available, sepsis can promptly be identified at the bedside with qSOFA score • Respiratory rate ≥22/min • Altered mentation • Systolic blood pressure ≤100 mm Hg
Septic shock It is a subset of sepsis in which underlying circulatory and cellular/metabolic abnormalities are profound Patients with septic shock can be identified with a clinical construct of sepsis with: • Persisting hypotension requiring vasopressors to maintain MAP (mean arterial pressure) ≥65 mm Hg and • Serum lactate level >2 mmol/L (18 mg/dL) despite adequate volume resuscitation Patients with septic shock has mortality of >40% in contrast to 10% for sepsis cases

Specimen Collection

- **Site:** Blood should always be collected in pair; from two separate peripheral lines. If a central line is present, then one sample from central line and one from peripheral line should be collected
- **Preparation of site:** To avoid contamination with skin flora, blood should be collected under strict aseptic conditions using sterile disposable syringe (Fig. 63.1)
- **Antiseptics:** Skin should be treated with 70% isopropyl alcohol and then a second antiseptic solution such as tincture iodine or chlorhexidine should be applied
- **Timing of collection:** Blood should be collected before starting antimicrobial therapy. If antimicrobial agent is already started, then the best time of collection is just before the next dose of antimicrobial agent
- **Blood volume:** Higher the volume of blood, greater is the yield of isolation (yield increases by 3.2% per mL of blood

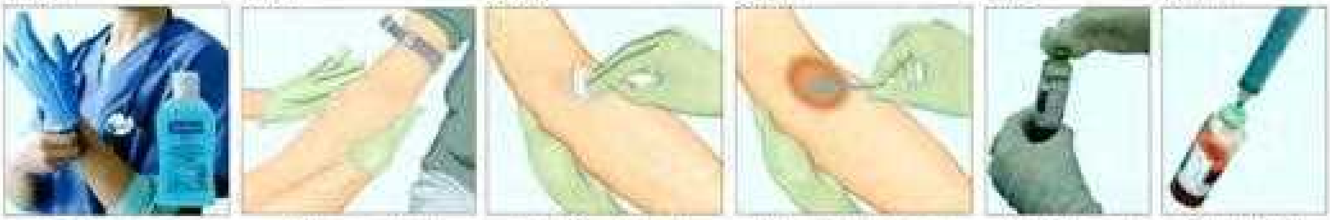


Fig. 63.1: Steps of collection of blood for blood culture

cultured). At least 8-10 mL per bottle for adult and 1-3 mL per bottle for pediatric individual is recommended

- **Number of blood cultures:** At least 2-3 blood culture sets (each set consists of two bottles: 1 aerobic and 1 anaerobic) are required to have good isolation chance (around 65%, 80% and 95% with one, two and three sets respectively).

Conventional Culture Medium

There are two types of conventional blood culture bottles.

1. **Monophasic medium:** It contains 50-100 mL of brain heart infusion (BHI) broth.
2. **Castaneda's biphasic medium:** It consists of BHI agar slope and BHI broth (50-100 mL).

Dilution: The blood is inoculated in the medium at a dilution of 1:5 so that the antibacterial components in the blood, if any, will get diluted.

SPS (sodium polyanethol sulfonate) is added to the medium as anticoagulant. It also counteracts the bactericidal action of blood.

Inoculation and Incubation

About 10-20 mL of fresh blood is directly injected at the bedside through the rubber cap of the bottle, rather than opening the bottle (to avoid contamination from the external environment) and then it is incubated at 37°C for up to 7 days.

Repeat Subcultures

- **From monophasic medium:** Repeat subcultures are made onto blood agar and MacConkey agar when the broth becomes turbid or periodically (blind subcultures) for one week. There is a risk of contamination due to opening of the cap of the bottle every time when subcultures are made
- **Biphasic medium** is preferred as the subcultures can be made just by tilting the bottles so that the broth runs over the agar slope. Bottle is incubated in the upright position.

If colonies appear over the agar slant, colonies are picked up and used for further identification.

Automated Culture Media

BACTEC and BacT/ALERT are the automated blood culture systems. The most advanced system is Bact/Alert Virtuo. In these systems, the growth is continuously monitored and reading is recorded every 15-20 min. When the growth is detected, the system gives a positive signal. Then the bottle is removed and processed similarly as done for conventional bottles. Automated systems are much superior to conventional bottles in terms of faster isolation and increased sensitivity. More so, it also helps in diagnosing CRBSI by estimating the differential time to positivity (see Table 63.3).

Identification

The isolated organism is identified by colony morphology, Gram staining, biochemical reactions and serological tests.

Antimicrobial Susceptibility Test

Antimicrobial susceptibility test by disk diffusion method is carried out for institution of appropriate therapy. However for endocarditis, determining the MIC (minimum inhibitory concentration) is the ideal method, especially when tested against penicillin.

TREATMENT **Sepsis/Blood Stream Infection**

Due to higher prevalence of multi-drug resistant bacteria (MDROs) and higher mortality in sepsis, antibiotics should be instituted at the earliest, as soon as sepsis is clinically suspected. De-escalation approach is usually followed which means:

- Empirical treatment consists of higher class of antimicrobials with both gram-negative and gram-positive coverage; e.g. carbapenem such as meropenem plus vancomycin.
- Definitive treatment can be tailored according to the culture sensitivity report.

FEVER OF UNKNOWN ORIGIN (FUO)

Fever of unknown origin (FUO) is a very common term used by clinicians to refer to any febrile illness without an initially obvious etiology.

- Most febrile illnesses either resolve before a diagnosis can be made or eventually show typical clinical features or positive for specific investigations that lead to arrive at a correct diagnosis. These group of febrile illness are not called as FUO
- The term FUO is reserved only for prolonged febrile illnesses without an established etiology despite of intensive evaluation and diagnostic testing.

Definitions

With the advent of modern diagnostic tools, the definition of FUO has changed over time.

Petersdorf and Beeson Classification

Petersdorf and Beeson had defined fever of unknown origin (FUO) in 1961 as patients having:

- Temperatures of more than 38.3°C (more than 101°F)
- For a duration of more than 3 weeks
- Failure to reach a diagnosis despite 1 week of inpatient investigation.

This classification has stood for more than 30 years, but later in 1990s, it was revised.

Durack and Street Classification

Durack and Street (1990) have proposed a revised system for classification of FUO that better accounts for non-endemic and emerging diseases and improved newer diagnostic facilities. This updated classification includes four types of FUO (Table 63.6).

Table 63.6: Classification of fever of unknown origin

Durack and Street definition of fever of unknown origin (FUO)
• **Classic FUO** (corresponds closely to the earlier definition of FUO): ➢ Temperatures of >38.3°C (>101°F) ➢ Duration of fever >3 weeks ➢ Three outpatient visits or 3 days in the hospital without elucidation of a cause or 1 week of intelligent and invasive ambulatory investigations
• **Nosocomial FUO:** ➢ Temperature of >38.3° C (101°F) develops in a hospitalized patient; in whom infection was not manifested or incubating on admission ➢ Three days of investigation*, including at least 2 days of incubation of cultures—reveals no source
• **Neutropenic FUO** is defined as: ➢ Temperature of >38.3°C (101°F) ➢ Neutrophil count is <500/μL ➢ Three days of investigation, including at least 2 days of incubation of cultures—reveals no source
• **HIV-associated FUO** is defined as: ➢ Temperature of >38.3°C (101°F) for >4 weeks for outpatients or >3 days for hospitalized patients ➢ HIV infection has been confirmed ➢ Three days of investigation, including at least 2 days of incubation of cultures—reveals no source

Abbreviations: FUO, fever of unknown origin; HIV, human immunodeficiency virus.

***Note:** Recently, the diagnostic workup (investigation) criteria for classical FUO is changed from quantitative criterion (diagnosis uncertain after 1 week of evaluation in Durak and street classification) to a qualitative criterion which requires the performance of a specific list of investigations before labeling a case as FUO.

Causes

- **Infections (36%):** This accounts for majority of FUO cases. All groups of microbial infections (both localized and systemic) can cause FUO (Table 63.7)
- **Neoplasms (19%):** For example, lymphoma, leukemia, myeloma, renal, colon and liver cancers, etc.
- **Non-infectious Inflammatory Diseases (19%):** For example, connective tissue disorders like rheumatoid arthritis, SLE (systemic lupus erythematosus), etc.
- **Miscellaneous Causes (19%):**
  - Granulomatous diseases
  - Inherited and metabolic diseases
  - Thermoregulatory disorders.
- Undiagnosed cases (7%).

Laboratory Diagnosis

Specimen Collection

Prior to specimen collection, a complete clinical history (including details of travel, immunization, exposure to any other patients) and physical examination should be carried out that may be helpful in choosing the appropriate specimen such as blood, urine, bone marrow aspirate, pus from abscesses, etc.

Microscopy

- **Blood microscopy:** Useful for detection of malaria parasites (ring forms and gametocytes), microfilariae, *Leishmania donovani* (LD bodies or amastigote forms), and trypanosomes (trypomastigote forms)
- **Stool wet mount:** For detection of cyst, trophozoite or ova of parasitic agent of FUO (e.g. *Entamoeba histolytica*)
- **Gram stain** of pus, sputum and other specimen can be carried out for detection of the causative agent
- **Ziehl-Neelsen stain:** For *M. tuberculosis*
- Periodic acid-schiff (PAS) or Gomori methenamine silver (GMS) stain for detection of fungal morphology.

