GOVERNMENT OF TAMIL NADU

HIGHER SECONDARY SECOND YEAR

MICROBIOLOGY

THEORY & PRACTICAL

A publication under Free Textbook Programme of Government of Tamil Nadu

Department of School Education

Untouchability is Inhuman and a Crime

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Government of Tamil Nadu

First Edition - 2019

Revised Edition - 2020, 2022

Reprint - 2021, 2023, 2024

(Published under New Education Syllabus)

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Content Creation

State Council of Educational Research and Training

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Printing & Publishing

Tamil NaduTextbook and Educational Services Corporation

www.textbooksonline.tn.nic.in

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Contents

Chapter	Topic	Page No	Month
1	Developments in Microbiology	01	June
2	Microscopy	10	June
3	Control of Microorganisms by Chemical Methods	26	June
4	Microbial Metabolism	42	July
5	Food Microbiology	65	July
6	Industrial Microbiology	82	July
7	Medical Bacteriology	110	August
8	Medical Parasitology	147	August
9	Medical Mycology	184	August
10	Medical Virology	202	October
11	Immunology	225	November
12	Microbial Genetics	242	December / January
	Practical	280	

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E-Book

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Assessment

TN_GOVT_XII_Micro_Biology_FM.indd 3 11-12-2021 22:29:01

HOW TO USE THE BOOK ?

Chapter Outline — Presents a complete overview of the chapter

Learning Objectives: — Goals to transform the classroom processes into learner centric with a list of bench marks



Amazing facts, Rhetorical questions to lead students to biological inquiry

Activity — Directions are provided to students to conduct activities in order to explore, enrich the concept.



To motivate the students to further explore the content digitally and take them to virtual world

Evaluation — Assess students to pause, think and check their understanding

Career corner — List of professions particular to that chapter

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Chapter

1

Developments in Microbiology

Learning Objectives

After studying this chapter the students will be able to,

- *Know the microbes in space*
- *Learn about genetically modified microbes*
- *Appreciate nanoparticles production using microbes*
- *Know the important of microbiome*
- *Understand the applications of automated machines in microbial identification*

Chapter Outline

1.1 Microbes in Space

1.2 Immunology

1.3 Molecular Biology and Genetic Engineering

1.4 Nanoparticles Production Using Microbes

1.5 Equipments

The field of microbiology, critical to human beings, not only due to the infectious diseases caused by these microbes but because "good" microorganisms are necessary to live on the planet and their extraordinary diversity of structure, function, habitat and applications are of paramount importance. Microorganisms (or microbes) inhabiting every corner of the globe, are indispensable to life on Earth and are responsible for some of the deadliest human diseases and form the basis of many industrial processes. This field of study could be considered as one of the most important areas of knowledge, considering that the bacteria in and on our bodies outnumber our own cells.

Microbiology, an organismal discipline concerned with the properties of small forms of life or microorganisms. Bacteria neatly fit this definition, but what about fungi and algae? These two groups each contain members that are far from microscopic. On the other hand, certain animals, such as nematode worms, can be microscopic, yet are not considered to be the domain of the microbiologist. Viruses represent another special case; they are most certainly microscopic (indeed, most are submicroscopic), but by most accepted definitions they are not living. The concept of microbiology is remarkably broad in covering bacteria, protozoa and the viruses, which differ profoundly their structural and biological properties. Microbiology is without aquestion, a

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branch of biology that possesses both unity and coherence.

The following list of specializations in microbiology will provide an insight on the significance of microbiology in the world today:

- Medicine
- Environmental science
- Food production
- Fundamental Research
- Agriculture
- Pharmaceutical Industry
- Genetic Engineering

The popular perception among the general public, however, remains one of infections and plagues. In reality, only a couple of hundred out of the half million or so known bacterial species, termed as pathogens with the potential to cause disease give rise to infections in humans and dominate the microbial world.

1.1 Microbes in Space

The majority of experiments on microorganisms in space were performed using Earth-orbiting robotic spacecraft, Example: the Russian ***Foton*** satellites and the ***European Retrievable Carrier*** (EURECA) (121), or human-tended spacecraft, such as space shuttles (106, 107) and space stations, Example: ***MIR*** and the ***International Space Station*** (ISS).

Only twice, during translunar trips of ***Apollo 16*** and ***17*** in the early 1970s, were microorganisms exposed to space conditions beyond Earth's magnetic shield, in the MEED (microbial ecology equipment device) facility and in the Biostack experiments. Arriving in space without any protection, *microorganisms* are confronted with an extremely hostile environment, characterized by an intense radiation field of galactic and solar origin, high vacuum, extreme temperatures, and microgravity.

HOTS

1. What are the sources of microorganisms in space?
2. How do bacteria survey in space?

Some bacteria were found in International Space Station and on the Mars Rover. Some bacteria and tiny microbes called tardigrades are able to survive for longer periods in space. This ability of surviving in extreme environmental condition leads to forward contamination. Sea planktons and other microorganisms have been identified in cosmonauts' spacewalk samples.

In July 2016, Kate Rubins was the first to sequence DNA in space. NASA astronaut Peggy Whitson amplified and sequenced the DNA of bacteria that grew as colonies in the petri plate on the surface on the space station. In June 2018, Professor George Fox and his team have isolated genus *Bacillus* from spacecraft assembly rooms at the Jet Propulsion Laboratory. They have sequenced the complete genomes of two strains, B. *safensis* FO-36bT and B. *pumilus* SAFR-032 and found that they are resistant to radiation.

Los Angeles in great news for India, scientists at NASA have named a new organism discovered by them after the much loved APJ Abdul Kalam. Till date, the new organism – a form of bacteria – has been found only on the International Space

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Station (ISS) and has been found on earth. Researchers at the Jet Propulsion Laboratory (JPL) the foremost lab of NASA for work on inter-planetary travel discovered the new bacteria on the filters of the international space station (ISS) and named it Solibacillus kalam to honour the late president, who was a renowned aerospace scientist.

HOTS

What could be source of microorganisms in space?

1.2 Immunology

Immunology is the study of the immune system and is a very important branch of the medical and biological sciences. The immune system protects us from infection through various lines of defence.

Important initial barriers to infection are physical (Example: the **skin**), enhanced by substances secreted by the body, such as saliva and tears, that contain molecules that can neutralise bacteria. The internal **mucosal tissues** (Example: **lungs & airways**, and the **gut**) are coated with mucus that is able to trap potential infectants. In the airways, mobile ciliate hairs work together to transport contaminants away from vulnerable areas. Tissues such as the skin, mucosal surfaces and airways also contain populations of immune cells that can respond to infectants that breach these physical defences.

In its most complex forms, the immune system consists of two branches: the **innate immune system** that utilises certain 'hard-wired' strategies to provide a rapid, general, response when alerted by certain typical signals of infection (essentially forming a first-line of defence); and the **adaptive immune system** that is able to develop highly specific responses (and a persistent 'immune memory') to target infection with extraordinary accuracy. Both systems work in close cooperation and, to an important extent, the adaptive immune system relies upon the innate immune system to alert it to potential targets, and shape its response to them.

Vaccines currently in development include

- A genetically-modified vaccine for the treatment of pancreatic cancer.
- A therapeutic vaccine that increases the immune response against the HIV virus.
- A vaccine that protects infants against meningococcal disease, a leading cause of meningitis.
- An immunotherapeutic vaccine for the treatment of Alzheimer's disease.
- A recombinant vaccine to prevent malaria.

Evolving science has increasingly enabled researchers to explore both promising therapeutic vaccines and new preventative agents for infectious diseases. Although the development process is extremely complex, advances in other scientific fields, such as genomics, are being leveraged in the development of new vaccines.

"Vaccines have been a major contributor in saving countless lives around the world," said Castellani. "Vaccinations contribute to the public

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health at large, and they make good economic sense. The many exciting candidates in the pipeline offer great hope for a healthier, more productive future."

1.2.1 Monoclonal Antibodies

mAb or moAb are identical immunoglobulins, generated from a single B-cell clone. These antibodies recognize unique epitopes, or binding sites, on a single antigen. Derivation from a single B-cell clones and subsequent targeting of a single epitope is what differentiates monoclonal antibodies from polyclonal antibodies

The traditional monoclonal antibody (mAb) production process usually starts with generation of mAb-producing cells (i.e. hybridomas) by fusing myeloma cells with desired antibody-producing splenocytes (Example: B cells). These B cells are typically sourced from animals, usually mice. After cell fusion, large numbers of clones are screened and selected on the basis of antigen specificity and immunoglobulin class (Figure 1.1).

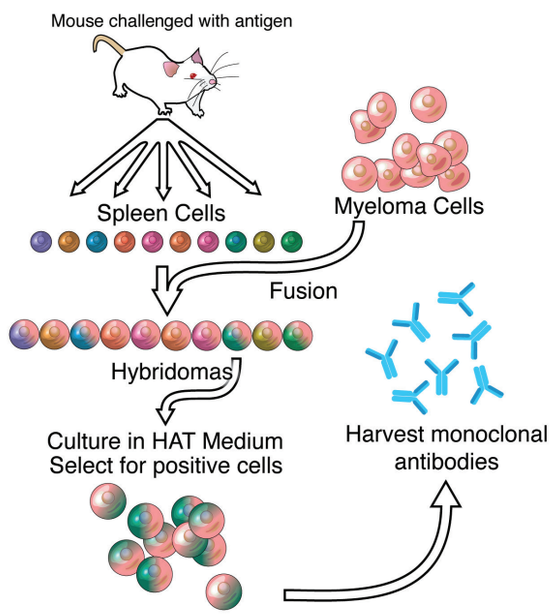

Figure 1.1 Monoclonal antibody

1.2.2 Stem Cell & Therapy

Stem cells are biological cells that can differentiate into other types of cells & they are found in multicellular organism. Stem cells are a class of undifferentiated cells that are able to differentiate into specialized cell types. Commonly, stem cells come from two main sources:

- Embryos formed during the blastocyst phase of embryological development (embryonic stem cells) and
- Adult tissue (adult stem cells).

Both types are generally characterized by their potency, or potential to differentiate into different cell types such as skin, muscle, bone, etc., (Figure 1.2).

Stem-cell therapy is the use of stem cells to treat or prevent a disease or condition. **Stem Cell Therapy** (SCT) is the treatment of various disorders, non-serious to life threatening, by using stem cells. These stem cells can be procured from a lot of different sources and used to potentially treat **more than 80 disorders**, including neuromuscular and degenerative disorders.

Hematopoietic disorders (Example: leukaemia, thallassemia, aplastic anemia, MDS, sickle cell anemia, storage disorders etc.) affect the bone marrow and manifest with various systemic complications. Stem cells from a donor (either from cord blood or bone marrow) are known to reconstitute the defective bone marrow and permanently overcome the disorder.

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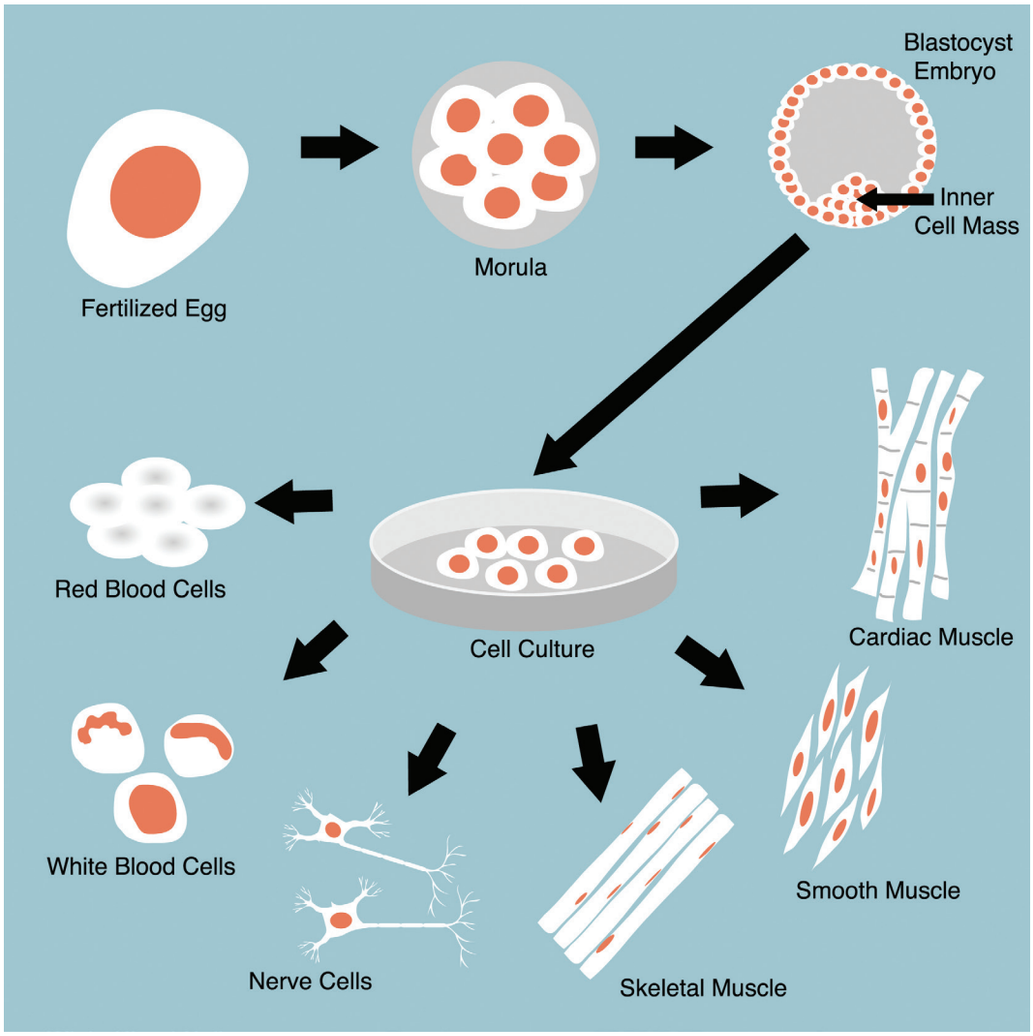

Figure 1.2 Stem cells

1.3 Molecular Biology and Genetic Engineering

Molecular biology- is the study of the structure, function & makeup of the molecular building blocks of life. It focuses on the interactions between the various system of a cell, including the interrelationship of DNA, RNA & Protein synthesis &how these interaction are regulated. Bioscience, Molecular biology closely interrelate with the fields of Biochemistry, Genetics & Cell biology.