Table 63.7: Causes of fever of unknown origin

Bacterial causes		Non-bacterial causes		
Localized pyogenic infections	**Systemic bacterial infections**	**Viral infections**	**Parasitic infections**	**Fungal infections**
• Appendicitis • Cholangitis • Cholecystitis • Localized abscess • Mesenteric lymphadenitis • Osteomyelitis • Pelvic inflammatory disease • Sinusitis • Suppurative thrombophlebitis • Intravascular infections	• Mycobacterial infections • Typhoid fever • Rickettsial infections • *Mycoplasma* infections • Chlamydial infections • Brucellosis • Melioidosis • Listeriosis • Bartonellosis • Spirochete infections: ➢ Syphilis ➢ Lyme disease ➢ Relapsing fever ➢ Leptospirosis	• Cytomegalovirus and EBV infection • Coxsackievirus group B infection • Viral hepatitis • HIV infection	• Malaria • Amoebiasis • Leishmaniasis • Chagas' disease • Toxoplasmosis • Strongyloidiasis	• Aspergillosis • Mucormycosis • Blastomycosis • Histoplasmosis • Coccidioidomycosis • Paracoccidioidomycosis • Candidiasis • Cryptococcosis • *Pneumocystis* infection • Sporotrichosis

Abbreviations: EBV, Epstein-Barr virus; HIV, human immunodeficiency virus.

Culture

- ❖ Blood culture is done for typhoid fever, brucellosis
- ❖ Culture on Lowenstein Jensen medium is done for *M. tuberculosis*
- ❖ Culture of pus and exudate specimen from the abscesses: for detection of the causative agent
- ❖ **Sabouraud dextrose agar (SDA) culture:** For fungal isolation
- ❖ **Cell line culture:** Culture in appropriate cell lines is useful for the isolation of virus, e.g. human diploid cell line for cytomegalovirus (CMV).

Serological Test

- ❖ ELISA and rapid tests for viral diseases such as hepatitis, HIV, CMV, EBV infections, etc.
- ❖ **Standard agglutination test:** For brucellosis
- ❖ **Microscopic agglutination test:** For leptospirosis
- ❖ **Cold agglutination test:** For *Mycoplasma*
- ❖ **Weil Felix test:** For rickettsial diseases
- ❖ **Paul-Bunnell test:** For infectious mononucleosis
- ❖ **Widal test:** For typhoid fever
- ❖ Microimmunofluorescence test or complement fixation test (CFT) for chlamydial infections
- ❖ **Rheumatoid arthritis (RA) factor:** For rheumatoid arthritis
- ❖ Antinuclear antibody detection by immunofluorescence or ELISA for diagnosis of SLE.

Molecular Tests

If the infective organism load is very low, PCR can be carried out to amplify the specific genes, even if few copies are present can be detected.

Other Tests

- ❖ **Complete blood count:** Increased neutrophil count indicates pyogenic infections
- ❖ **Raised ESR (erythrocyte sedimentation rate):** It may indicate tuberculosis
- ❖ Histopathological examinations of the biopsies obtained from tumors may suggest the underlying etiology
- ❖ **Imaging methods:** Chest X-ray (for diagnosis of tuberculosis) and CT or MRI scan to identify the malignant tumors and their extension
- ❖ ECG for rheumatic fever and infective endocarditis.

TREATMENT — FUO

Treatment of FUO depends upon the etiological diagnosis and then it is managed accordingly.

EXPECTED QUESTIONS

I. Essay:

1. Describe in detail the clinical types, etiological agents and laboratory diagnosis of blood stream infections.

II. Write short notes on:

1. Fever of unknown origin (FUO).
2. CRBSI (catheter-related blood stream infection).
3. Infective endocarditis.

Respiratory Tract Infection

64
CHAPTER

CASE SCENARIOS

Case scenario-1 (URTI: Sore throat): A 5-year-old child with history of fever and throat pain for 3 days, was brought to ENT OPD. On examination, he was febrile (101°F) and throat examination revealed pustules over the tonsils. His throat swab was sent to the microbiology laboratory and was subjected to culture, and antimicrobial susceptibility testing.

Case scenario-2 (LRTI: pneumonia): A 9-year-old boy was admitted with complaints of productive cough, chest pain, and shortness of breath for past 3 days. Clinical examination revealed dullness over left sixth intercostal space on percussion and crepitations and rales with reduced breath sounds over left sixth intercostal space on auscultation. Chest X-ray showed homogeneous ground glass opacification in the left lower lobe. Her sputum was collected and subjected to microscopy and culture.

Questions

1. What is your clinical diagnosis in both the cases?
2. What are the etiological agents, pathogenesis and clinical manifestations of these conditions?
3. Describe in detail about your approach for the laboratory diagnosis?
4. What are the treatment modalities for these clinical conditions?

Explanation:

Case scenario-1: Child with fever, throat pain and pustules over the tonsils suggests that this is a case of upper respiratory tract infection (URTI), to be specific, a case of sore throat.

Case scenario-2: This is a case of lobar pneumonia. The points in favor are:

- Child presented with productive cough, chest pain, and shortness of breath (dyspnea) for past 3 days
- Dullness on percussion over left intercostal space indicates denser tissue, such as consolidation
- On auscultation—crepitations and rales with reduced breath sounds were heard over left sixth intercostal space, i.e. the left lower lobe area
- Chest X-ray showed homogeneous opacification in the left lower lobe.

Explanation to all other questions has been described in detail below.

RESPIRATORY TRACT INFECTION

Infections involving the respiratory tract can be further classified into: upper and lower respiratory tract infections.

Upper Respiratory Tract Infections

Upper respiratory tract infections (URI or URTI) includes infections of the airway above the glottis or vocal cords. This includes the nose, sinuses, pharynx, and larynx.

- Typical infections of the upper respiratory tract include tonsillitis, pharyngitis, laryngitis, sinusitis, otitis media, and rhinitis
- Symptoms of URIs can include cough, sore throat, running nose, nasal congestion, headache, low-grade fever, facial pressure and sneezing.

Common etiological agents of URTI are listed in Table 64.1.

Lower Respiratory Tract Infection

The infections of trachea, bronchi, bronchioles, and the lungs are called as lower respiratory tract infections (LRI or LRTI). These include bronchitis, bronchiolitis, pneumonia, and lung abscesses (Table 64.2).

Pneumonia

Pneumonia refers to inflammation of lungs which can be classified into: (1) community acquired—patients acquire the organisms in the community, (2) hospital acquired—patients acquire the organisms in the hospital setting.

Community-acquired Pneumonia (CAP)

The agents causing CAP are enlisted in Table 64.2. *Streptococcus pneumoniae* followed by *Mycoplasma*

Table 64.1: Microorganisms causing upper respiratory tract infections (URTI) and associated symptoms

Rhinitis or common cold	Pharyngitis (sore throat), and tonsillitis	Laryngitis	Laryngotracheobronchitis (or croup)
Mostly caused by viruses: • Rhinovirus • Coronavirus • Adenovirus • Influenza virus • Parainfluenza virus • Human metapneumovirus • Respiratory syncytial virus **Sinusitis** Inflammation of the sinuses (due to allergy or infections) **Symptoms:** • Headache/facial pain • Thick nasal mucus, • Plugged nose **Agents of acute sinusitis:** Viruses (most common cause): • Rhinoviruses, • Influenza viruses, • Parainfluenza viruses **Bacterial agents:** • *Streptococcus pneumoniae* • *Haemophilus influenzae* • *Moraxella catarrhalis* • *Pseudomonas* and other gram- negative bacilli (nosocomial sinusitis) **Agents of chronic sinusitis:** ❑ Obligate anaerobes ❑ *Staphylococcus aureus*	**Symptoms:** Pharynx and/or tonsils become inflamed, red, swollen, and show exudate, and sometimes a membrane is formed **Viruses:** (most common cause) • Influenza virus • Parainfluenza virus • Coxsackievirus A • Rhinovirus • Coronavirus • Epstein-Barr virus • Adenoviruses **Bacteria:** • *Streptococcus pyogenes* (most common bacterial cause) • *Streptococcus* groups C and G • *Arcanobacterium* species • *Corynebacterium diphtheriae* and *C. ulcerans* • *Mycoplasma pneumoniae* • Vincent angina ➢ *Treponema vincentii* ➢ *Leptotrichia buccalis* **Fungal:** *Candida albicans*	**Symptoms:** • Hoarseness of voice • Lowering and deepening of voice **Mostly viral agents:** • Influenza virus • Parainfluenza virus • Rhinovirus • Adenovirus • Coronavirus • Human metapneumovirus **If membrane or exudate present:** • *Streptococcus pyogenes* • *C. diphtheriae* • Epstein-Barr virus	Age—Children, <3 years age **Symptoms:** • Inspiratory stridor (high-pitched breath sounds resulting from turbulent air flow in the larynx) • Hoarseness • Fever • Cough (harsh, barking non-productive) **Agents:** • Parainfluenza virus (most common) • Influenza virus • Respiratory syncytial virus • Adenoviruses **Epiglottis** Edema and inflammation of epiglottis and soft tissue above vocal cords **Age:** children 2–6 years **Symptoms:** • Fever, • Difficulty in swallowing • Inspiratory stridor **Most common agent:** *Haemophilus influenzae* type b

pneumoniae are the most common agents of CAP. The prognosis of CAP can be predicted by a scoring system called CURB65. If the score >1, patient should be hospitalized; else the treatment can be given on outpatient basis (Table 64.3).

Hospital-acquired Pneumonia (HAP)

Hospitalized patients have increased risk of developing pneumonia; most of which are ventilator-associated pneumonia. Various risk factors for developing VAP are discussed in Chapter 53. VAP can be clinically diagnosed by Clinical Pulmonary Infection (CPIS) (Table 64.4). The likelihood of VAP is higher when total CPIS is >6.

Clinical Manifestations of Pneumonia

The common manifestations include fever, chills, chest pain and cough. Based on area of lungs involved, and type of cough produced, pneumonia can be grouped into:

❖ **Lobar pneumonia** infecting lung parenchyma (alveoli): It is characterized by consolidation and productive cough with purulent sputum (refer case scenario-2). It is mostly caused by pyogenic organisms such as:
  - Pneumococcus
  - *Haemophilus influenzae*
  - *Staphylococcus aureus*
  - Gram-negative bacilli.

❖ **Interstitial or atypical pneumonia** infection occurs in interstitial space of lungs. Cough is characteristically non-productive. It is mostly caused by organisms such as:
  - *Chlamydophila pneumoniae*
  - *Mycoplasma pneumoniae*
  - Viral pneumonia
  - *Legionella* species.

Bronchitis

Bronchitis is characterized by inflammation of bronchus, which occurs either as an extension of upper respiratory tract infection such as influenza or may be caused directly by bacterial agents such as *Bordetella*. Common symptoms include fever, cough, sputum production, and rarely croup-like features

Table 64.2: Organisms causing lower respiratory tract infections (LRTI)

Pneumonia		Bronchitis
Community acquired	**Hospital acquired**	**Bacterial agents:** • *B. pertussis* • *B. parapertussis* • *Mycoplasma pneumoniae* • *Chlamydophila pneumoniae* **Viral agents:** • Influenza viruses • Adenoviruses • Rhinoviruses • Coronaviruses
No co-morbidity: • *Streptococcus pneumoniae* (most common) • Atypical pathogens: ➢ *Chlamydophila pneumoniae* and *C. psittaci* ➢ *Legionella* and *Mycoplasma* ➢ *Coxiella burnetii* (Q fever) ➢ Viral pneumonia (influenza, adenovirus, parainfluenza, RSV) **Co-morbidity:** • Alcoholism: *S. pneumoniae*, *H. influenzae* • COPD: *H. influenzae*, *M. catarrhalis*, *S. pneumoniae* • Post-CVA-aspiration: *S. pneumoniae* • Post-obstruction of bronchi: *S. pneumoniae*, anaerobes • Post-influenza: *S. pneumoniae*, *S. aureus*	**Bacterial agents:** • Gram-negative bacilli (most common) ➢ MDR non-fermenters (*Pseudomonas* and *Acinetobacter*) ➢ MDR Enterobacteriaceae (*E. coli*, *Klebsiella* and *Enterobacter*) • *Staphylococcus aureus* (both MRSA and MSSA) • *S. pneumoniae* (rarely, in early stage) **Viral agents:** • Influenza, adenovirus, parainfluenza, RSV	**Bronchiolitis** **Viral agents:** • Respiratory syncytial viruses • Parainfluenza viruses • Rhinoviruses • Influenza viruses • Adenoviruses • Enterovirus • Human metapneumovirus

Abbreviations: CVA, cerebrovascular accident; MSSA, methicillin sensitive *S. aureus*; MRSA, methicillin resistant *S. aureus*; MDR, multidrug resistant.

Table 64.3: Prediction of prognosis in community-acquired pneumonia (CAP)

The prediction of prognosis of CAP in an adult is done by **CURB-65** score.
• **C** (Confusion) = 1 point • **U** (blood urea nitrogen >19 mg/dL) = 1 point • **R** (respiratory rate >30 min) = 1 point • **B** (BP <90/60) = 1 point • 65 (Age ≥65 years) = 1 point Higher the score, greater is the mortality If the score ≤1, outpatient therapy is indicated If the score >1, patient should be hospitalized

Bronchiolitis

Bronchiolitis is inflammation of the smaller airways (bronchioles). It presents as an acute viral infection that primarily occurs in children less than 2 years

- It is characterized by acute onset of wheeze, dyspnea, cough, rhinorrhea, and respiratory distress
- Respiratory syncytial viruses account for 40–80% of the infections.

Oral Cavity Infections

- **Stomatitis:** Inflammation of the mucus membrane of oral cavity:
  - Most common agent: Herpes simplex virus
  - Symptom: Painful vesicular ulcers.
- **Oral thrush:** It is characterized by whitish patch of exudate in the oral cavity.
  - Agent: *Candida albicans*
  - Risk factor: Immunosuppression.
- **Periodontal infections:** Root canal infections and perimandibular infections mainly caused by:

Table 64.4: Clinical Pulmonary Infection Score (CPIS) used for ventilator-associated pneumonia (VAP)

Parameter(s)	Score 0	Score 1	Score 2
Temperature (°C)	≥36.5°C and ≤38.4°C	≥38.5°C and ≤38.9°C	≥39°C and ≤36.4°C
Leukocytosis	≥4000 and ≤11,000	<4000 and >12000	
Tracheal aspirate	None	Non-purulent	Purulent
Oxygenation (PaO_2/FiO_2, mm Hg)	>250 or ARDS		≤250 and no ARDS
Chest radiograph	No opacity	Diffuse (patchy) opacity	Localized opacity
Progressive radiological progression	No radiological progression		Radiological progression
Culture of tracheal aspirate	Pathogenic bacteria Light or no growth	Pathogenic bacteria Moderate or heavy growth	
Culture-gram stain correlation	Different morphology than Gram stain	Same morphology as Gram stain	

Note: The likelihood of VAP is higher when total CPIS is >6.

- Anaerobic bacteria (*Bacteroides fragilis*)
- Streptococci (*S. sanguis*)
- *S. aureus*
- *Eikenella corrodens*

❖ **Salivary gland infections:** Parotitis is characterized by painful tender swelling of the parotid glands. The most common cause is *Staphylococcus aureus*, whereas most common viral agent is mumps.

Laboratory Diagnosis of Respiratory Tract Infections

Specimen Collection

Depending upon the nature of the disease, various respiratory specimens collected are:

❖ **For URTI**:
- Throat swab: Two swabs should be collected, one for direct examination, other one for culture
- A part of the membrane, if present
- Nasopharyngeal aspirate for viral diagnosis or for *B. pertussis*.

❖ **For LRTI:** Sputum, induced sputum, tracheal aspirate, bronchoalveolar lavage (BAL).

Microscopy

❖ **Albert staining** of the throat swab may demonstrate the presence of metachromatic granules in the ends of the bacilli—suggestive of *C. diphtheriae*

❖ **Gram staining** of the sputum or other specimens is done to:
- Detect the quality of the sputum: If many pus cells are present (>25/low power field) and less epithelial cells are present (<5/low power field), such samples are regarded as good quality sputum, where the chance of recovery of the pathogen is more
- Identify bacteria based on their morphology—for example, gram-positive cocci, pair, lanceolate shaped—suggestive of pneumococcus

❖ **Acid fast staining** of sputum by Ziehl-Neelsen technique is performed to demonstrate the acid fast bacilli, e.g. *M. tuberculosis*

❖ **GMS stain** (Gomori methenamine silver stain) is used to demonstrate *Pneumocystis jirovecii*

❖ **Immunofluorescence microscopy** of nasopharyngeal aspirate is performed to detect the presence of antigens of respiratory viruses.

Culture

❖ **For bacteriological culture:** Specimens are inoculated onto blood agar, chocolate agar and MacConkey agar and incubated overnight

❖ **For isolation of *C. diphtheriae*:** Loeffler's serum slope and potassium tellurite agar are used

❖ **For *M. tuberculosis*:** Specimen should be inoculated onto LJ medium and incubated for up to 6-8 weeks

❖ **For fungal pathogen isolation:** Sabouraud dextrose agar is used

❖ **Appropriate cell lines** are sometimes used for the isolation of the respiratory viruses.

Identification

❖ Specific bacterial identification is done based on colony morphology, and biochemical reactions (Table 64.5)

❖ Viral agents in the cell lines can be detected by demonstration of cytopathic effect or detection of viral antigens by immunofluorescence test.

Serology

This is important for detection of antibodies:

❖ ***Mycoplasma*:** Cold agglutination test, complement fixation test (CFT) and ELISA formats are available

❖ **Chlamydial antibodies in serum:** Micro-IF and CFT are used (Table 64.5)

❖ Most viral infections.

Molecular Test

Multiplex PCR assays are available where multiple primers targeting the genes specific for each of the suspected agents of URTI/LRTI are used.