Molecular biology is a specialised branch of biochemistry, the study of the chemistry of molecules which are specifically connected to living processes. Importance to molecular biology are the nucleicacids (DNA and RNA) and the proteins which are constructed using the genetic instructions encoded in those molecules. Other biomolecules, such as carbohydrates and lipids may also be studied for the interactions they have with nucleic acids and proteins. Molecular biology is often separated from the field of cell biology, which concentrates on cellular structures (organelles and the like), molecular pathways within cells and cell life cycles.

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Genetic Engineering

Genetic Engineering is the act of modifying the genetic makeup of an organism. Modification can be generated by methods such as gene therapy, nuclear transplantation, transfection of synthetic chromosome or viral insertion.

The manipulation of genetic make up of living cells by inserting desired genes through a DNA vector, is the genetic engineering. The gene is a small piece of DNA that encodes for a specific protein. The gene is inserted into a `vector DNA so that a new combination of vector DNA is formed. The DNA formed by joining DNA segments of two different organisms is called recombinant DNA. The organism whose genetic make up is manipulated using recombinant DNA technique, is called genetically manipulated organism (GMO).

Genetic engineering has many application in agriculture, animal science, industry and medicines (Figure 1.3).



Figure 1.3 Genetic Engineering

Genetically Modified Organism (GMO)

Organism genome has been engineered in the laboratory in order to favour the expression of desired physiological traits or the production of desired biological products. In conventional livestock production, crop farming, and even pet breeding, it has long been the practice to breed select individuals of a species in order to produce offspring that have desirable traits. In genetic modification, however, recombinant genetic technologies are employed to produce organisms whose genomes have been precisely altered at the molecular level, usually by the inclusion of genes from unrelated species of organisms that code for traits that would not be obtained easily through conventional selective breeding.

GMOs are produced through using scientific methods that include recombinant DNA technology and reproductive cloning. In reproductive cloning, a nucleus is extracted from a cell of the individual to be cloned and is inserted into the enucleated cytoplasm of a host egg. The process results in the generation of an offspring that is genetically identical to the donor individual. The first animal produced by means of this cloning technique with a nucleus from an adult donor cell (as opposed to a donor embryo) was a sheep named Dolly, born in 1996. Since then a number of other animals, including pigs, horses, and dogs, have been generated by reproductive cloning technology. Recombinant DNA technology, on the other hand, involves the insertion of one or more individual genes from an organism of one species

TN_GOVT_XII_Micro_Biology_CH01.indd 6 07-12-2021 14:59:50

into the DNA (deoxyribonucleic acid) of another. Whole-genome replacement, involving the transplantation of one bacterial genome into the "cell body," or cytoplasm, of another microorganism, has been reported, although this technology is still limited to basic scientific applications.

1.4 Nanoparticles Production Using Microbes

Particles with one or more dimensions of the order of 100 nm or less. There are a large number of physical, chemical, biological, and hybrid methods available to synthesize different types of nanoparticles. Although physical and chemical methods are more popular in the synthesis of nanoparticles, the use of toxic chemicals greatly limits their biomedical applications, in particular in clinical fields. Therefore, development of reliable, nontoxic, and eco-friendly methods for synthesis of nanoparticles is of utmost importance to expand their biomedical applications. One of the options to achieve this goal is to use microorganisms to synthesize nanoparticles.

Nanoparticles are biosynthesized when the microorganisms grab target ions from their environment and then turn the metal ions into the element metal through enzymes generated by the cell activities. It can be classified into intra-cellular and extracellular synthesis according to the location where nanoparticles are formed. The intracellular method consists of transporting ions into the microbial cell to form nanoparticles in the presence of enzymes. The extracellular synthesis of nanoparticles involves trapping the metal ions on the surface of the cells and reducing ions in the presence of enzymes.

The biosynthesized nanoparticles have been used in a variety of applications including drug carriers for targeted delivery, cancer treatment, gene therapy and DNA analysis, antibacterial agents, biosensors, enhancing reaction rates, separation science, and magnetic resonance imaging (MRI). Many microorganisms can produce inorganic nanoparticles through either intracellular or extracellular routes. This section describes the production of various nanoparticles via biological methods following the categories of metallic nanoparticles including gold, silver, alloy and other metal nanoparticles, oxide nanoparticles consisting of magnetic and nonmagnetic oxide nanoparticles, sulfide nanoparticles, and other miscellaneous nanoparticles (Figure 1.4).

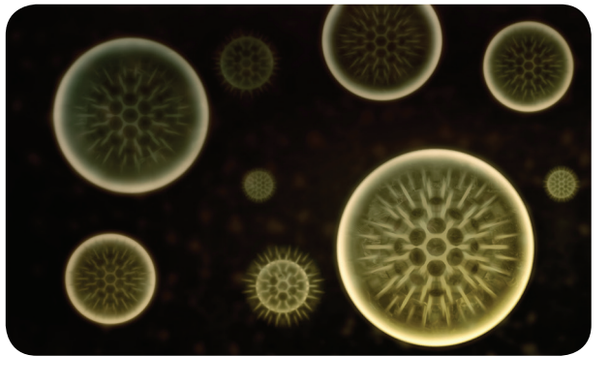

Figure 1.4: Advanced Nano particles

1.5 Equipments

1.5.1 Confocal Microscopy

Confocal microscopy offers several advantages over conventional optical microscopy, including shallow depth of field, elimination of out-of-focus glare, and the ability to collect serial optical sections from thick specimens. In the biomedical sciences, a major application

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Microbiome

The human microbiome is composed of communities of bacteria, viruses and fungi that have a greater complexity than the human genome itself. Genome sequencing technologies and metagenomic analysis has helped in our understanding of human microbiome. This is useful in manipulation of gut microbiome to be used in the treatment of childhood diseases.

of confocal microscopy involves imaging either fixed or living cells and tissues that have usually been labeled with one or more fluorescent probes.

Confocal microscopy, most frequently **confocal laser scanning microscopy (CLSM)** or **laser confocal scanning microscopy (LCSM)**, is an optical imaging technique for increasing optical resolution and contrast of a micrograph by means of using a spatial pinhole to block out-of-focus light in image formation. Capturing multiple two-dimensional images at different depths in a sample enables the reconstruction of three-dimensional structures (a process known as optical sectioning) within an object. This technique is used extensively in the scientific and industrial communities and typical applications are in life sciences, semiconductor inspection and materials science.

Light travels through the sample under a conventional microscope as far into the specimen as it can penetrate, while a confocal microscope only focuses a smaller beam of light at one narrow depth level at a time. The CLSM achieves a controlled and highly limited depth of focus.

1.5.2 DNA Sequencing System

Sequencing means finding the order of nucleotides on a piece of DNA. Nucleotide order determines amino acid order, and by extension, protein structure and function (proteomics). An alteration in a DNA sequence can lead to an altered or non functional protein, and hence to a genetic disorder. DNA sequence is important to detect the type of mutations in genetic diseases and offer hope for the eventual development of treatment DNA.

Methods of sequencing

1. Sanger dideoxy (primer extension/ chain-termination) method

Most popular protocol for sequencing, very adaptable, scalable to large sequencing projects.

2. Maxam-Gilbert chemical cleavage method

DNA is labelled and then chemically cleaved in a sequence dependent manner. This method is not easily scaled and is rather tedious.

It provides an important tool for determining the thousands of nucleotide variations associated with specific genetic diseases, like Huntington's, which may help to better understand these diseases and advance treatment.

Summary

Microorganisms (or microbes) inhabiting every corner of the globe, are indispensable to life on Earth and are responsible for some of the deadliest human diseases and form the basis of many industrial processes. Microbiology, an organismal discipline concerned with the properties of small forms of life or

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microorganisms. Microbes could help solve crimes. Arriving in space without any protection, microorganisms are confronted with an extremely hostile environment, characterized by an intense radiation field of galactic and solar origin, high vacuum, extreme temperatures, and microgravity. Emerging infection is defined as those infections whose incidence in humans has increased in the past two decades or will increase in the future. It can be new, reemerging, drug resistant infections. This condition associated with many chronic diseases, including diabetes, Lupus multiple sclerosis symptoms of leaky gut are bloating, gas, cramps, inflammatory bowel disease, fatigue, food sensitivities, join pain, moodiness, irritability, sleepless, skin problem and eczema, psoriasis.

(https://www.sciencedaily.com/releases/2018/06/180627160249.htm)

(http://tass.com/science/977591)

Evaluation

Multiple choice questions

1. Size of the Nono particles varies from
   a. Less than 10nm
   b. 100nm or less
   c. 100nm or more
   d. none of these
2. _____ is an example for optical imaging technique
   a. CLSM b. LCSM
   c. both a and b d. TEM
3. First genetically produced animal by cloning technique is _____
   a. Shally b. Dolly c. bally d. Vally
4. ISS stands for _____
   a. International space station
   b. Indian space station
   c. Indian standard system
   d. None of these

Answer the following

1. Short note on Microbes in space.
2. Brief account on Monoclonal antibody.
3. What is r.DNA technology?
4. Discuss on Emerging microbes.
5. Describe about Nano particles and its importance and its important in the field of medicine?
6. Give the importance of stem cells?
7. List out the various instruments used in Diagnostic microbiology.
8. Short note on genetically modified foods.
9. Brief note on sequencing methods.
10. Write about Vaccines and its importance.

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Chapter 2

Microscopy

Learning Objectives

After studying this chapter the students will be able to,

- *Identify the principle components of Phase Contrast, Fluorescence and Electron Microscope.*
- *Understand the optics in different light microscope and image formation mechanism.*
- *Know the principle, working mechanism of Phase Contrast, Fluorescent Microscope and Electron Microscope.*
- *Differentiate Light and Electron Microscope.*
- *Appreciate the applications of Phase Contrast, Fluorescence and Electron Microscopes.*

Chapter Outline

2.1 Phase Contrast Microscope

2.2 Fluorescence Microscope

2.3 Electron Microscope

Microscopes are specialized optical instruments designed to produce magnified visual or photographic images of objects or specimens that are too small to be seen with naked eye. Today, more sophisticated compound light microscopes are routinely used in microbiology laboratories. In the previous year, we have learnt about light microscopes that includes bright field and Dark-field microscopes. This year we are going to learn about other types of light microscopes such as phase contrast and fluorescence microscopes. Yet another well advanced microscope which uses electron as source rather than light – the electron microscope is also discussed in detail in this chapter.

2.1 Phase Contrast Microscope

Frits Zernike a Dutch Physicist invented the Phase Contrast Microscope and was awarded Nobel Prize in 1953. It is the microscope which allows the observation of living cell. This microscopy uses special optical components to exploit fine differences in the refractive indices of water and cytoplasmic components of living cells to produce contrast.

2.1.1 Principle

The phase contrast microscopy is based on the principle that small phase changes in the light rays, induced by differences in the thickness and refractive index of the different parts of an object, can be transformed into

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differences in brightness or light intensity. The phase changes are not detectable to human eye whereas the brightness or light intensity can be easily detected.

2.1.2 Optical Components of Phase Contrast Microscope (PCM)

The phase contrast microscope is similar to an ordinary compound microscope in its optical components. It possesses a light source, condenser system, objective lens system and ocular lens system (Figure 2.1).

A phase contrast microscope differs from bright field microscope in having,

i. Sub-stage annular diaphragm (phase condenser)

   An annular aperture in the diaphragm is placed in the focal plane of the sub-stage which controls the illumination of the object. This is located below the condenser of the microscope. This annular diaphragm helps to create a narrow, hollow cone of light to illuminate the object.

ii. Phase – plate (diffraction plate or phase retardation plate)

   This plate is located at the back focal plane of the objective lenses. The phase plate has two portions, in which one is coated with light retarding material (Magnesium fluoride) and the other portion devoid of light retarding material but can absorb light. This plate helps to reduce the phase of the incident light (Figure 2.2).

2.1.3 Working Mechanism of Phase Contrast Microscopy

The unstained cells cannot create contrast under the normal microscope. However, when the light passes through an unstained cell, it encounters regions in the cell with different refractive indexes and thickness. When light rays pass through an area of high refractive index, it deviates from its normal path and such light rays experience phase change or phase retardation (deviation). Light rays pass through the area of less refractive index

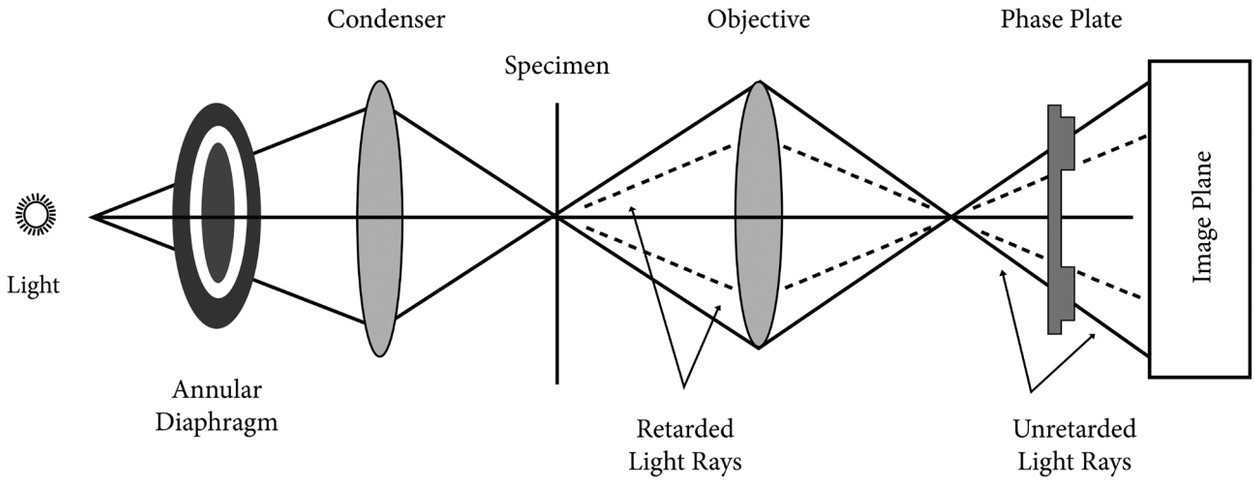

Figure 2.1: Components of phase contrast microscope

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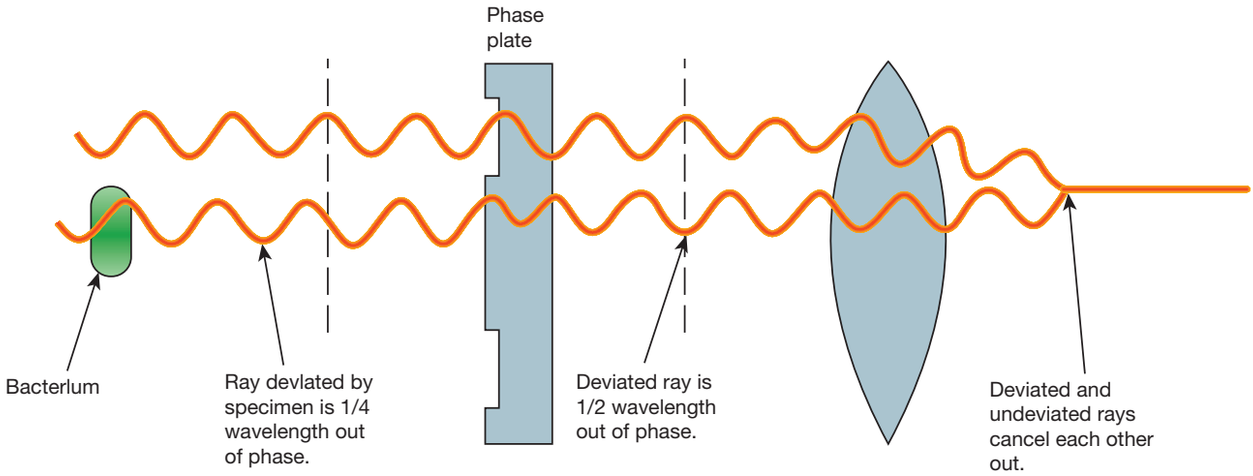

Figure 2.2: Production of contrast in phase contrast microscopy by phase plate

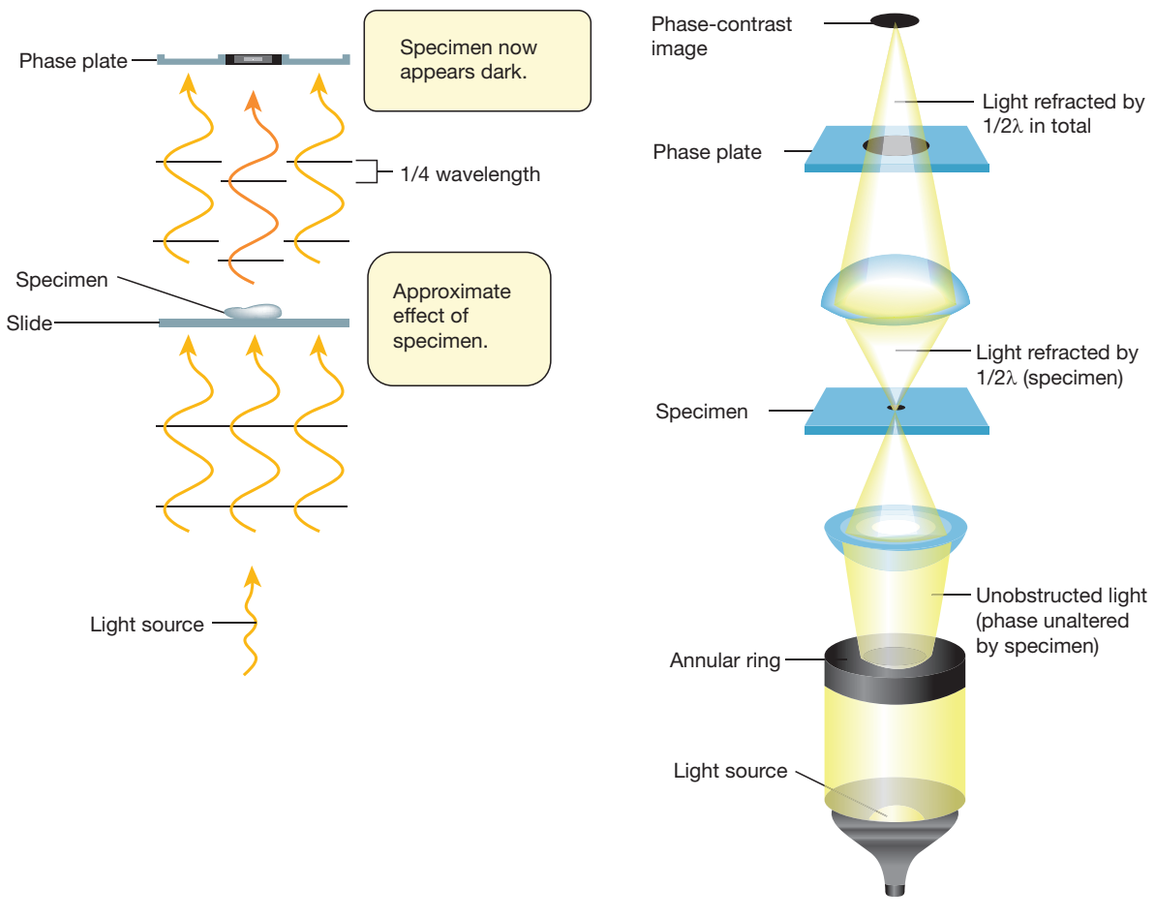

Figure 2.3: Optical path of Phase contrast microscopy

remain non-deviated (no phase change). Figure 2.3 shows the light path in phase contrast microscope.

HOTS

How does phase contrast microscope differ from Bright Field microscope?

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The difference in the phases between the retarded (deviated) and un-retarded (non-deviated) light rays is about ¼ of original wave length (i.e., λ/4). Human eyes cannot detect these minute changes in the phase of light. The phase contrast microscope has special devices such as annular diaphragm and phase plate, which convert these minute phase changes into brightness (amplitude) changes, so that a contrast difference can be created in the final image. This contrast difference can be easily detected by human eyes.

Infobits

Whenever light (or any wave in general) goes from one medium to another, some of the energy of the wave is "reflected" back through the first medium cut the same angle as the incident wave and some of the energy is refracted (bent). Through the second medium when light goes from a low refractive index medium to a high refractive index medium such as air to water the reflection undergoes a 180° phase change. Light waves that are in phase (that is, their peaks and valleys exactly coincide) reinforce one another and their total intensity increases.

Light waves that are out of phase by exactly one-half wavelength cancel each other and result in no intensity. That is darkness wavelengths that are out of phase by any amount will produce some degree of cancellation and result in brightness less than maximum, but more than darkness. Thus, contrast is provided by differences is light intensity that result from differences in refractive indices in parts of the specimen that put light waves indices in parts of the specimen that put light waves more or less out of phase. As a result, the specimen appears as various levels of darks against a bright background.

In phase contrast microscope, to get contrast, the diffracted waves have to be separated from the direct waves. This separation is achieved by the sub-stage annular diaphragm.

The annular diaphragm illuminates the specimen with a hollow cone of light. Some rays (direct rays) pass through the thinner region of the specimen and do not undergo any deviation and they directly enter into the objective lens. The light rays passing through the denser region of the specimen get regarded and they run with a delayed phase than the non-deviated rays. Both the deviated and non deviated light has to pass through the phase plate kept on the back focal plane of the objective to form the final image. The difference in phase (Wavelength) gives the contrast for clear visibility of the object. Figure 2.4 Microscopic image comparing phase and bright field microscopy.

2.1.4 Applications

- Phase contrast microscope enables the visualization of unstained living cells.
- It makes highly transparent objects more visible.

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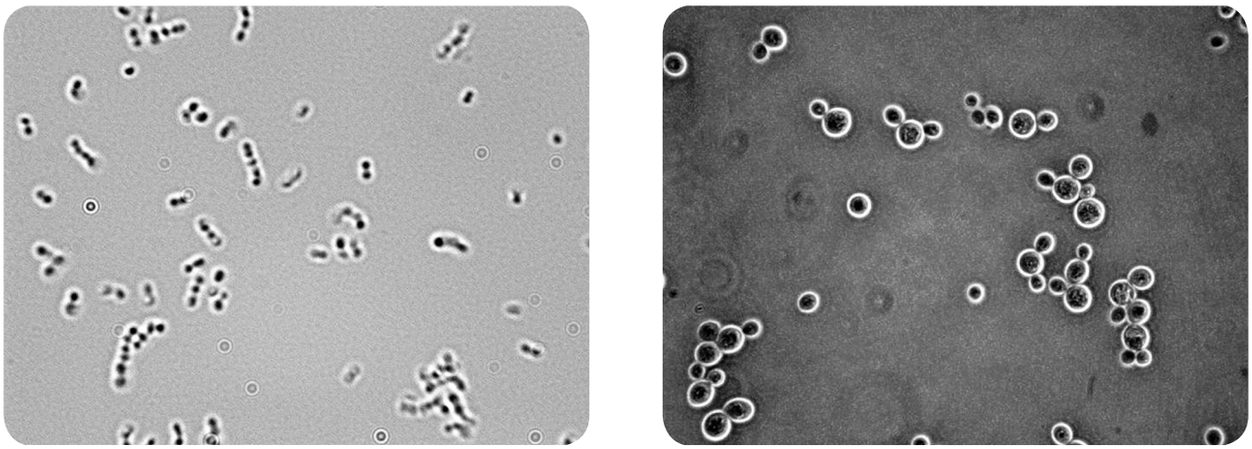

Figure 2.4: **(a)** *Saccharomyces* under bright field microscope **(b)** *Saccharomyces* under phase contrast microscope

- It is used to examine various intracellular components of living cells at relatively high resolution.
- It helps in studying cellular events such as cell division.
- It is used to visualize all types of cellular movements such as chromosomal and flagellar movements.

2.2 Fluorescence Microscope

Fluorescence microscope is a very powerful analytical tool that combines the magnifying properties of light microscope with visualization of fluorescence.

Fluorescence microscope is a type of light microscope which instead of utilizing visible light to illuminate specimens, uses a higher intensity (lower wavelength) light source that excites a fluorescent molecule called a fluorophore (also known as fluorochrome). Fluorescence is a phenomenon that takes place when the substances (fluorophore) absorbs light at a given wavelength and emits light at a higher wavelength. Thus, fluorescence microscopy combines the magnifying properties of the light microscope with fluorescence technology.

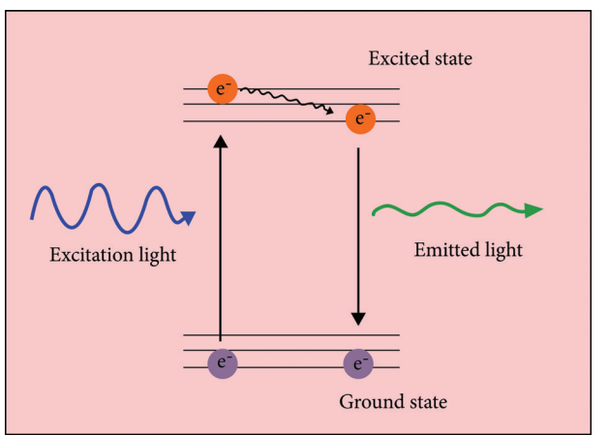

Figure 2.5: Principle of fluorescence

Infobits

British scientist Sir George G. Stokes first described fluorescence in 1852. He observed that the fluorophore emitted red light, when it was illuminated by ultraviolet excitation. Stokes noted that fluorescence emission always occurred at a longer wavelength than of the excitation light. This shift towards longer wavelength is known as stokes shift.

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The fluorophore absorbs photons leading to electrons moving to a higher energy state (excited state). When the electrons return to the ground state by losing energy, the fluorophore emits light of a longer wavelength (Figure 2.5). Three of the most common fluorophores used are Diamidino – phenylindole (DAPI) (emits blue), Fluorescein isothiocyanate (FITC) (emits green), and Texas Red (emits red).

2.2.1 Principle

Light source such as Xenon or Mercury Arc Lamp which provides light in a wide range of wavelength, from ultraviolet to the infrared is directed through an exciter filter (selects the excitation wavelength). This light is reflected toward the sample by a special mirror called a dichroic mirror, which is designed to reflect light only at the excitation wavelength. The reflected light passes through the objective where it is focused onto the fluorescent specimen. The emissions from the specimen are in turn, passed back up through the objective where magnification of the image occurs and through the dichroic mirror.

This light is filtered by the barrier filter, which selects for the emission wavelength and filters out contaminating light from the arc lamp or other sources that are reflected off from the microscope components. Finally, the filtered fluorescent emission is sent to a detector where the image can be digitized.

2.2.2 Components of Fluorescence Microscope

The main components of the fluorescent microscope resemble the traditional light microscope. However, the two main difference are the type of light source used and the use of the specialized filter elements (Figure 2.6).

Light source

Fluorescence microscopy requires a very powerful light source such as a Xenon or Mercury Arc Lamp. The light emitted from the Mercury Arc Lamp 10–100 times brighter than most incandescent lamps and provides light in a wide range of wavelengths from ultra-violet to the infrared. Lasers or high-power LEDs were mostly used for complex fluorescence microscopy techniques.

Filter elements

A typical fluorescence microscope consists of three filters: excitation, emission and the dichroic beam splitter.

Excitation filters: It is placed within the illumination path of a fluorescence microscope. Its purpose is to filter out all

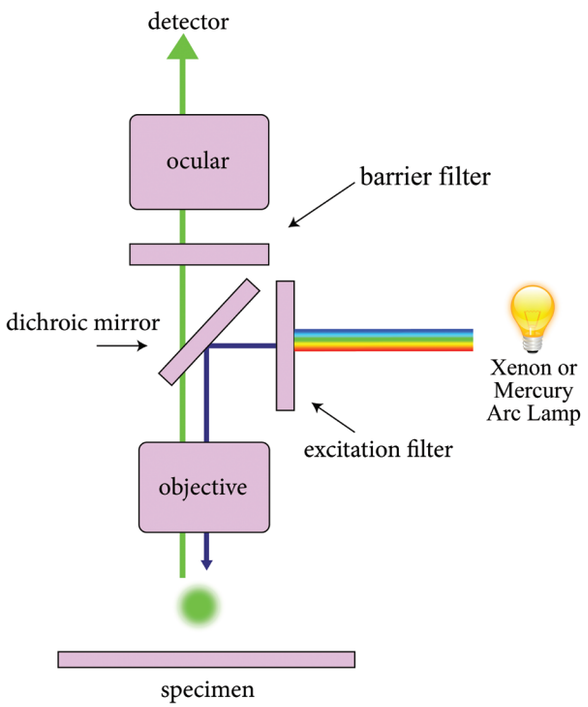

Figure 2.6: Components of fluorescence microscope

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wavelength of the light source, except for the excitation range of the fluorophore in the sample or specimen of interest.

Emission filters: The emission filter is placed within the imaging path of a fluorescence microscope. Its purpose is to filter out the entire excitation range and to transmit the emission range of the fluorophore in the specimen.

Dichroic filter or beam splitter: The dichroic filter or beam splitter is placed in between the excitation filter and emission filter, at 45° angle. Its purpose is to reflect the excitation wavelength towards the fluorophore in the specimen, and to transmit the emission wavelength towards the detector.