TREATMENT — URTI

Depends up on type of URTI and the organism suspected.

TREATMENT — Pneumonia

Community-acquired pneumonia (CAP)

Empiric regimen in CAP is determined by presence of co-morbidity (see Table 64.2) and prediction of prognosis by CURB-65 scoring system (see Table 64.3).

CAP, hospitalized (if CURB65 score >1):
- IV ceftriaxone plus azithromycin or
- IV levofloxacin.
- Add vancomycin if CA-MRSA suspected.

CAP, outpatient (if CURB-65 score ≤1):
- If no comorbidity present: Oral azithromycin or azithromycin plus amoxyclav.
- If comorbidity present: Oral levofloxacin.

Hospital-acquired pneumonia (HAP)
- Empirical therapy: comprises of both gram-negative (e.g. piperacillin-tazobactam or meropenem) *plus* gram-positive coverage (e.g. vancomycin).
- Definitive therapy: The empirical treatment should be tailored based on the organism isolated and its sensitivity pattern.

Table 64.5: Identification features/detection methods of common organisms causing pneumonia

Agents of pneumonia	Direct demonstration in sputum	Culture identification
Lobar pneumonia		
Streptococcus pneumoniae (Figs 22.9, 22.10)	Pus cells >25/LPF and epithelial cells <5/LPF gram-positive cocci in pair, lanceolate shaped	Alfa hemolytic, draughtsman-shaped colonies on blood agar Sensitive to optochin Bile soluble, ferments inulin
Haemophilus influenzae (Fig. 33.2)	Pus cells >25/LPF and epithelial cells <5/LPF Pleomorphic gram-negative bacilli	Satellitism on blood agar with *S. aureus* streak line
Staphylococcus aureus (Figs 21.2 to 21.4)	Pus cells >25/LPF and epithelial cells <5/LPF gram-positive cocci in clusters	BA- golden yellow hemolytic colonies Catalase positive, coagulase positive
Gram-negative bacilli E. coli, Klebsiella, Pseudomonas, etc.) (Figs 29.1 to 29.3, 32.1C)	Pus cells >25/LPF and epithelial cells <5/LPF gram-negative bacilli	Identification is based on: • Growth on MacConkey agar (LF or NLF colonies) and • Biochemical reactions (ICUT: indole, citrate, urease, TSI)
Interstitial or atypical pneumonia		
Chlamydophila pneumoniae	Direct immunofluorescence test Antigen detection by enzyme immunoassay Nucleic acid amplification test (NAAT) detecting specific genes	Serology-antibody detection by • CFT using LPS antigen • ELISA using recombinant LPS antigen • Micro-IF test using outer membrane protein antigen
Mycoplasma pneumoniae	Direct immunofluorescence test Capture ELISA-detecting antigen (P1 adhesin) PCR targeting P1 adhesin gene	Culture-fried egg colonies on PPLO agar Antibody detection • Non-specific test (cold agglutination test) • Specific test (e.g. ELISA)
Legionella pneumophila (Fig. 36.3)	Pus cells >25/LPF and epithelial cells <5/LPF Detection of specific antigen in sputum, urine	Growth on BCYE medium
Viral pneumonia	Detection of specific viral antigen in sputum Detection of specific viral genes in sputum (PCR)	

Abbreviations: LPF, low power field; PCR, Polymerase chain reaction; CFT, complement fixation test; LF, lactose fermenting; NLF, non-lactose fermenting; LPS antigen, Lipopolysaccharide antigen; ELISA, enzyme-linked immunosorbent assay; IF, Immunofluorescence; BCYE, Buffered charcoal yeast extract; PPLO; pleuro pneumonia-like organism.

EXPECTED QUESTIONS

I. Essay:

1. Describe in detail the clinical types, etiological agents and laboratory diagnosis of lower respiratory tract infections.

II. Write short notes on:

1. Ventilator-associated pneumonia.
2. Treatment of pneumonia.
3. Upper respiratory tract infections.

III. Multiple Choice Questions (MCQs):

1. CURB65 score is used for:
   a. Ventilator-associated pneumonia
   b. Upper respiratory tract infections
   c. Community-associated pneumonia
   d. Tuberculosis

2. Clinical-pulmonary infection score (CPIS) is used for:
   a. Ventilator-associated pneumonia
   b. Upper respiratory tract infections
   c. Community-associated pneumonia
   d. Tuberculosis

3. Sputum specimen sent for culture is considered good quality if it contains:
   a. Pus cells <5/low power field and epithelial cells >25/low power field
   b. Pus cells >25/low power field and epithelial cells <5/low power field
   c. Pus cells >25/low power field and epithelial cells >25/low power field
   d. Pus cells <5/low power field and epithelial cells <5/low power field

Answers

1. c **2.** a **3.** b

Miscellaneous Infectious Syndromes

65 CHAPTER

CASE SCENARIO

Skin and Soft Tissue Infections (SSTIs): A 72-year-old male diabetic patient admitted to the hospital with complaints of swelling in the arm with pus discharge. On physical examination, the local area was found to be red, warm and tender. The swelling has a feel of fluid-filled when pressed. Pus was aspirated and was sent to microbiology laboratory.

Questions:

1. What is the clinical diagnosis and list etiological agents causing this condition?
2. How will you manage this case?
3. What are the various types of skin and soft tissue infections?

Explanation:

Pus filled painful swelling, red, warmth—indicates that the clinical condition is an abscess. This has to be managed by incision and drainage and send the pus for culture and sensitivity. Empirical antibiotic therapy can be started as cotrimoxazole or cephalexin which can be tailored later according to culture sensitivity report.

The explanation to all other questions has been described in detail below.

SKIN AND SOFT TISSUE INFECTIONS (SSTI)

Approximately 15% of all patients who seek medical attention have some skin diseases or lesions, and many of which are infectious.

- Skin and soft tissue infections (SSTIs) can arise from invasion of organism through skin or from organisms that reach the skin from blood as a part of systemic infection
- Skin comprises of epidermis, dermis and subcutaneous tissues. Hair follicles and sweat glands originate in the subcutaneous tissues. Infection can involve any of these layers of skin (Table 65.1).

Clinical types of SSTIs

Skin infections can be subdivided into primary and secondary lesions:

- **Primary lesion:** An area of tissue with impaired structure/function due to damage by trauma or disease
- **Secondary lesion:** A lesion arising as a consequence of any primary infection.

Agents implicated in surgical site infections and burn wound infections are listed in Tables 65.2 and 65.3 respectively.

Laboratory Diagnosis

Specimen Collection

Appropriate specimens include:

- Pus from the wound collected by sterile swab
- Pus from abscess collected by incision and drainage, or needle aspiration
- Vesicle or bulla fluid, collected by needle aspiration or sterile swab
- Subcutaneous infections: Sample collected from the base of the lesion or biopsy of the deep tissues
- Skin scrapings, plucked hair or nail clippings in suspected fungal infections.

Microscopy

- Gram staining of the specimen may demonstrate the morphology of the causative organisms
- KOH mount is done for suspected fungal infections (e.g. dermatophyte)
- Tzanck smear of the vesicle fluid suspected of herpes simplex or varicella virus infections.

Culture

- For the culture of aerobic bacteria, specimens are inoculated onto blood agar and MacConkey agar and incubated overnight at 37°C
- **For culture of atypical *Mycobacterium*:** Lowenstein Jensen medium may be used
- **For dermatophytes:** Sabouraud's dextrose agar is used
- **For anaerobic organisms:** Robertson's cooked meat broth and BHIS (brain heart infusion agar with supplements) should be used. The plates should be incubated anaerobically.

Table 65.1: Infective skin manifestations and their common causative agents

Skin lesions	Description	Common etiological agents
Macule	Flat, non-palpable discoloration of skin (<5 cm size). If size exceeds 5 cm, is called as patch	Dermatophytes Viral rashes (e.g. enterovirus)
Papule	Elevated lesions usually <5 mm in size that can be felt or palpated	Molluscum contagiosum Scabies (*Sarcoptes scabiei*) Warts (Human Papilloma virus)
Plaque	Multiple papules my become confluent to form plaque which are palpable lesions >5 mm	
Nodule	Firm lesions >5 cm size	*Staphylococcus aureus* *Sporothrix* *Mycobacterium marinum*
Vesicle	Fluid-filled lesions with a diameter less than 0.5 cm	Herpes simplex virus, varicella-zoster virus
Bulla	Fluid-filled lesions with a diameter more than 0.5 cm	*Clostridium* Herpes simplex virus *Staphylococcus aureus*
Pustule	A fluid-filled vesicle containing neutrophils (i.e. pus) and is less than 0.5 cm in diameter	*Candida* *Staphylococcus aureus* *Streptococcus pyogenes*
Abscess	A fluid-filled lesion containing neutrophils and is more than 0.5 cm in diameter	
Secondary lesions		
Scale	Excess dead epidermal layer	Dermatophytes *Streptococcus pyogenes*
Ulcer	Break in epithelial lining extending into the epidermis/dermis	*Bacillus anthracis* decubitus ulcers of leprosy
Erysipelas	Painful, red, indurated swollen lesion involving dermis with a well-marked raised border Associated fever and lymphadenopathy	*Streptococcus pyogenes* Other streptococci
Impetigo	Erythematous lesions which may be bullous or non-bullous with exudates and golden-yellow crusts	Non-bullous: *Streptococcus pyogenes* Bullous: *Staphylococcus aureus*
Cellulitis	Diffuse spreading infection involving deep layers of dermis Ill-defined flat red, painful lesions Associated fever and lymphadenopathy	*Streptococcus pyogenes* *Staphylococcus aureus*
Hidradenitis	Chronic infection of obstructed sweat glands	*Staphylococcus aureus* *Streptococcus anginosus* group
Hair follicle infections		
Folliculitis	Superficial infection of single hair follicle, presents as pustule	*Staphylococcus aureus*
Furuncle	Deeper infections of the hair follicles, presents as abscess, spread deeply into dermis and subcutaneous tissues	
Carbuncle	Represents the coalescence of a number of furuncles	
Infection of fascia and muscles		
Necrotizing fasciitis	Rapidly spreading infection of fascia	*Streptococcus pyogenes*
Pyomyositis	Pus formation in the muscle layer	*Staphylococcus aureus* *Streptococcus pyogenes*
Myonecrosis	Extensive necrosis of the muscle layer with gangrene formation	Clostridial myonecrosis Other anaerobic infections