Fluorescence is called "cold light" because it does not come from a hot source like an incandescent light bulb.

2.2.3 Working Mechanism

The specimen to be observed are stained or labeled with a fluorescent dye and then illuminated with high intensity ultra violet light from mercury arc lamp. The light passes through the exciter filter that allows only blue light to pass through. Then the blue light reaches dichroic mirror and reflected downward to the specimen. The specimen labeled with fluorescent dye absorbs blue light (shorter wavelength) and emits green light. The emitted green light goes upward and passes through dichroic mirror, reflects back blue light and allows only green light to pass the objective lens, then it reaches barrier filter which allows only green light. The filtered fluorescent emission is sent to a detector where the image can be digitized Figure 2.7.

Infobits

The Two Types of Fluorescence Microscopes includes diascopic fluorescene and episcopic fluorescene.

Diascopic Fluorescence: K. Reichert and O. Heimstadt demonstrated a fluorescence microscope using auto fluorescent specimens in 1911.

This first type of fluorescence microscopy used transmitted light. Light from the illumination source first passes through an excitor filter and subsequently to the specimen through a dark field condenser. This eliminates most of the excitation light from the imaging side of the system.

Episcopic Fluorescence: In episcopic fluorescence microscopy, the excitation light comes from above the specimen through the objective lens. This is the most common form of fluorescence microscopy today. In this microscope, objective lens acts as both condenser and objective. Quartz objective lenses are required for deep ultraviolet excitation.

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2.2.4 Application

- Fluorescence microscope has become one of the most powerful techniques in biomedical research and clinical pathology.

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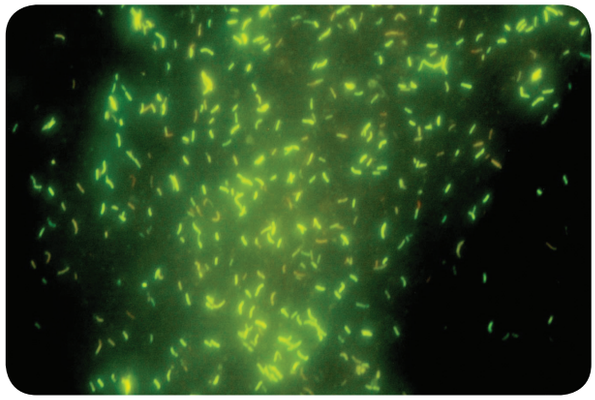

Figure 2.7: (a) Fluorescence microscope **(b)** Tubercule bacilli stained using Fluorescent dye observed under Fluorescence microscope

- Fluorescence microscope allows the use of multicolour staining, labeling of structures within cells, and the measurement of the physiological state of a cell.
- Fluorescence microscope helps in observing texture and structure of coal.
- To study porosity in ceramics, using a fluorescent dye.
- To identify the Mycobacterium tuberculosis.

2.3 Electron Microscope

Examining the ultra structure of cellular components such as nucleus, plasma membrane, mitochondria and others requires 10,000X plus magnification which was just not possible using Light Microscopes. This is achieved by Electron microscopes which have greater resolving power than light microscopes and can obtain higher magnifications.

In an electron microscope, a focused electron beam is used instead of light to examine objects. Electrons are considered as radiation with wavelength in the range 0.001–0.01 nm compared to 400–700 nm wavelength of visible light used in an optical microscope. Optical microscopes have a maximum magnification power of 1000X, and resolution of 0.2 µm compared to resolving power of the electron microscope that can reach 1,000,000 times and resolution of 0.2 nm. Hence, electron microscopes deliver a more detailed and clear image compared to optical microscopes. Table 2.1 differentiate electron microscope from light microscope.



In 1924, a French scientist, Dr. De Broglie, showed that an electron beam behaved like waves and had a wavelength much shorter than the sizes of molecules and atom when accelerated.

Types of Electron Microscopes

- Transmission electron microscopes (TEM)
- Scanning electron microscopes (SEM)
- Scanning transmission electron microscopes (STEM)

The electron microscope was invented in 1931 by two German scientists, Ernst Ruska and Max Knoll. Ernst Ruska later

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Table 2.1: Difference between light and electron microscope

S.No	Light microscope	Electron microscope
1.	Light is the illuminating source	The beam of electrons is the electron source
2.	Specimen preparation takes usually few minutes to hours. Live or dead specimen may be seen	Specimen preparation takes usually takes a few days. Only dead or dried specimen are seen
3.	Condenser, objective and eye piece lenses are made up of glasses	All lenses are electromagnetic
4.	Specimen is stained by coloured dyes	Specimen is coated with heavy metals in order to reflect electrons
5.	It has low resolving power (0.25µm to 0.3 µm). It has a magnification of 500X to 1500X	It has high resolving power (0.001µm), about 250 times higher than light microscope. It has a magnification more than 100,000X
6.	Vacuum is not required	Vacuum is essential for its operation
7.	Image is seen by eyes through ocular lens	Image is produced on fluorescent screen or photographic plate

received Nobel Prize for his work in 1986. The Transmission Electron Microscope (TEM) was the first type of Electron Microscope to be developed.

2.3.1 Principle

The fundamental principle of electron microscope is similar to light microscope. In electron microscope, a high velocity beam of electrons is used instead of photons. In the electron gun, electrons are emitted from the surface of the cathode and accelerated towards the anode by high voltage to form a high energy electron beam. All lenses in the electron microscope are electromagnetic. Charged electrons interact with the magnetic fields and magnetic force focuses an electron beam. The condenser lens system controls the beam diameter and convergence angles of the beam incident on a specimen. The image is formed either by using the transmitted beam or by using the diffracted beam. The image is magnified and focused onto an imaging device, such as a fluorescent screen, on a layer of photographic film, or to be detected by a sensor.

Sample Preparation

Preparation of specimens is the most complicated and skillful step in EM. The material to be studied under electron microscopy must be well preserved, fixed, completely dehydrated, ultrathin and impregnated with heavy metals that sharpen the difference among various organelles.

The material is preserved by fixation with glutaraldehyde and then with

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osmium tetroxide. The fixed material is dehydrated and then embedded in plastic (epoxy resin) and sectioned with the help of diamond or glass razor of ultra-microtome.

In TEM, sample sections are ultrathin about 50–100 nm thick. These sections are placed on a copper grid and exposed to electron dense materials like lead acetate, uranylacetate, phosphotungstate. In SEM, samples can be directly imaged by mounting them on an aluminum stub.

Electron–Sample Interactions

Interaction of electron beam with the sample results in different types of electrons: Elastic scattered electrons, Inelastic scattered electrons, secondary electrons and backscattered electrons. Almost all types of electron interactions can be used to retrieve information about the specimen. Depending on the kind of radiation or emitted electrons which are used for detection, different properties of the specimen such as topography, elemental composition can be concluded. Figure 2.8 shows the interaction of the electron beam with the specimen.

In Transmission electron microscope (TEM), a beam of electrons is transmitted through an ultrathin specimen, interacting with the specimen as it passes through. An image is formed from the interaction of the transmitted unscattered electrons through the specimen.

Secondary electrons are mainly used in scanning electron microscope (SEM) for imaging the surface topography of biological specimens. The interaction of electron beam with samples results in secondary electrons and backscattered electrons that are detected by standard SEM equipment.

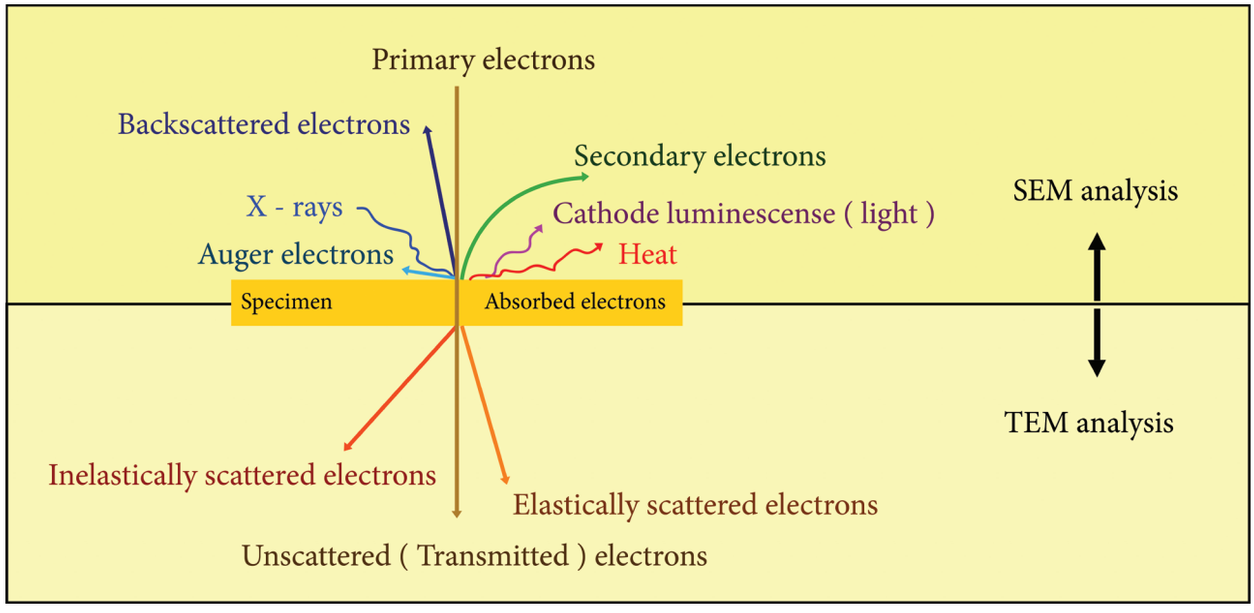

Figure 2.8: Interaction between electron beams with specimen

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2.3.2 Working Principle and Instrumentation of TEM

The optics of the TEM is similar to conventional transmission light microscope. A transmission electron microscope has the following components,

1. Electron gun
2. Condenser lens
3. Specimen stage
4. Objective lens and projector lens
5. Screen/photographic film/Charged Coupled Device (CCD) camera

Electron Gun consists of a tungsten filament or cathode that emits electrons on receiving high voltage electric current (50,000–100,000 volts). A high voltage between the electron source (cathode) and an anode plate is applied leading to an electrostatic field through which the electrons are accelerated.

The emitted electrons travel through vacuum in the microscope column. Vacuum is essential to prevent strong scattering of electrons by gases. Electromagnetic condenser lenses focus the electrons into a very thin beam. Electron beam then travels through the specimen and then through the electromagnetic objective lenses. In a TEM microscope, the sample is located in the middle of the column. At the bottom of the microscope, unscattered electrons hit the fluorescent screen giving image of specimen with its different parts displayed in varied darkness, according to their density. The image can be studied directly, photographed or digitally recorded. Figure 2.9 show the arrangement of components for transmission electron microscope.

Information that can be obtained using TEM include,

- Topography: surface features, texture
- Morphology: shape and size of the particles
- Crystallographic arrangement of atoms
- Composition: elements and the their relative amounts

2.3.3 Working Principle and Instrumentation of SEM

It is first built by Knoll in 1935. It is used to study the three dimensional images of the surfaces of cells, tissues or particles. The SEM allows viewing the surfaces of specimens without sectioning. The specimen is first fixed in liquid propane at-180°C and then dehydrated in alcohol at-70°C. The dried specimen is then coated with a thin film of heavy metal, such as platinum or gold, by evaporation in a vacuum provides a reflecting surface of electrons. In SEMs, samples are positioned at the bottom of the electron column and the scattered electrons (back-scattered or secondary) are captured by electron detectors.

In SEM, there are several electromagnetic lenses, including condenser lenses and one objective lens. Electromagnetic lenses are for electron probe formation, not for image formation directly, as in TEM. Two condenser lenses reduce the crossover diameter of the electron beam. The objective lens

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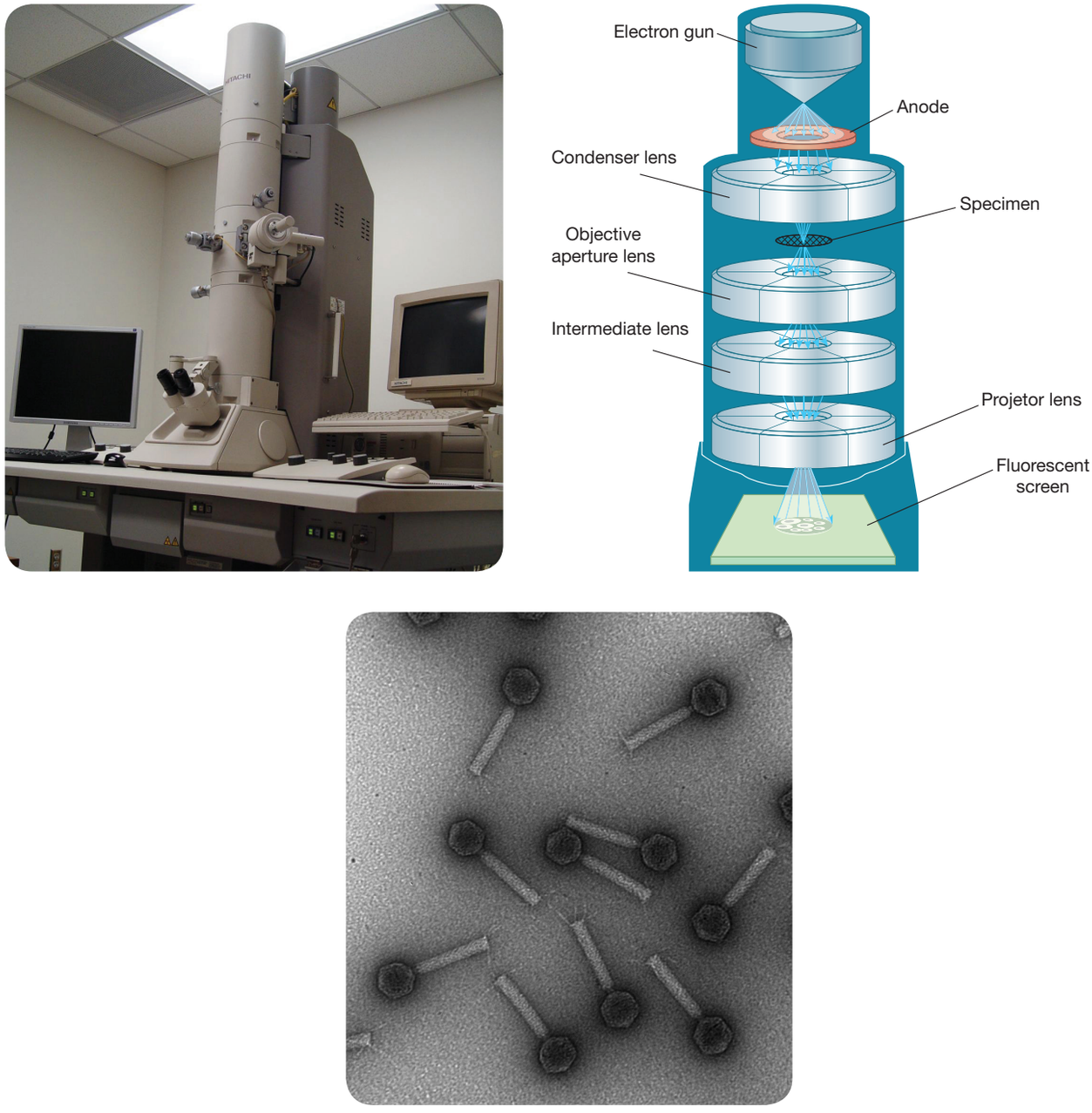

Figure 2.9: **(a)** Transmission microscope **(b)** Components of TEM **(c)** image under TEM

further reduces the cross-section of the electron beam and focuses the electron beam as probe on the specimen surface (Figure 2.10). Objective lens thus functions like a condenser. This is in contrast to TEM where objective lens does the magnification. Major difference between SEM and TEM are given in Table 2.2. SEMs are equipped with an energy dispersive spectrometer (EDS) detection system which is able to detect and display most of the X-ray spectrum. The detector normally consists of semiconducting silicon or germanium.

Scanning transmission electron microscopy (STEM) combines the principles of transmission electron microscopy and scanning electron microscopy and can be performed on either type of instrument. Like TEM,

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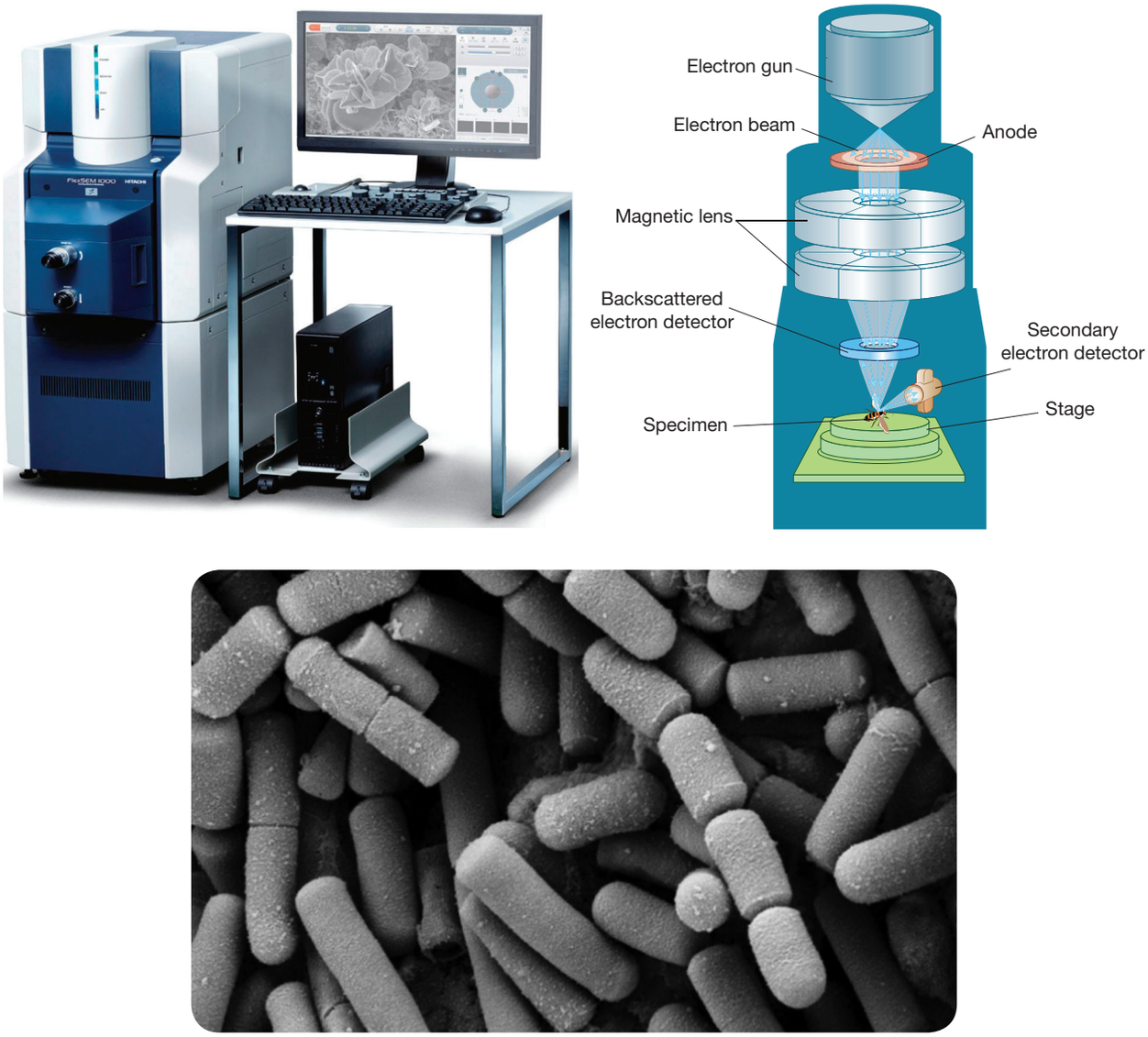

Figure 2.10: **(a)** Scanning electron microscope **(b)** Components of SEM **(c)** image under SEM



Foldscope – origami based paper microscope

A foldscope is an optical microscope that can be assembled from simple components, including a sheet of paper and a lens. It was developed by an Indian Manu Prakash. It consists of the following parts which are as follows: Lens stage, sample stage, panning guide, ramp, lens and magnetic cuppler. It has the magnification of 140X and maximum of 2400X

STEM requires very thin samples and the primary electron beam is transmitted by the sample. One of its principal advantages over TEM is in enabling the use of other of signals that cannot be spatially correlated in TEM, including secondary electrons, scattered beam electrons, characteristic X-rays, and electron energy loss.

Summary

Microscopes are specialized optical instruments designed to produce magnified visual or photographic images of objects or specimens that are too small to be seen with

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Table 2.2: Difference between SEM and TEM

S.no	Properties	SEM	TEM
1.	Types of electrons	It is based on scattered electrons that are emitted from the surface of a specimen	It is based on transmitted electrons
2.	Sample preparation	Sample can be of any thickness and is coated with a thin layer of a heavy metal such as gold or palladium and mounted on an aluminum slab	Laborious sample preparation is required. The sample has to be cut into thin sections so as to allow electrons to pass through it and are supported on TEM grids
3.	Resolution	The resolution is up to 20nm	TEM has much higher resolution than SEM. It can resolve objects as close as 1nm
4.	Magnification	The magnifying power of SEM is up to 100,000X	The magnifying power of TEM is up to 5,000,000X
5.	Image formation	SEM provides a 3 dimensional image. Secondary or back scattered electrons are captured, detected and displayed on computer screen	TEM provides a 2 dimensional image. Transmitted electrons hit a fluorescent screen giving rise to a shadow image. The image can be studied directly by the operator or photographed with a camera
6.	Application	SEM is used to study the topography and atomic composition of specimens	TEM is used to study the interior of cells, the structure of protein molecule, the organization of molecules in viruses and cytoskeletal filaments and the arrangement of protein molecules in cell membranes

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naked eye. Frits Zernike a Dutch Physicist invented the Phase Contrast Microscope and was awarded Nobel Prize in 1953. It is the first microscopic method which allows the observation of living cell. The image of the aperture is formed at the rear focal plane of the objective. In this plane there is a phase shifting element or phase plate. Deviated rays from object form structures due to different refractive index. Light waves that are in phase (that is, their peaks and valleys exactly coincide) reinforce one another and their total intensity increases. Light waves that are out of phase by exactly one-half wavelength cancel each other and result in no intensity. Fluorescence microscopy is a very powerful analytical tool that combines the magnifying properties of light microscopy with visualization of fluorescence. Examining the ultra structure of cellular components such as nucleus, plasma membrane, mitochondria and others requires 10,000x plus magnification which was just not possible using Light Microscopes. It is first built by Knoll in 1935. It is used to study the three dimensional images of the surfaces of cells, tissues or particles. The SEM allows viewing the surfaces of specimens without sectioning. The specimen is first fixed in liquid propane at-180°C and then dehydrated in alcohol at-70°C. **Scanning transmission electron microscopy (STEM)** combines the principles of transmission electron microscopy and scanning electron microscopy and can be performed on either type of instrument. One of its principal advantages over TEM is in enabling the use of other of signals that cannot be spatially correlated in TEM, including secondary electrons, scattered beam electrons, characteristic X-rays, and electron energy loss.

Evaluation

Multiple choice questions

C526J

1. Who invented phase contrast microscope?
   a. Robert Koch
   b. Frits Zernike
   c. George Strokes
   d. Alexander Fleming
2. The component that makes the difference between phase contrast microscope and Bright Field microscope is
   a. Objective b. Phase plate
   c. Condenser d. Occular
3. Tumor cells can be diagnosed by
   a. PCM
   b. BFM
   c. Light Microscope
   d. Electron Microscope
4. In electron microscope light source is
   a. Electric light b. Electron Beam
   c. Sun light d. Fluorescent light
5. What is the medium used in electron microscope?
   a. Air b. Water
   c. Vacuum d. Light
6. _____ are mainly used in scanning electron microscope
   a. Transmitted electrons
   b. Primary electrons
   c. Secondary electrons
   d. Elastically scattered electrons
7. Lenses used in TEM
   a. Objective lens
   b. Electromagnetic lens
   c. Glass lenses
   d. Condensor lenses

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8. _____ is used to illuminate specimen in fluorescent microscope

   a. Mercury arc lamp b. Sunlight

   c. Tungsten lamp d. LED

9. Which among the following help us in getting a 3-D picture of specimen?

   a. TEM

   b. SEM

   c. Compound microscope

   d. Simple microscope

10. Dye used to stain specimen in fluroscent microscopic view

    a. Acridine dye b. Rezazurin

    c. Methylene Blue d. Flurochrome

Answer the following

1. Principle of PCM
2. Write about the special features of TEM
3. List out the dyes used for fluorescence microscopy
4. What are the different types of filters used in fluorescence microscopy
5. Define flurochromes.
6. Write the functions of Barrier filter
7. Write the components of PCM
8. What are the Application of EM
9. Write about TEM – Introduction and Principle
10. Write a brief account on the application of fluorescent microscopy
11. Explain in detail about the principle construction and working of PCM.
12. Explain the Principle, Components and Mechanism of EM.

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Chapter 3

Control of Microorganisms by Chemical Methods

Learning Objectives

After studying this chapter the students will be able to,

- *Defines the terms disinfectants, antiseptics and antibiotics*
- *Describe major groups of antimicrobial chemical agents and uses of disinfectants.*
- *Describe the factors related to effective disinfectants.*
- *Discuss the classification of antibiotics and their mode of action.*
- *Know the procedure used in antimicrobial susceptibility testing in clinical laboratory.*
- *Know the resistance mechanisms developed by pathogens against antibiotic or chemotherapy drugs.*

Chapter Outline

3.1 Disinfectants, Antiseptics and Antibiotics

3.2 Factors Influencing the Antimicrobial Activity of Chemical Agents

3.3 Mode of Action of Chemical Agents

3.4 Major Groups of Antimicrobial Chemical Agents

3.5 Evaluation of Antimicrobial Chemical Agents

3.6 Antibiotics

3.7 Antimicrobial Susceptibility Testing

3.8 Drugs Resistance Mechanisms

WYLDH

Control of microorganisms is essential in order to prevent the transmission of diseases, infection, spoilage and to prevent unwanted microbial contamination. Microorganisms are controlled by means of physical agents and chemical agent. In 11th standard, we learnt different physical methods of sterilization. Control by chemical agents refers to the use of disinfectants, antiseptics, antibiotics and chemotherapeutic antimicrobial chemicals. This chapter describes various chemical agents, their mode of action, and their evaluation.

Use of chemicals to sterilize objects and to control microbial pathogen from causing diseases has been in practice since centuries. A large number of chemicals are now available for this purpose. Commercial products which incorporate these chemicals are used in a variety of conditions and they usually differ in their mode of action. No single chemical agent is best for any and all purposes. Hence several classes of chemicals have been identified and new compounds are developed that possess destructive properties in terms of their suitability for practical application.

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3.1 Disinfectants, Antiseptics and Antibiotics

Disinfection is the elimination of microorganisms from inanimate objects or surfaces. The term disinfectant is used for an agent used to disinfect inanimate objects or surfaces but is generally toxic to use on human tissues. Antiseptic refers to an agent that kills or inhibits growth of microorganisms but is safe to use on human tissues.

Antibiotics produced by microorganisms which kill or inhibit the growth of other microbes.

Following Table gives few examples of antimicrobial chemical agents that destroy unwanted microorganisms.

Disinfectants	Antiseptics	Antibiotics
Chlorine, Copper	Phenol, Tincture Iodine	Penicillin, Streptomycin

Basic terms used in chemical control of microorganism are mentioned in Table 3.1 and Table 3.2 Describes the difference between Bactericidal and Bacteriostatic agents.

Table 3.1: Basic terms used in Chemical sterilization

Term	Meaning
Disinfection	The selective elimination of certain undesirable microorganisms to prevent their transmission directed against their metabolism or structure; applies to the use directly on inanimate objects.
Antisepsis	Prevention of the growth or activity of microorganisms by inhibition or killing; applies to the use of chemicals on living tissue
-cide	Suffix used to denote agents, usually chemical, that kill. Commonly used terms are bactericide, fungicide, virucide, and algicide. The term germicide is used if the agents kill pathogens but not necessarily spores. An agent that kills bacterial spores is a sporicide.
-static	Suffix used to denote agents, usually chemical, that prevents growth but do not necessarily kill the organism or bacterial spores. Commonly used terms include bacteriostatic and fungistatic.