Table 65.2: Agents causing surgical site wound infection

Bacterial agents	Fungi
For most clean wounds: • *Staphylococcus aureus* • Coagulase-negative staphylococci • *Enterococcus*	*Candida albicans*
If bowel integrity is compromised: • Gram-negative flora like *E. coli* and • Anaerobic organisms like *Bacteroides, Prevotella*, etc.	

Table 65.3: Agents causing burn wound infections

Bacteria	Fungi
Staphylococcus aureus (may be MRSA) *Pseudomonas aeruginosa* Coagulase-negative staphylococci (*e.g. S. epidermidis*)	*Candida albicans*

Abbreviation: MRSA, methicillin resistant *staphylococcus aureus.*

Quantitative Culture

As the degree of bacterial contamination of the wound, is directly related to the chance of development of wound sepsis, hence quantitative culture may be performed to determine the number of colony forming units/gram of the tissue collected from the wound.

Identification

Accurate identification of the causative agent is done based on colony morphology, culture smear, and biochemical re-actions.

Antimicrobial Susceptibility Test

It helps in initiation of appropriate therapy.

TREATMENT — SSTIs

Skin and soft tissue infections are treated by both surgically (incision and drainage or surgical debridement) and medically (antibiotics) (Table 65.4).

SEXUALLY TRANSMITTED INFECTIONS (STIs)

CASE SCENARIO—1

Urethritis

A 32-year-old male with history of frequent sexual contact with a commercial sex worker presented with urethral discharge. Urethral discharge was collected and sent to microbiology laboratory.

Questions:

1. What is the clinical diagnosis?
2. List the various etiological agents associated with this clinical condition.
3. What are the various modalities of laboratory diagnosis?
4. How will you manage such case?

Explanation:

Adult male with history of frequent sexual contact presented with urethral discharge is suggestive of urethritis.

Urethritis can be of two types gonococcal and non-gonococcal urethritis.

The explanation to all other questions has been described in the chapter subsequently.

Table 65.4: Treatment of skin and soft tissue infections (Source: IDSA guideline)

	Definition	Surgical treatment	Empirical antibiotic **
For purulent SSTIs (abscess, furuncle, carbuncle)			
Mild	Purulent infection without systemic signs of infection	Incision and drainage	No
Moderate	Purulent infection with systemic signs of infection	Incision and drainage and send for culture sensitivity	Oral cotrimoxazole or cephalexin or any other orally effective agent
Severe	Failed treatment for moderate SSTIs Immunocompromised patient Severe systemic features*	Incision and drainage and send for culture sensitivity	IV vancomycin
For non-purulent SSTIs (necrotizing infection, cellulitis, erysipelas)			
Mild	Typical cellulitis/erysipelas with no focus of purulence and no systemic signs of infection		Oral cephalosporins or dicloxacillin
Moderate	Typical cellulitis/erysipelas with systemic signs of infection		IV penicillin or ceftriaxone
Severe	Failed oral antibiotic treatment Immunocompromised patient Severe systemic features* Following present: bullae, skin sloughing, hypotension, or evidence of organ dysfunction	Emergency surgical debridement	Vancomycin *plus* piperacillin/ tazobactam

*Severe systemic signs of infection such as temperature >38°C, tachycardia (heart rate >90 beats per minute), tachypnea (respiratory rate >24 breaths per minute) or abnormal white blood cell count (>12,000 or <400 cells/μL).

**Empirical treatment is modified based up on the culture and sensitivity report

Abbreviation: IDSA: Infectious Disease Society of America.

CASE SCENARIO—2

Genital Ulcerative Diseases

A 29-year-old male with a history of extramarital sexual contact presented with localized, painless indolent ulcers with hard base on penis. On examination, the inguinal lymph nodes were found to be enlarged, hard, and non-tender. The serum sample from the patient was collected and sent to microbiology laboratory for serological test.

Questions:

1. What is the clinical diagnosis and its causative organism?
2. What are the various types of genital ulcerative diseases and their causative agents? How will you clinically differentiate them?
3. What are the various modalities of laboratory diagnosis?
4. How will you treat this condition?

Explanation:

This is a case of genital ulcerative disease. The various agents causing this condition are listed in Tables 65.5 and 65.6.

History of painless genital ulcers with hard, and non-tender inguinal lymph nodes is suggestive of syphilis.

The laboratory diagnosis and management of such case has been described in the chapter subsequently.

SEXUALLY TRANSMITTED INFECTIONS

The sexually transmitted infections (STIs) are a group of communicable diseases which are transmitted by sexual contact.

Causative agents of STIs may be classified into two groups:

1. Agents causing local manifestations such as:
   - Genital ulcers
   - Urethral discharge
   - Vaginal discharge
   - Genital warts
   - Pelvic inflammatory diseases.
2. Agents transmitted by sexual route, producing only systemic manifestations and do not cause local manifestations (e.g. HIV).

Laboratory Diagnosis of STIs

Specimen Collection

- Discharge from the infected area such as vaginal or urethral discharge are collected in a sterile container
- **Sterile swabs may be used to collect the discharge** (if scanty): Charcoal impregnated swabs are used for suspected gonococcal infection
- Fluid from the vesicles (genital herpes).

Microscopy

- **Wet mount examination:** It is carried out for the vaginal discharge:
  - In trichomoniasis: Pus cells along with motile trophozoites are seen
  - In candidiasis: Yeast cells along with pseudohyphae are seen.
- **Gram-stained smear** of the discharge or the swab is useful for:
  - Bacterial vaginosis—clue cells are seen, which are vaginal epithelial cells studded with gram variable pleomorphic coccobacilli; suggestive of *Gardnerella vaginalis*
  - In gonorrhea—intracellular kidney-shaped diplococci are seen
  - In candidiasis—gram-positive budding yeast cells along with pseudohyphae are seen.
- **Giemsa stain** is done for:
  - *Klebsiella granulomatis* to detect the presence of Donovan's bodies (macrophage filled with bipolar stained bacilli)
  - *Chlamydia trachomatis* inclusion bodies.
- **Dark field microscopy** and silver impregnation methods—in syphilis, reveals characteristic spirally coiled bacilli.

The microorganisms causing STIs are listed in Table 65.5 and the important features of STIs producing genital ulcers are compared in Table 65.6.

Culture

Specimens are inoculated onto the appropriate culture media or cell line for the isolation of the causative organism.

- Thayer-Martin medium—for *N. gonorrhoeae*
- Chocolate agar added with isovitalex and vancomycin—for *H. ducreyi*
- McCoy cell line—for *Chlamydia trachomatis*
- Sabouraud's dextrose agar (SDA)—for *Candida* species
- Cell lines such as Vero cells, monkey kidney cell line for herpes simplex virus.

Serology

Serological tests such as venereal disease research laboratory (VDRL) or rapid plasma reagin (RPR) test can be performed for the diagnosis of syphilis.

Molecular Test

Multiplex PCR and real-time PCR have been developed for simultaneous detection of pathogens causing STIs such as *C. trachomatis* (opacity protein gene or 16s or 23s rRNA), *N. gonorrhoeae* (16s or 23s rRNA gene), *T. pallidum* (47 kDa *tpp* gene or *polA* gene), *H. ducreyi* (16s rRNA) and HSV (*TSK3 gene*), etc.

TREATMENT urethritis

Combination of ceftriaxone + azithromycin is the recommended regimen to ensure cure and to prevent further development of resistance. Ceftriaxone will act against gonococcus and azithromycin will treat *C. trachomatis*; as these are the common causative agents of urethritis. Treatment to both the sexual partners is needed.