Term	Action	Examples
Algicide	Agent that kills algae	Copper sulfate
Bactericide	Agent that kills bacteria	Chlorohexidine, ethanol
Biocide	Agent that kills living organisms	Hypochlorite (bleach)
Fungicide	Agent that kills fungi	Ethanol
Germicide	Chemical agent that specifically kills pathogenic microorganisms	Formaldehyde, silver, mercury
Sporicide	Agent that kills bacterial endospores	Glutaraldehyde
Virucide	Inactivates viruses so that they lose the ability to replicate	Cationic detergents (quaternary ammonium salts of acetates, chlorides)

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Table 3.2: Difference between Bactericidal and Bacteriostatic

Bactericidal	Bacteriostatic
Bactericidal refers to agents that kill bacteria	Bacteriostatic refers to agents that prevent the growth of bacteria
Action is irreversible	Action is reversible
Inhibit the cell wall formation of bacteria	Inhibit DNA replication and protein synthesis of bacteria
Do not work with the immune system of the host	Work with the immune system of the host to prevent the growth and reproduction of bacteria
Minimal Bactericidal Concentration (MBC) refers to the concentration of the drug required to kill 99.99% of the bacterial population	Minimal Inhibitory Concentration (MIC) is the minimum drug concentration which inhibits the bacterial growth
Examples include betalactam antibiotics, cephalosporins, and vancomycin	Examples include tetracyclines, spectinomycin, chloramphenicol, sulfonamides, etc.

3.2 Factors Influencing the Antimicrobial Activity of Chemical Agents

The following factors will affects the activity of a disinfectant or antiseptic and these should be borne in mind during use.

a. The Concentration and kind of a chemical agent used

The higher the concentration of the germicide the greater will be the rate of killing. This is particularly important with the phenolic group of compounds, whose activity falls off very rapidly with dilution.

b. Time of exposure to the agent

In general germicidal activity is increased with time and a sufficient exposure is imperative for efficient disinfection.

c. Temperature at which the agent is used

An increase of temperature will also raise the rate of killing.

d. Presence of Organic matter

Most germicides are reduced in activity by the presence of organic matter and particularly by the presence of proteins such as those in body fluids.

e. Number of organisms present

The larger the number of organisms, the greater will be the time required for disinfection.

f. The kinds of microorganisms present - Presence of spores

Spores are exceptionally resistant to the great majority of disinfection..

3.3 Mode of Action of Chemical Agents

Chemical agents act on microorganisms by:

- They may damage the lipids and proteins of the cytoplasmic membrane of microorganisms.
- They may denature microbial enzymes and other proteins usually by disrupting the hydrogen and disulfide bonds that give the protein its 3-D shape. This blocks metabolism function.

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3.4 Major Groups of Antimicrobial Chemical Agents

A large number of chemical agents are in common use. Some of the more common groups are listed below.

1. Phenol and Phenolics

Phenol was the first widely used chemical antiseptic and disinfectant. In 1867, Joseph Lister employed carbolic spray to reduce the risk of infection in surgical theatres. Phenol derivatives called phenolics contain altered molecules of phenol useful as antiseptics and disinfectants. The phenolics damage cell membranes and inactivate enzymes of microorganisms, while denaturing the proteins. Phenolics includes cresols, such as Lysol, as well as several bisphenols, such as hexachlorophene. Today phenol and phenolics such as cresol, xylenol, and orthophenyl phenol are used as disinfectants in laboratories and hospitals.

The commercial disinfectant Lysol is made of mixture of phenolics. Phenolics are tuberculocidal, effective in the presence of organic material, and remain active on surfaces long after application. However, they have a disagreeable odour and can cause skin irritation.

Hexachlorophene is one of the most popular antiseptics because it persists on the skin once applied and reduces skin bacteria for a long period. It is mainly used in soaps and creams. It is an ingredient of various dermatological preparation used for skin disorders.

2. Alcohols

Alcohols are among the most widely used disinfectant and antiseptic. They are bactericidal and fungicidal but not sporicidal. Alcohols can destroy the lipid component of enveloped viruses. The two most popular alcoholic germicides are ethanol and isopropanol. They act by denaturing proteins and dissolving membrane lipids. The recommended optimum concentration of ethanol is 70%, but concentration between 60% and 95% are employed to kill germs as well. Thermometers and small instruments are disinfected by immersing in alcohol for 10 to 20 minutes.

3. Halogens

Halogen compounds are broad spectrum compounds that are considered low toxicity, low cost and easy to use. Among the halogens, iodine and chlorine are important antimicrobial agents. Small quantities of drinking water can be disinfected with halazone tablets.

a. Iodine

Iodine compound are broad spectrum and considered effective for a variety of bacteria, mycobacteria, fungi and viruses. The alcoholic tincture of iodine is highly active against gram positive organisms and so is used as a skin antiseptic. It stains the skin. Iodine combines with microbial protein and inhibits their function.

b. Chloride

Chloride is also used as a gas to maintain a low microbial count in drinking water. Chlorine together with ammonia called chloramines are used to sanitize glasswall and eating utensils. Sodium hypochlorite (NaOCl) is one of the most widely used chlorine containing disinfectants. Low concentrations (2-500ppm) are active against vegetative bacteria, fungi and most

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viruses. Rapid sporicidal action can be obtained around 2500ppm, however this concentration is very corrosive so should be limited in its use. High concentrations are also irritating to the mucous membranes, eyes and skin. Chlorine compounds are rapidly inactivated by light and some metals so fresh solutions should always be used. Hypochlorites should never be mixed with acids or ammonia as this will result in the release of toxic chlorine gas.

c. Iodophores

The combinations of iodine and organic molecules are called Iodophores. They include wescodine, betadine and previdone. These iodophore contains surface active agents. They cause less irritation to the skin than free Iodine and do not stain. They are used for cleaning wounds and as a general purpose laboratory disinfectant for discarded jars.

4. Heavy Metals

For many years the ions of heavy metals such as mercury, silver, arsenic, zinc, and copper were used as germicides. More recently these have been superseded by other less toxic and more effective germicides. Many heavy metals are more bacteriostatic than bactericidal. There are a few exceptions. 1% solution of Silver nitrate is often applied to the eyes of infants to prevent ophthalmic gonorrhea. Silver sulfadiazine is used on burns. Copper sulfate is an effective algicide used in lake and swimming pools to retard the growth of algae.



In many hospitals, Erythromycin is used instead of Silver nitrate because it is effective against *Chlamydia* as well as *Neisseria*.

Heavy metals combine with sulfhydryl (SH) groups of proteins and inactivate them. High concentration of metallic salts, particularly those of mercury, silver and copper coagulate cellular proteins that results in damage or death of the microbial cell. The most toxic heavy metals are the mercury, silver, and copper.

5. Quaternary Ammonium Compounds (Quats)

The most widely used surface active agents are the cationic detergents, especially the quaternary ammonium compounds (quats).

Quaternary Ammonium compounds are strongly bactericidal against Gram positive bacteria and less active against gram negative bacteria. These include agents such as cetrimide, bromide and benzalkonium chloride. Their antibacterial activity is antagonized by soaps and certain organisms like *Pseudomonas*. They are useful for washing cutlery in catering industry and for cleaning wounds in hospitals. Savlon, a popular antiseptic, is a mixture of cetrimide and chlorohexidine and is active against Gram negative bacteria. They are used as skin disinfectants and as a preservative of ophthalmic solution.

The combined properties of germicidal activity and low toxicity, high solubility in water, stability in solution and non-corrosiveness have resulted in many applications of quaterneries as disinfectants and sanitizing agents.

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Quats are also fungicidal, amoebicidal, and virucidal against enveloped viruses. They do not kill endospores or mycobacteria.

Infobits

If you mouthwash bottle fills with foam when shaken, the mouthwash probably contains a quat.

6. Aldehydes

Aldehydes are highly effective, broad spectrum disinfectant. The most which typically achieve its anitimicrobial action by denaturing proteins and disrupting nucleic acids. Commonly used aldehydes are formaldehyde and glutaraldehyde. Formaldehyde is usually dissolved in water or alcohol before use. Formaldehyde is used as a surface disinfectat and a fumigant and has been used to decontaminate in animate objects. A concentration of 2% glutaraldehyde is an effective disinfectant. It is less irritating than formaldehyde and is used to disinfect hospital and laboratory equipments. Glutaraldehyde usually disinfects objects about 10 minutes but may require as long as 12 hours to destroy all spores.

Infobits

Disinfection of Rooms

Fumigation with gaseous disinfectants was at one time commonly performed after a room had been occupied by a patient with an infections disease. Sulphur-di-oxide, generated by burning sulphur was the popular agent for this purpose but it is effective only if the relative humidity is 60 percent or more.

These are highly reactive molecules that combine with nucleic acids and proteins and inactivate them. They disrupt the function of cell organelles and kill the cells probably by cross linking and alkylating the molecules. These are sporicidal and can be used as chemical sterilants.

7. Gaseous Sterilization

Gaseous disinfectants (alkylating agents) are used for the sterilization or disinfection of hospital equipment that is bulky or heat labile. The most widely used gases are ethylene oxide, formaldehyde and β Propiolactone.

Ethylene oxide (EtO)

Ethylene oxide has a boiling point of 10.8°C. It is highly inflammable and explosive in pure form, but is safe to handle when mixed with Carbon dioxide. It is powerful in the killing of all bacteria, including tubercule bacilli and spores. It is an effective sterilizing agent because it rapidly penetrates packing materials, even plastic wrappers. To be potent, however, the humidity and temperature must be carefully controlled within narrow limits.

It is highly toxic on contact with the skin or mucous membrane. Materials that have been sterilized with ethylene oxide must be set aside in detoxification chambers for a few days to allow the gases to dissipate. It is frequently used to sterilize heart lung machines and plastic items like catheters.

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Recently vapour – phase hydrogen peroxide has been used to decontaminate biological safety cabinets.

Formaldehyde

It is highly bactericidal. Formaldehyde is used as 40% formalin with humidity at around 50%. It causes irritation. It is used occasionally to fumigate rooms and disinfect respirators.

Betapropiolactone (BPL)

This is occasionally employed as a sterilizing gas in the liquid form. It has been used to sterilize vaccines, tissue grafts, surgical instrument and enzyme as a sterilants of blood plasma, water, milk and as a vapour – phase disinfectant in enclosed spaces, short-term inhalation exposure to betapropiolactone causes severe irritation of the eyes, nose, throat and respiratory tract in humans.

BPL decomposes to an inactive form after several hours and is therefore not difficult to eliminate. It destroys microorganisms more readily than ethylene oxide but does not penetrate materials well and may be carcinogenic. For these reasons, BPL has not been used as extensively as EtO.

3.5 Evaluation of Antimicrobial Chemical Agents

Testing of antimicrobial agents is a complex process regulated by two different federal agencies.

The U.S. Environmental Protection Agency regulates disinfectants, where as agents used on humans and animals are under the control of the Food and Drug Administration.

Testing of antimicrobial agents often begins with an initial screening test to see if they are effective and at what concentrations.

Laboratory techniques for the evaluation of antimicrobial chemical agents are conducted by one of the following three general procedures. In each procedure, the chemical agent is tested against a specific microorganisms referred to as the test organism.

Agar Plate Method

A plate of agar medium is inoculated with the test organism and the chemical agent is placed on the surface of the medium. The chemical solution is first impregnated in absorbent papers or confined by a hollow cylinder placed on the agar surface. Following incubation, the plate is observed for a zone of inhibition around the chemical agent. This is particularly suitable for semisolid preparations.

Tube Dilution Methods

Appropriately diluted water soluble liquid substances are dispensed into sterile test tubes and are inoculated with a measured amount of the test organism. At specified intervals, a transfer is made from this tube into tubes of sterile media that are then incubated and observed for the appearance of growth. It is necessary in this type of procedure to ascertain whether the inhibitory action is bactericidal and not bacteriostatic. This approach can also be used to determine the number of organisms killed per unit time by performing a plate count on samples taken at appropriate intervals.

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Phenol Coefficient Test

Phenol coefficient is a measure of the bactericidal activity of a chemical compound in relation to phenol. Phenol coefficient is calculated by dividing the concentration of test disinfectant at which it kills the organism in 10 minutes and not in 5 minutes under the same conditions. This method is used for evaluating the efficiency of water-miscible disinfectants.

Series of 10 test tubes with 2ml of distilled water is taken (Figure 3.1). Phenol is added to first test tube and dilution is made by transferring 1ml to next tube up to 5 dilutions. Similarly commercial disinfectant is also diluted. Pure culture of test organisms, such as *Staphylococcus aureus* or *Salmonella typhi*, is added to test tubes. Subcultures from these tubes incubated at 37°C for 48 hours are examined for the presence or absence of growth at intervals of 5, 10 and 15 minutes. The highest dilution that kills the bacteria after 10 minutes, but not after 5 minutes is used to calculate the phenol coefficient (Table 3.3),

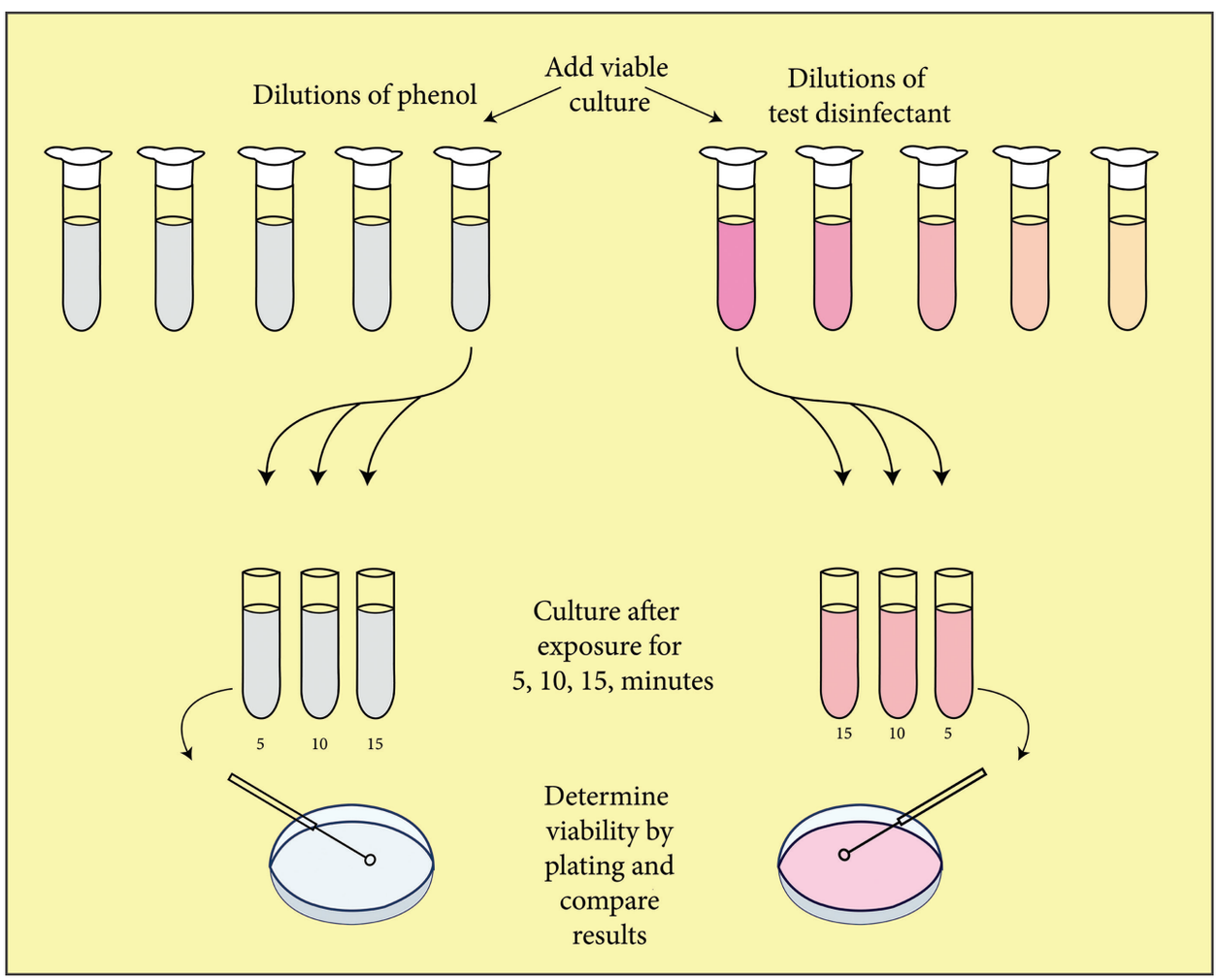

Figure 3.1: Phenol coefficient test

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Table 3.3: Illustration of phenol coefficient determination

Chemical Agent and Dilution	Presence of Growth in Subcultures (minutes)		
	5	10	15
Phenol			
1:80	-	-	-
1:90*	+	-	-
1:100	+	+	-
Test Chemical			
1:400	-	-	-
1:450+	+	-	-
1:500	+	+	-

Phenol dilution of 1:90 showed growth at 5 minutes but no growth at 10 minutes Test Chemical dilution of 1:450 showed growth at 5 minutes but no growth at 10 minutes phenol coefficient of test chemical as 450/90=5.

3.6 Antibiotics

The term 'antibiotic' was derived from 'antibiosis' which refers to the suppression of microorganisms due to secretion of toxic or inhibitory compounds by other microorganisms. Although antibiosis has been observed by many scientific workers fairly frequently towards the end of the nineteenth century, it was not until the discovery and development of Penicillin that a truly wide ranging search for antibiotics was initiated.



Antibiotics are not effective against viral infections such as the common cold.

Historical Development

The first chemotherapeutic agent, discovered by Paul Ehrlich, was Salvarsan, used to treat syphilis.

Alexander Fleming discovered the first antibiotic, penicillin, in 1929; its first clinical trails were done in 1940.

Antibiotics are produced by species of Streptomyces, Bacillus, Penicillium and Cephalosporium.

Infobits

1904 Ehrlich found that the dye trypan red was active against the trypanosome that causes African sleeping sickness and could be used therapeutically.

Drugs such as the sulfonamides are sometimes called antibiotics although they are synthetic chemotherapeutic agents which are not synthesized using microbes.

Classification of Antibiotics

The antibiotics are usually classified on the basis of:

- Target group of microorganisms
- Antimicrobial spectrum and
- Mode of action

❖ Classification based on target group of microorganisms

Based on the target group, the antibiotics can be classified as antibacterial, antifungal and antiviral.

❖ Classification based on Antimicrobial spectrum

Antimicrobial spectrum or antibiotic spectrum refers to the range of

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effectiveness of antibiotics on different kind of microorganisms, i.e. the range of different kind of microorganisms that can be inhibited, killed, or lysed by a particular type of antibiotic.

The susceptibility of microorganisms to individual antibiotic varies significantly and on account of this, the antibiotics can be classified in two groups as,

Broad – spectrum antibiotics

These attack different kinds of microbial pathogens and therefore find wider medical use. Antibacterial antibiotics of broad – spectrum are effective against both Gram positive and Gram negative bacteria. They also attack pathogens belonging to *Mycobacteria*, *Rickettsia*, and *Chlamydia*. Similarly, broad – spectrum antifungal antibiotics attack different type of fungal pathogens.

Narrow – spectrum antibiotics

Narrow – spectrum antibiotics are categorized as those that are effective only against a limited variety of microbial pathogens. These antibiotics are quite valuable for the control of microbial pathogens that fail to respond to other antibiotics. For example, vancomycin is a narrow spectrum glycopeptide. It is an effective bactericidal agent for gram – positive penicillin resistant bacterial pathogens belonging to genera *Staphylococcus, Bacillus,* and *Clostridium*.

3.6.1 Mode of Action of Antibiotics

The mode of action of antibiotics varies as they damage pathogens in several ways (Flowchart 3.1). Some of the important actions of therapeutic drugs in microbial pathogens are as follows.

Cell wall synthesis, Protein synthesis, Nucleic acid synthesis, Cell membrane disruption and Metabolic pathways blockage.

1. Inhibition of Cell Wall Synthesis

The most selective therapeutic antibiotics are those that interfere with the synthesis of bacterial cell walls. These drugs posses a high therapeutic index because bacterial cell walls have a unique structure which is not found in eukaryotic cells. The important cell wall attacking drugs are Penicillin, Cephalosporin, Ampicillin, Methicillin and Vancomycin.

2. Inhibition of Protein Synthesis

Many therapeutic antibiotics discriminate between prokaryotic and eukaryotic ribosomes and inhibit protein synthesis. The therapeutic index of these drugs is fairly high, but not as favourable as that of cell wall synthesis inhibitors. Several of these drugs are medically useful and effective research tools because they block individual steps in protein synthesis. Some therapeutic drugs bind to 30S while others attach to 50S ribosomal subunits. Example Streptomycin, Chloramphenicol, Tetracyclin and Erythromycin



A chemotherapeutic agent destroys or inhibit the intracellular parasite by penetrating the cells and tissues of the host in effective concentrations.

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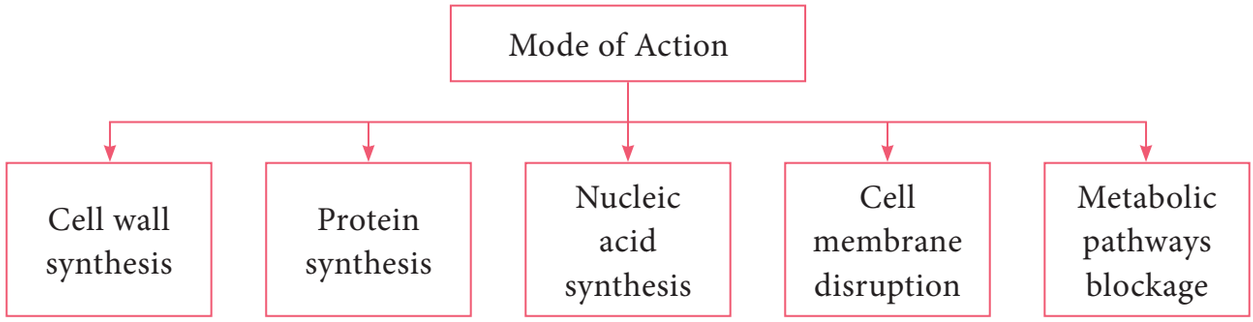

Flowchart 3.1: Mode of action of antibiotics

3. Inhibition of Nucleic Acid Synthesis

Some antimicrobial drugs or antibiotics inhibit nucleic acid synthesis. These are not selectively toxic as other drugs. This is due to the fact that prokaryotic and eukaryotic nucleic acid synthesis mechanisms do not vary greatly. Example Quinolones, Novobiocin, Actinomycin and Rifampin

4. Disruption of Cell Membrane

There are some antimicrobial drugs or antibiotics that act as cell membrane disorganizing agents. Polymyxins are such drugs of clinical importance.

E.g. Polymyxin B and Polymyxin E (colistin)

5. Blocking Metabolic Pathways

Some therapeutic drugs act as antimetabolites and block the functioning of metabolic pathways. They competitively inhibit the key enzymes in the metabolic pathway. Example Sulfonamides,

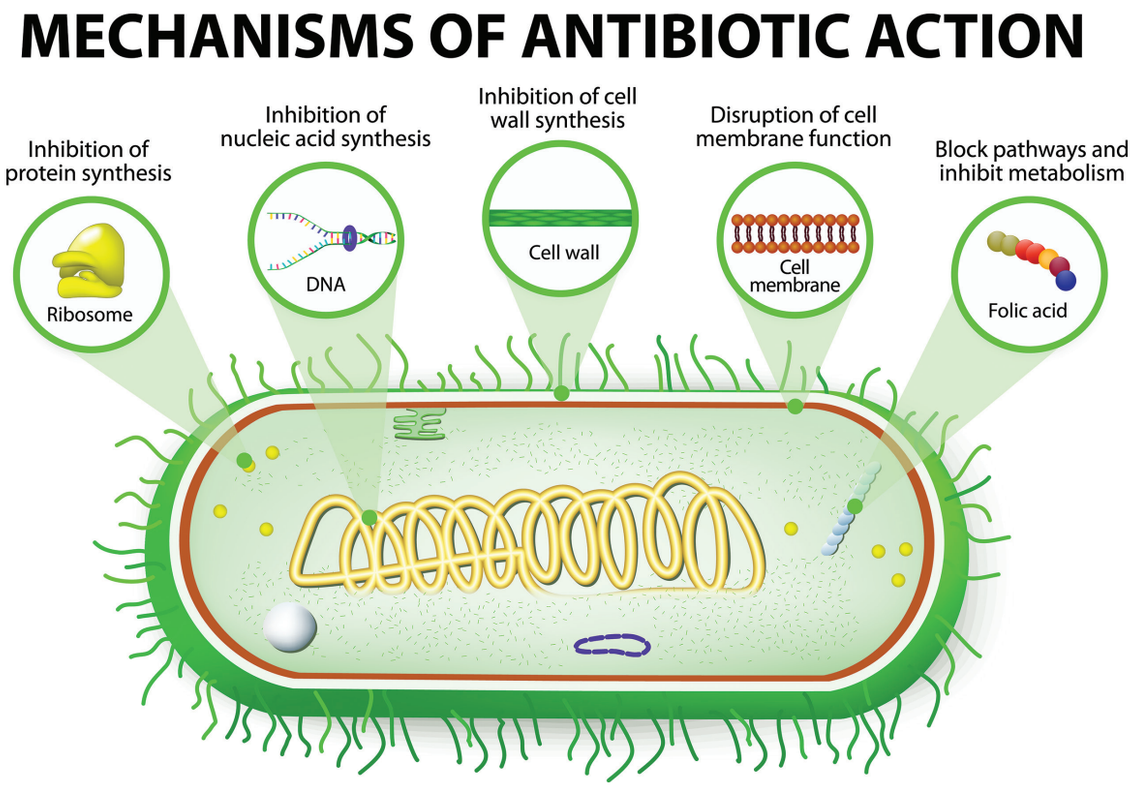

Figure 3.2: Mechanism of Antibiotic action

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Trimethoprim, Dapsone and Isoniazid (Figure 3.2).

3.7 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility tests are used to determine the type and quantity of antimicrobial agents used in chemotherapy. One of the most important functions of a clinical laboratory is to determine the antimicrobial susceptibility. Antimicrobial susceptibility of pathogens refers to the limitation of pathogens to grow in the presence of effective antibiotics. There are two methods that can be used to determine the susceptibility of a potential pathogen to antimicrobial agents. They are:

- Disk diffusion method
- Tube dilution method

Disc Diffusion Method (Kirby – Bauer Test)

William Kirby and Alfred Bauer, in 1966 first introduced the principle of measuring zones of inhibition around antibiotic discs to determine antimicrobial agent susceptibilities. It is a rapid, convenient method to determine the susceptibilities of microorganisms to antimicrobial agents and a most common procedure used in susceptibility testing in clinical laboratory.

Filter paper discs containing known concentrations of antimicrobial agents are placed onto the surface of an agar plate (Muller – Hinton agar medium) inoculated with the test bacterium (Figure 3.3). The plate is incubated for 16 to 18 hours, and the zones of inhibition are read around each paper disc. During the incubation periods, the antimicrobial agent diffuses through the agar, and a concentration gradient of agent is established. At some point in this gradient, growth of the susceptible bacteria is suppressed, and no growth is observed within a circular zone around disc. The size of a zone of inhibition must be compared to a standard Table for that particular drug before accurate comparisons can be made. Thus, enabling to classify pathogens as susceptible (S), intermediate or resistant (R) to a drug. The procedure is highly regulated and controlled by the clinical and laboratory standards institute (CLSI) and must be accompanied by a rigorous quality assurance program including performance by certified and/or licensed personnel when the results are to be reported in clinical settings.

Minimal Inhibitory Concentration (MIC) Test

The potency of an effective antimicrobial agent is expressed in terms of **minimal inhibitory concentration (MIC)**. It is the minimum concentration of drug that will **inhibit** the growth of pathogen. The MIC is determined by serial dilutions of antimicrobial agents in tubes with standard amount of bacteria. Turbidity (cloudiness) after incubation indicates bacterial growth and lack of turbidity indicates that the growth of bacteria is inhibited.

E – test

This is another test to determine the minimum inhibitory concentration where a plastic strip containing a gradient of the antimicrobial agent is used (Figure 3.4). An elliptical zone of inhibitory concentration can be noted with the help of a scale printed on the strip.