Table 65.5: Causative agents of sexually transmitted infections (STIs)

Agents causing local manifestations	
Genital ulcers	
Syphilis	*Treponema pallidum*
Herpes genitalis	Herpes simplex viruses
Chancroid	*Haemophilus ducreyi*
Lymphogranuloma venereum	*Chlamydia trachomatis*
Donovanosis	*Klebsiella granulomatis*
Urethral discharge	
Gonorrhea	*Neisseria gonorrhoeae*
Non-gonococcal urethritis (NGU)	*Chlamydia trachomatis* (D-K) *Ureaplasma urealyticum* *Mycoplasma genitalium* *Mycoplasma hominis* Herpes simplex virus *Candida albicans* *Trichomonas vaginalis*
Vaginal discharge	
Vulvovaginal candidiasis	*Candida albicans* Non-albicans *Candida* species
Bacterial vaginosis	*Gardnerella vaginalis* *Mobiluncus* species
Trichomonal vaginitis	*Trichomonas vaginalis*
Genital warts	
Condyloma acuminata	Human papilloma viruses
Agents causing systemic manifestations	
Pelvic inflammatory diseases (PID)	*Neisseria gonorrhoeae* *Chlamydia trachomatis*
No genital lesions but only systemic manifestations	HIV Hepatitis B virus (HBV) Hepatitis C virus (HCV)

CONGENITAL INFECTIONS

Vertical transmission refers to the spread of infections from mother-to-baby. These infections may occur by transplacental route (congenital infection), during delivery, or after delivery.

Congenital Infection

A congenital infection is an infection that crosses the placenta to infect the fetus. They often lead to defects in fetal development or even death.

TORCH is an acronym used for some common congenital infections. These are:

- **T**oxoplasmosis
- **O**ther infections (congenital syphilis, hepatitis B, Coxsackie virus, Epstein-Barr virus, varicella-zoster virus, *Plasmodium falciparum* and human parvovirus)
- **R**ubella
- **C**ytomegalovirus (CMV)
- **H**erpes simplex virus.

Perinatal Infections (During Delivery)

Perinatal infections occur while the baby moves through an infected birth canal. These infections are usually caused by the agents of STIs. These also include the infections transmitted through contamination with fecal matter during delivery. Common examples of agents causing perinatal infections include:

- Cytomegalovirus
- *Neisseria gonorrhoeae*
- *Chlamydia* species
- Herpes simplex virus
- Human papilloma virus (genital warts)
- Group B streptococci.

Postnatal Infections (After Delivery)

These infections spread from mother to baby following delivery, usually during breastfeeding. Some examples of postnatal infections are: CMV, HIV and group B streptococci.

Table 65.6: Comparison of sexually transmitted infections (STIs) producing genital ulcer

Feature	Syphilis	Herpes	Chancroid	LGV	Donovanosis
Incubation period	9–90 days	2–7 days	1–14 days	3 days–6 weeks	1–4 weeks (up to 6 months)
Genital ulcer	Painless, indurated, single	Multiple, painful	Painful, soft Single or multiple	Painless	Painless beefy-red ulcer
Lymphadenopathy	Painless, moderate swelling (no bubo)	Absence or moderate swelling (no bubo)	Painful, soft, marked swelling leads to bubo formation	Painful and soft	Absent (pseudobubo may be present due to subcutaneous swelling)

Abbreviation: LGV, Lymphogranuloma venereum.

EYE INFECTIONS

In general, ocular infections are grouped into:

- **Infections involving external structures of the eyes:** such as eyelid (blepharitis), conjunctiva (conjunctivitis), cornea (keratitis) and sclera (scleritis)
- **Infections involving internal structures:** Retina (retinitis), uvea (uveitis) and aqueous humor or vitreous humor (endophthalmitis).

The list of the organisms causing various ocular infections is given in Table 65.7.

EAR INFECTIONS

Common ear infections are (Table 65.8)

- **Otitis externa:** Inflammation, irritation, or infection of the outer ear and ear canal.
  - Also called as swimmer's ear—swimming in contaminated water is one of the reasons of contracting swimmer's ear.
  - Symptoms—itchy ear canal, Inflammation of ear canal's skin and pus formation in ear canal and earache that is aggravated when the ear lobe is pulled.
- **Otitis media:** Infections of middle ear; characterized by earache and ear discharge.
  - It often begins with an infection that causes a sore throat, cold or respiratory problem and eventually spread to the middle ear.
  - Symptoms include: Intense earache, headache, fever and nausea and leaking of discharge from ear following rupture of the tympanic membrane.

Table 65.7: Ocular infections and their causative agents

Infections	Organisms
Blepharitis (infection of eyelids)	*Staphylococcus aureus*
Conjunctivitis (infection of conjunctiva)	*Haemophilus influenzae* *Staphylococcus aureus* *Chlamydia trachomatis* *Neisseria gonorrhoeae* *Moraxella lacunata* (angular conjunctivitis) Adenovirus, Herpes simplex virus
Keratitis (infection of cornea)	*Staphylococcus aureus* *Streptococcus pneumoniae* *Fusarium, Candida* *Acanthamoeba*
Scleritis (infection of sclera)	*Staphylococcus aureus*
Chorioretinitis and uveitis (infection of choroid, retina, and uvea)	*Mycobacterium tuberculosis* *Treponema pallidum* *Borrelia burgdorferi* Cytomegalovirus *Toxoplasma gondii*
Endophthalmitis (infection of aqueous humor or vitreous humor)	*Staphylococcus aureus* *Streptococcus pneumoniae* *Pseudomonas aeruginosa* Other gram-negative bacilli Herpes simplex virus, *Candida*

Table 65.8: Organisms causing ear infections

Otitis externa: Infection of external ears
Acute otitis externa • *Staphylococcus aureus* (most common) • *Streptococcus pyogenes* • *Pseudomonas* (malignant otitis externa) • Other gram-negative bacilli • *Aspergillus* species • *Candida* species
Chronic otitis externa • Anaerobes (most common) • *Pseudomonas*
Otitis media: (middle ear infections)
Acute otitis media *Streptococcus pneumoniae*: Most common (33%, in children) *Haemophilus influenzae* type b (second most common) *Moraxella catarrhalis* *Streptococcus pyogenes* Respiratory syncytial virus Influenza virus
Chronic otitis media Anaerobes (most common)

EXPECTED QUESTIONS

I. Write short notes on:

1. TORCH infections.
2. Skin and soft tissue infections.
3. Sexually transmitted infections.

II. Multiple Choice Questions (MCQs):

1. **Which of the following sexually transmitted infection produces painful genital ulcers and painful lymph nodes?**
   a. Syphilis b. Chancroid
   c. Herpes d. Donovanosis
2. **The agent of malignant otitis externa is:**
   a. *Staphylococcus aureus*
   b. *Pseudomonas* species
   c. *Streptococcus pyogenes*
   d. *Candida* species

Answers

1. b **2.** b

Annexures

Section Outline

Annexure-1

EMERGING AND RE-EMERGING INFECTIONS

Definitions

Emerging Infections

They are the infectious diseases; whose incidence in humans has increased in the past two decades or threatens to increase in the near future (Tables A1.1 and A1.2). These diseases, with respect to no national boundaries, include:

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.

Re-emerging Infections

They are old infections; which were clinically silent or reduced in incidence, have again re-emerged in the community, either as a result of—(1) Antimicrobial resistance in known agents or (2) Breakdown in public health measures. Chikungunya virus re-emergence in 2005 is the classical example (Table A1.2).

Drug Resistance and Re-emergence

The re-emerging infections that have increased in frequency in the last decade as a result of development of antimicrobial resistance include:

- MDRTB (Multi drug resistant tuberculosis)
- XDRTB (Extensively drug resistant tuberculosis)
- MRSA (Methicillin resistant *Staphylococcus aureus*)
- VRE (Vancomycin resistant enterococci)
- VRSA (Vancomycin resistant *Staphylococcus aureus*)
- Beta lactamase producers
  - ESBL (Extended spectrum beta lactamase producers)
  - Carbapenemase producers
  - Amp C beta lactamase producers

WHO's Top 8 Emerging Diseases

In 2015, the World Health Organization has published the top 8 emerging diseases that are likely to cause severe outbreaks and major epidemics in the near future. These diseases include:

1. Crimean Congo haemorrhagic fever
2. Ebola virus disease
3. Marburg virus disease
4. Lassa fever
5. MERS coronavirus disease
6. SARS coronavirus disease
7. Nipah virus disease
8. Rift Valley fever

This list provides a basis for working on research and development preparedness (R&D) to try and control potential future outbreaks.

Three other diseases were designated as 'serious', requiring action by WHO to promote R&D as soon as possible; these were:

- Chikungunya
- Severe fever with thrombocytopenia syndrome
- Zika virus disease.

Table A1.1: Emerging infections in the world since 1975

Year	Organism
1975	*Parvovirus* B-19
1976	*Cryptosporidium parvum*
1977	Ebola virus
1977	*Legionella pneumophila*
1977	Hantavirus
1977	*Campylobacter jejuni*
1980	Human T-lymphotropic virus I (HTLV-I)
1981	Toxin producing strains of *Staphylococcus aureus*
1982	*Escherichia coli* O157:H7
1982	Human T-lymphotropic virus II (HTLV-II)
1982	*Borrelia burgdorferi*
1983	Human immunodeficiency virus (HIV)
1983	*Helicobacter pylori*
1985	*Enterocytozoon bieneusi*
1986	*Cyclospora cayatanensis*
1988	Hepatitis E virus
1989	*Ehrlichia chafeensis*
1989	Hepatitis C virus

Contd...

Contd...

Year	Organism
1991	Guanarito virus
1991	*Encephalitozoon hellem*
1991	New species of *Babesia*
1992	*Vibrio cholerae* O139
1992	*Bartonella henselae*
1993	Sin Nombre virus
1993	*Encephalitozoon cuniculi*
1994	Sabia virus
1995	Human herpes virus 8 (HHV-8)
1999	Nipah virus
2002	SARS coronavirus (Severe acute respiratory syndrome coronavirus)
2003	Influenza A (H5N1)
2009	Influenza A (H1N1)
2012	Novel coronavirus or MERS-CoV (Middle East respiratory syndrome coronavirus)
2013	Severe fever with thrombocytopenia syndrome (SFTS) virus

Table A1.2: Emerging and re-emerging infections in India, 1990–2011

Year	Organism	Place
1992	*V. cholerae* O139	Chennai
1994	Plague	Surat
2000	Diphtheria	Delhi
2001	Nipah virus	Siliguri
2002	Plague	Shimla
2004	Plague	Uttrakhand
2003	Chandipura	Andhra Pradesh
2004	Chandipura	Gujrat
2005	Chikungunya	Hyderabad
2007	Chandipura	Maharashtra
2009	H1N1	Almost all states
2011	Crimean-Congo hemorrhagic fever	Gujrat
2018	Nipah virus	Calicut (Kerala)

Annexure-2

BIOTERRORISM—BIOLOGICAL WARFARE

Definition

Bioterrorism is a form of terrorism (unlawful use of weapon against mankind) where there is intentional and deliberate release of biological agents (bacteria, viruses, fungi or their toxins) to cause mass illness or death of people, animals, or plants.

Biologic Agents used as Bioweapons (Table A2.1)

The biologic agents used as bioweapons should have the following key features:

- Should produce high morbidity and mortality in the community
- Potential for person-to-person spread
- Should be of low infective dose
- Should be highly infectious by aerosol
- Lack of rapid diagnostic facilities
- Effective vaccine should not be available globally
- Potential to cause anxiety
- Availability of pathogen and feasibility of production
- Environmental stability—should have the potential to be "weaponized".

History of Bioterrorism Attacks

The use of biological agents as weapons is not a new concept. They have been used since ancient time.

- The first bioweapon used was the fungus *Claviceps purpurea* (rye ergot) by the Assyrians, in the sixth century B.C
- The plague bacilli were used in 14th century
- During World War I—Anthrax was used by Germany to infect the mules and horses of enemies
- During World War II—Japanese forces used anthrax and plague bacilli against prisoners
- **Sverdlovsk anthrax leak:** This is the largest biological weapon accident occurred so far. The spores of anthrax were accidentally released from a military research facility of Sverdlovsk (now called as Yekaterinburg) in 1979. This accident is also referred to as "**biological Chernobyl**", which resulted in approximately 100 deaths
- **2001 USA World Trade center attacks**—Anthrax spores were mailed to US media and government offices during a terrorist attack. There were 22 cases with five deaths.

Prevention and Preparedness

To strengthen the area of bio-defence, US government passed the 'Bioterrorism Act of 2002' soon after the 2001 anthrax attack. The emergency preparedness and response network has been made, which aims at:

- Prevention of a bioterrorism attack
- Detection of a bioweapons with efficient laboratory systems with newer diagnostic facilities
- Quick relief during a bioterrorism attack.

Globally various agencies are working hard to curb such problems in future. In India however, such a network is still lacking.

Table A2.1: Classification of bioweapons

Category A: These agents are the highest priority pathogens which pose the greatest risk to national security	
• These agents can be easily disseminated or transmitted from person to person • Result in high mortality and have the potential for major public health impact • Might cause public panic and social disruption • Require special action for public health preparedness.	• Anthrax (*Bacillus anthracis*) • Botulism (*Clostridium* botulinum toxin) • Plague (*Yersinia pestis*) • Tularemia (*Francisella tularensis*) • Small pox (Variola major) • Hemorrhagic viruses ➢ Arenaviruses: Old World virus (Lassavirus), New World viruses (Machupo, Junin, Guanarito, and Sabia) ➢ Bunyaviridae: Crimean-Congo virus ➢ Filoviridae: Ebola, Marburg virus
Category B: These agents are the second highest priority pathogens	
• Moderately easy to disseminate • Result in moderate morbidity rates and low mortality rates • Require specifically enhanced diagnostic capacity	• Melioidosis (*Burkholderia pseudomallei*) • Glanders (*Burkholderia mallei*) • Brucellosis (*Brucella* species) • Psittacosis (*Chlamydophila psittaci*) • Q fever (*Coxiella burnetii*) • Typhus fever (*Rickettsia prowazekii*) • Toxin: Ricin, *S. auerus* enterotoxin B, Epsilon toxin of *Clostridium perfringens* • Viral encephalitis [alphaviruses (e.g., Venezuelan, eastern, and western equine encephalitis)] • Food threats: *Salmonella*, *Shigella*, *E. coli* O157 • Water threats: *Vibrio cholerae*, *Cryptosporidium*
Category C: These agents are the third highest priority pathogens. They are the emerging pathogens, to which the general population lacks immunity	
• These agents could be engineered for mass dissemination in future because of availability, ease of production, and ease of dissemination • They have a potential for high morbidity and mortality rates	• Nipah virus • Hantavirus • SARS and MERS coronavirus • Pandemic influenza virus • MDRTB • Yellow fever virus

Adapted from Centers for Disease Control and Prevention (CDC)

Annexure-3

LABORATORY-ACQUIRED INFECTIONS

Laboratory acquired infections (LAIs) are defined as all infections acquired through laboratory or laboratory-related activities, regardless whether they are symptomatic or asymptomatic in nature.

LAIs result from occupational exposure to infectious agents. The most common route of exposure and accidental inoculation are the following:

- Inhalation (see aerosols)
- Percutaneous inoculation (needle and syringe, cuts or abrasions from contaminated items and animal bites)
- Contact between mucous membranes and contaminated materials (hands or surfaces)
- Ingestion (aspiration through a pipette, smoking or eating).

The risk-based classification of potential organisms responsible for LAIs are summarized in Table A3.1.

Table A3.1: Risk based classification of agents causing laboratory-acquired infections

Group	Definition	Bacteria	Virus	Fungi	Parasite
Group-1	Biological agents that are unlikely to cause human disease	Non-pathogenic organisms	–	–	–
Group-2	Biological agents that can cause human disease and may be hazard to workers; but are unlikely to spread to community; effective treatment or prophylaxis is usually available	• *Bacillus* species (except *B. anthracis*) • *Clostridium* species • *Corynebacterium diphtheriae* • Enterobacteriaceae • *Staphylococcus* • *Streptococcus* • *Mycobacterium* (except *M. tuberculosis*)	• Adenovirus • Calicivirus • *Coronavirus* (not SARS-CoV) • Herpesvirus • Influenza virus	• *Cryptococcus* • *Candida* • Dermatophytes • *Aspergillus*	All clinically important parasites
Group-3	Biological agents that can cause severe human disease and are a serious hazard to workers They may spread to the community; but effective treatment or prophylaxis is usually available	• *B. anthracis* • *Brucella* species • *Coxiella burnetii* • *Francisella tularensis* • *M. tuberculosis*	• Prion • LCM virus (Lymphocytic choriomeningitis) • Hantavirus • SARS-CoV • Encephalitis virus such as: ➢ St Louis ➢ Japanese ➢ West Nile ➢ Western equine	–	–
Group-4	Same as group 3 except that effective treatment or prophylaxis is usually not available	–	• Lassa virus • Ebola virus • Marbug virus • Herpes simiae virus	–	–

Annexure-4

ZOONOSES

Zoonoses has been defined as infectious diseases that are primary infections of vertebrate animals, which can be naturally transmitted to humans.

Classification (Table A4.1)

Zoonotic diseases can be classified in terms of their reservoir hosts as:

- **Anthropozoonoses:** Infections transmitted to man from lower vertebrate animals
- **Zooanthroponoses:** Infections that are transmitted from man to lower vertebrate animals
- **Amphixenoses:** Infections that are maintained in both animals and lower vertebrate animals that may be transmitted in either direction.

More than 150 zoonotic diseases have been recognized, and the important ones are given in the Table A4.1.

Table A4.1: Important zoonotic infections affecting human beings and their usual sources

Bacteria	Animals	Viruses	Animals	Fungi	Animals	Parasites	Animals
Anthrax	Herbivores	Rabies	Dogs	Zoophilic dermatophytoses		*Toxoplasma*	Cats
Plague	Rat	Yellow fever	Monkeys	*Trichophyton equinum*	Horse	*Leishmania*	Dogs
Brucellosis	Sheep, goat, camel	Japanese encephalitis	Pigs	*Trichophyton simii*	Dogs, poultry	*Taenia*	Pigs, cattle
				Microsporum canis	Dogs	*Echinococcus*	Dog
Leptospirosis	Rodents	Kyasanur forest disease	Monkeys	*Microsporum equinum*	Horse	*Cryptosporidium*	Cattle
Salmonellosis	Poultry			*Sporothrix*	Cats	*Fasciolopsis buski*	Pigs, cattle
Bovine tuberculosis	Cow	Chikungunya	Monkeys	*Malassezia*	Dogs, cats	*Trichinella*	Pigs
		Monkey pox	Monkeys	*Cryptococcus*	Wide variety of animals, birds	Hookworms and Roundworms	Dogs and cats
Endemic typhus	Rodents	Prion diseases	Cattle			*Dirofilaria* and zoonotic *Brugia* species	Dogs, cats, raccoons etc.
Tularaemia	Rabbits	Hemorrhagic fevers	Rodents, cattle, wild animals	*Penicillium marneffei*	Bamboo rats		
		Influenza	Pigs, birds	*Lacazia loboi*	Dolphins		
				Conidiobolus	Horses		
				Histoplasma	Cattle, sheep		
				Coccidioides	Dogs		
				Paracoccidioides	Dogs		
				Blastomyces	Dogs		
				Pneumocystis jirovecii	Rodents		

Annexure-5

QUALITY CONTROL IN MICROBIOLOGY

Microbiological investigations play an important role in the diagnosis of infectious diseases and while taking decision on selecting appropriate antimicrobial agents for patient care. It is, therefore, essential that the test reports are relevant, reliable, rapid, and interpreted correctly. Quality is the meeting of pre-determined user requirement for a particular substance or service, which can be met by having quality assurance programme in place.

Quality Assurance (QA)

According to World Health Organization (WHO), the quality assurance has been defined as the total process whereby the quality of laboratory reports can be guaranteed every time when the test is performed.

- It denotes delivery of right result, on the right specimen, by the right method, at the right time, by right interpretation, and at the right price
- QA has to be dealt in three phases:
  1. Pre-analytical stage—involves sample collection, labeling of specimen, transportation etc.
  2. Analytical stage—involves performing the test in actual
  3. Post-analytical—involves reporting and interpretation of the test results.

QA also encompasses monitoring and quality assessment (described later). Components of quality assurance programme is given in highlight box below.

Components of quality assurance

- Personnel with adequate competence
- Proper specimen collection, storage and transport
- Use of precise and accurate techniques
- Appropriate processing of test results
- Good quality equipment and reagents
- Methods of detecting errors and corrective steps
- Preventive maintenance of equipment
- Continual staff training
- Documentation and timely feed back.

Quality Control (QC)

The term QC covers that part of QA, which refers to institution of appropriate checks during the performance of tests and verification of test results; that is the analytical phase of QA. QC must cover all aspects of every procedure within the department.

- All materials, procedures and equipment must be adequately controlled
- However, it is mainly restricted to quality control of stains, media, reagents, antibiotic disks etc. It is not synonymous with quality assurance.

Standard Operating Procedures (SOPs)

Every laboratory must maintain their own SOPs. SOP is a laboratory bench manual, which describes the step-wise process and technique of performing a routine or repetitive activity in the laboratory. The laboratory staff refer the SOPs while performing the tests.

- **Objectives:** SOPs help in maintaining uniformity while performing the laboratory tests, regardless of who is performing the test. It is also helpful in training the new staff
- **Preparation of SOPs**: SOPs must be prepared by a qualified laboratory officer in a language clearly understood by users. Each SOP must be given a title and identification number with version and date. It should be signed by an authorised signatory
- **Review:** It should be reviewed at least annually or whenever the procedure is changed. SOPs should include only those tests which are currently in use
- The SOP should include requirements for pre-analytic, analytic and post-analytic phases, keeping the quality issue in view.

Effective QA detects errors early, even before they could lead to incorrect test results. Laboratory personnel need to be aware of the errors that can occur while sample collection (pre-analytical stage), performing the tests (analytical stage) and reporting and interpreting the test results (post-analytical). For factors influencing quality refer Table A5.1.

Table A5.1: Factors influencing quality

Pre-analytical	Analytical	Post-analytical
Right investigation	Equipment reliability	Accurate recording
Right sample	Reagents stability, integrity and efficiency	Biological reference intervals
Right collection	Adequate calibration	Age and sex-related variations
Right technique	Correct interpretation	Turnaround time
Right laboratory	Procedural reliability using SOP	Availability of guidance
Right background milieu	Proficiency of personnel	Authorized release of results
Right transportation	Right technique for available reagents	Archiving of specimen
Right quantity	Internal quality control	Transcribing results from worksheet to report forms
Right labelling	External quality assessment	

Adapted from World Health Organization.

Quality indicators

Quality must be measurable, if it has to be managed. It is said that 'If you can't measure it, you can't improve it.'

- Quality indicators (QI) help the health laboratory to define and measure the progress
- The measurement of quality indicators leads to early detection of system failure, which includes all three stages - pre-analytic, analytic and post-analytic; so that corrective actions can be taken promptly (see Table A5.2).

Assessment of Quality

Monitoring is an important and vital component of a quality system. Main objectives of monitoring are (i) to confirm consistency, (ii) to identify opportunities for improvement, and (iii) to assess the impact of changes in procedures.

The periodic and retrospective assessment of quality can be undertaken by an independent external agency or internally by a designated staff on behalf of the laboratory management. Quality can be thus assessed by an on-site inspection by trained professionals (auditors) or by processing of the material sent by a designated institution. Assessment is of two types as follows:

- **Material driven:** Internal quality assessment and External quality assessment scheme (EQAS)
- **Man-driven:** Internal audit and External audit (described later).

External Quality Assessment Scheme (EQAS)

External Quality Assessment Schemes vary as per the test method and number of participating laboratories.

- It should include testing for major pathogens; should not be too complicated, costly, or time consuming
- They are considered as powerful tool to monitor laboratory performance, to identify errors, for inter-laboratory comparability and reliability of future testing, to stimulate staff motivation, to assure clients that test results are reliable and promote high standards of good laboratory practices
- Participation in EQA schemes should always be considered as additional to internal QC. It is retrospective and periodic (at least four times a year); conducted by independent agency, ensures inter-laboratory comparability and improve the performance of participating laboratories
- A scoring system is used in EQAS to stimulate performance and to enhance inter-laboratory concordance. Participants, need to be clearly told whether their test results are in consensus and any corrective action to be taken.

Table A5.2: Examples of quality indicators

Pre-analytic indicators	Analytic indicators	Post-analytic indicators
• Incomplete requisitions • Phlebotomy efficiency • Specimen acceptability/ rejection rates • Accuracy of sample accessioning • Specimen transport time	• Internal and external control failures • Performance in EQAS • Frequency of unscheduled service and repairs of equipment • On-time performance – Calibration and maintenance of equipment • Vendor evaluation (supplier performance) • Inventory – emergency orders, Outdating • Misinterpretation of results • Availability of back-up services	• Compliance to turnaround time (TAT) • Errors/ Incomplete test reports • Availability of archived samples **Other Quality Indicators** • Number and type of laboratory accidents • Training and competency evaluation of personnel • Document problems discovered – outdated, incomplete, incorrect • Customer feedback and complaints – physicians, other health care staff, patients. • Numbers and types of non-conformances • Physician satisfaction with report format and content

Abbreviation: EQAS, External quality assessment scheme.
Adapted from World Health Organization.

Internal Quality Assessment (IQA)

IQA is similar to EQAS, except that the material preparation, distribution, evaluation and result assessment is done internally.

- Its main objective is to release of reliable results on day to day basis. It is a continuous, concurrent process, which is performed by laboratory staff
- Each laboratory should have an internal quality assessment scheme and can be carried out by regular use of certified reference material, split sampling, replicate testing, retesting of retained items and correlation of results
- If the discrepancies are observed, they are recorded and analysed by a senior professional in discussion with designated quality manager and possible corrective and preventive actions are taken.

Assurance

When both IQA and EQAS are in place, a laboratory can assertion a level of assurance. QA can be simply stated as the sum of quality control, internal quality assessment and external quality assessment.

Quality Audit

Quality audit is defined as a planned and documented activity performed in accordance with written procedures and check-lists to verify the examination and evaluation of applicable elements of a quality assurance programme have been developed, documented and implemented.

There are two types of audits, **internal** and **external**.

Internal Audits

They are performed by laboratory staff to inspect their own system and is done by members who are either trained in audit techniques. Internal audit allows better understanding of the day-to-day work and, through the review process, allows to make better decisions and provides a mechanism for continual quality improvement (CQI).

External Audits

External audits can be of two types.

Second Party External Audits

They are normally supplier audits performed to ensure that goods supplied are of the required standard. They are needed if there is change of suppliers *or* on a planned basis for regular suppliers.

Third Party External Audits

They are also called as **accreditation** and are normally performed by regulatory/statutory bodies; can be voluntary or mandatory. Accreditation is an approved procedure by which an authorized body or regulatory authorities accord formal recognition to a laboratory to undertake specific tasks, provided that predefined standards are met by the laboratory. Standards for laboratory accreditation have been developed by the **International Organization for Standardization (ISO)**. For example, ISO 15189:2012 specifies requirements for quality and competence in medical laboratories.

Index

Page numbers followed by *f* refer to figure and *t* refer to table.

Q

R

S

T