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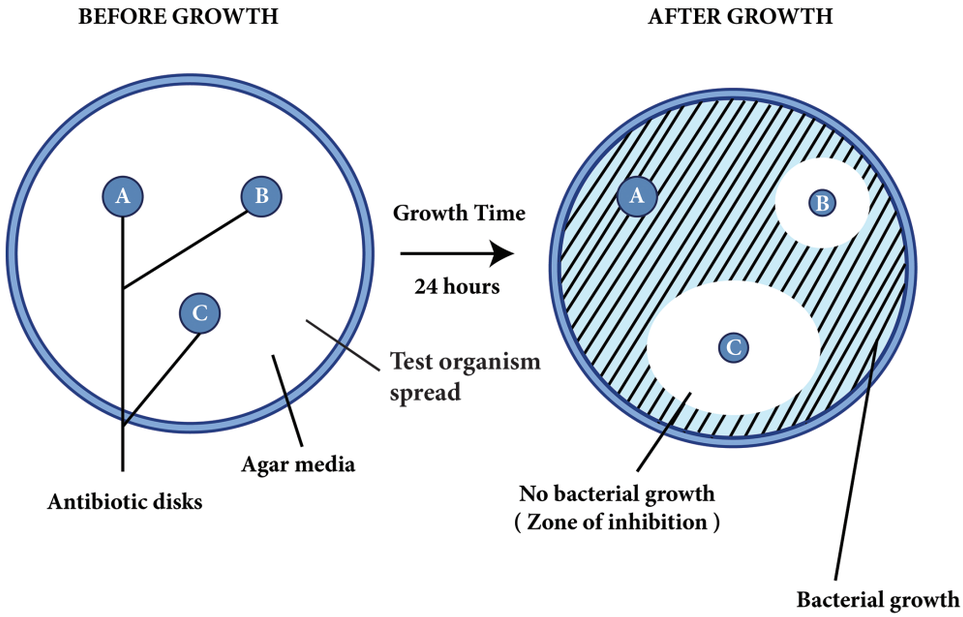

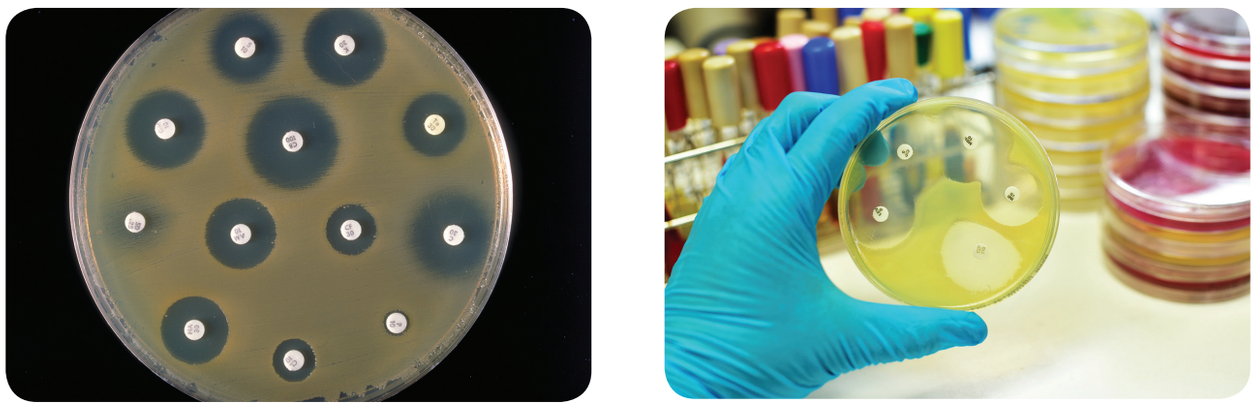

Figure 3.3: Microorganisms in Petri plate showing sensitivity and resistance towards antibiotics

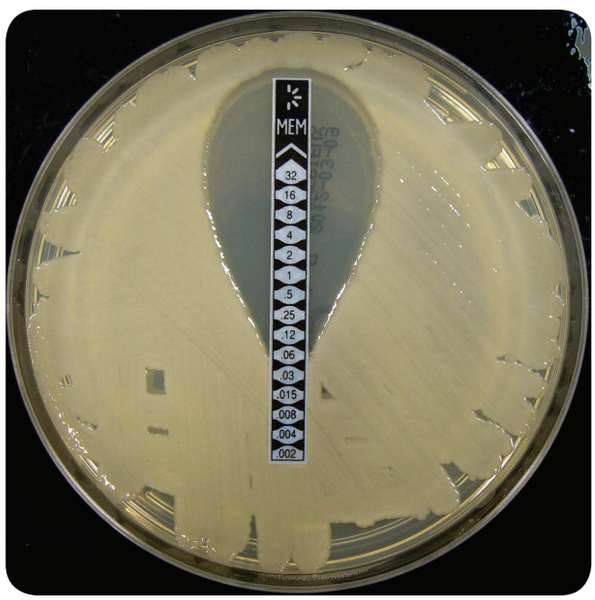

Figure 3.4: E – test

The Minimal Bactericidal Concentration (MBC) Test

MBC test is similar to MIC, the minimal bactericidal concentration test is used to determine the amount of antimicrobial agent required to rather **kill** the pathogen. In MBC test, samples taken from MIC tubes are transferred to drug free plates. Bacterial growth in these subcultures indicates that some bacterial cells have survived antimicrobial drug. The lowest concentration of drug for which no growth occurs is the minimum bactericidal concentration.

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The tube dilution method is considered accurate for determining susceptibility of a pathogen to precise quantities of antimicrobial agent. However, the method is time consuming, expensive, and not practical for use in most clinical laboratories for routine susceptibility testing.

Infobits

What is CRE?

CRE, which stands for carbapenem resistant Enterobacteriaceae, is the most fearsome family of germs because it is resistant even to last-resort antiboitics.

3.8 Drugs Resistance Mechanisms

Some microbes respond predictably to certain drugs making selection of treatment easy. Other microbes may vary in their responses, and laboratory tests are usually required to ensure that the selected therapy is appropriate. Chemotherapeutic effectiveness depends upon the sensitivity of the pathogen to the agent. Antibiotic resistance, however, may develop in microbes within the population. In fact, the history of chemotherapy has been closely paralleled by the history of drug resistance.

None of the therapeutic drugs (antibiotic) inhibits all microbial pathogens and some microbial pathogens possess natural ability to resist to certain antibiotics. Bacteria become drug resistant using several different resistance mechanisms. A particular type of resistance mechanism is not confined to a single class of drugs. Two bacteria may employ different resistance mechanisms to counter the same antibiotic. However, bacteria acquire drugs resistance using resistance mechanisms such as reduced permeability to antibiotic, efflux (pumping) antibiotic out of the cell, drug inactivation through chemical modification, target modification and development of a resistant biochemical pathway (Figure 3.5).

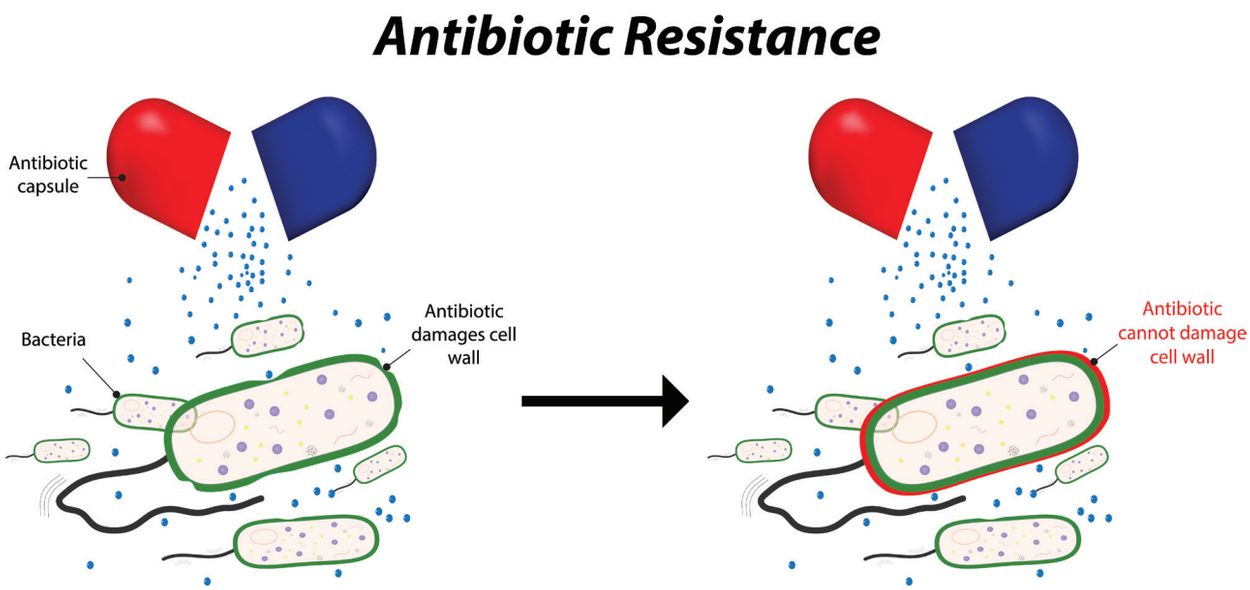

Figure 3.5: Drugs resistance mechanisms

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Methicillin-resistant staphylococcus aureus (MRSA) is a bacteria that is resistant to many antiobiotics. Staph and MRSA can cause a variety of problems ranging from are skin infections and sepsis to pneumonia to blood stream infections.

Summary

Chemical control refers to the use of disinfectants, antiseptics, antibiotics and chemotherapeutic antimicrobial chemicals. Disinfection is the elimination of microorganism, but not necessarily endospores, from inanimate objects or surface. A disinfectant is an agents used to disinfect inanimate objects but generally to toxic to use on human tissues. An antibiotic is a metabolic product produced by one microorganisms that inhibits or kills other microorganism. Synthetic chemicals that can be used therapeutically.

An agent that is static in action inhibits the growth of microorganism. An agent that is cidal in action kills microorganism. Selective toxicity means that the chemical being used should inhibit or kill the intended pathogen without seriously harming the host. A broad spectrum agent is one generally effective against a variety of gram positive & gram negative bacteria. A narrow spectrum agent generally works against just Gram positive, gram negative or only a few bacteria.

Evaluation

Multiple choice questions

1. Identify the term that describes a disinfectant that can kill bacteria:

   a. Bactericidal
   b. Bacteriostatic
   c. Pathogenic
   d. Bacteriosis

2. Which of the following is not a disinfectant containing a heavy metal?

   a. Silver b. Mercury
   c. Zinc d. Chlorine

3. Which of the following is most effective for sterilizing mattresses and plastic petriplates?

   a. Chlorine
   b. Ethylene oxide
   c. Glutaraldehyde
   d. Ultraviolet radiation

4. _____ is used to prevent infection by killing or inhibiting pathogen growth on animal tissue.

   a. Bacteriostatic agent b. Sanitizer
   c. Disinfectant d. Antiseptic

5. In the disk-diffusion assay, a large zone of inhibition around a disk to which a chemical disinfectant has been applied indicates _____ of the test microbe to the chemical disinfectant.

   a. Susceptibility or Sensitivity
   b. Resistant
   c. Intermediate
   d. None of these

TN_GOVT_XII_Micro_Biology_CH03.indd 40 07-12-2021 15:06:40

6. Which of the following agents are used as a preservative in ophthalmic solution?
   a. Alcohol
   b. Quaternary ammonium salts
   c. Phenol
   d. Aldehydes
7. Which of the following chemical lack penetrating power?
   a. Phenol
   b. Iodine
   c. Ethylene oxide
   d. Beta-propiolactone
8. Polymyxins inhibits the growth of the microbes by carrying get which of the following actions?
   a. Inhibition of cell-wall synthesis
   b. Damage to cell membrane
   c. Inhibition of nucleic acid and protein synthesis
   d. Inhibition of specific enzyme systems
9. All of the following are sporicidal except
   a. Glutaraldehyde b. Ethylene oxide
   c. Formaldehyde d. Alcohol

Answer the following

1. Define Disinfectants/antiseptics/ antibiotics.
2. Difference between Bacteriostatic and Bactericidal ?
3. What is Iodophores ?
4. Explain the mode of action of chemical agents against microorganisms?
5. Listout the major groups of antimicrobial chemical agents with an example.
6. Give examples of antimicrobial chemical agent which act as both disinfectant and antiseptics ?
7. Give an account on Gaseous sterilization ?
8. Describe the test is used to evaluate antimicrobial agent ?
9. How antibiotics the therapeutic drugs acts on target microorganisms?
10. Through disc diffusion method how an antibiotic sensitivity of microorganism is evaluated and explain the test ?
11. What is E – test?

TN_GOVT_XII_Micro_Biology_CH03.indd 41 07-12-2021 15:06:40

Chapter
4

Microbial Metabolism

Learning Objectives

After studying this chapter the students will be able to,

- *Identify the role of ATP in cellular activities.*
- *Define metabolism and describe the fundamental differences between catabolism and anabolism.*
- *Explain oxidation – reduction reaction.*
- *List and provide examples of three types of phosphorylation reactions that generates ATP.*
- *Describe the Carbohydrate, Lipid, Protein and its pathways (Glycolysis, Krebs cycle, electron transport chain)*
- *Electron transport chain and chemiosmotic model for ATP generation.*
- *Understand about the types of fermentation and its products.*
- *Describe the mechanism of enzymatic activity and significance of microbial enzymes.*

Chapter Outline

4.1 Metabolism

4.2 Energy of Chemical Reaction

4.3 Generation of ATP

4.4 Carbohydrate Catabolism

4.5 Tricarboxylic Acid Cycle

4.6 Electron Transport Chain

4.7 Lipid Catabolism

4.8 Protein Metabolism

4.9 Fermentation

4.10 Enzymes



All living organisms are constantly in need of energy to function. The life support activity of even the most structurally simple organism involves a large number of complex biochemical reactions. Living cells carry out three major types of processes namely **Chemical Process, Transport Process** and **Mechanical Process**. In chemical processes, energy is required to synthesize complex biological molecules from much simpler molecules. Transport processes require energy to take up nutrients, eliminate waste, and maintain ion balance. Mechanical processes require energy to change the physical location of structures within cells. Even during resting state, a substantial amount of energy is needed for fundamental functions of cells. All living system obeys the laws of thermodynamics. This law analyzes energy

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changes in a collection of matter called system (a cell or a plant).

The energy exchanges between the system and the surrounding balance each other. All chemical reactions in cells involve energy transformation. (For example: Photosynthetic bacteria transform radiant energy into chemical energy). In living cells thermodynamic changes are essential for biological function such as growth, reproduction, photosynthesis and respiration. Microorganisms obtain energy and nutrients for their survival and reproduction through metabolism. The microbial species and ecological niche can often be differentiated from each other based on metabolic characteristics. The metabolic reaction often allows the use of micro organisms in fermentation process and biogeochemical cycle.



Three fourth of the energy is derived from carbohydrate that we consume and Glucose is the major fuel for all living organisms.

4.1 Metabolism

The term Metabolism refers to the sum of all bio chemical reactions that occur within a living cell. Chemical reaction either release energy or require energy. Metabolism can be viewed as an energy balancing act. It can be divided into two classes of chemical reactions namely **Catabolism** and **Anabolism**.

Catabolism: It is called catabolic or degradative reactions because complex organic compounds are broken down into simples ones. Catabolic reactions are generally hydrolytic reactions. It is enzyme regulated chemical reaction that release energy and they are exergonic Example: Break down of sugar into Carbon dioxide and water in cells.

Anabolism: It is called anabolic or biosynthetic reactions because complex organic molecules are formed from simples ones. Anabolic process often involves dehydration, are bio-synthetic reactions (Figure 4.1). It is enzyme regulated energy requiring reaction and they are endergonic. Examples: Formation of proteins from amino acids.

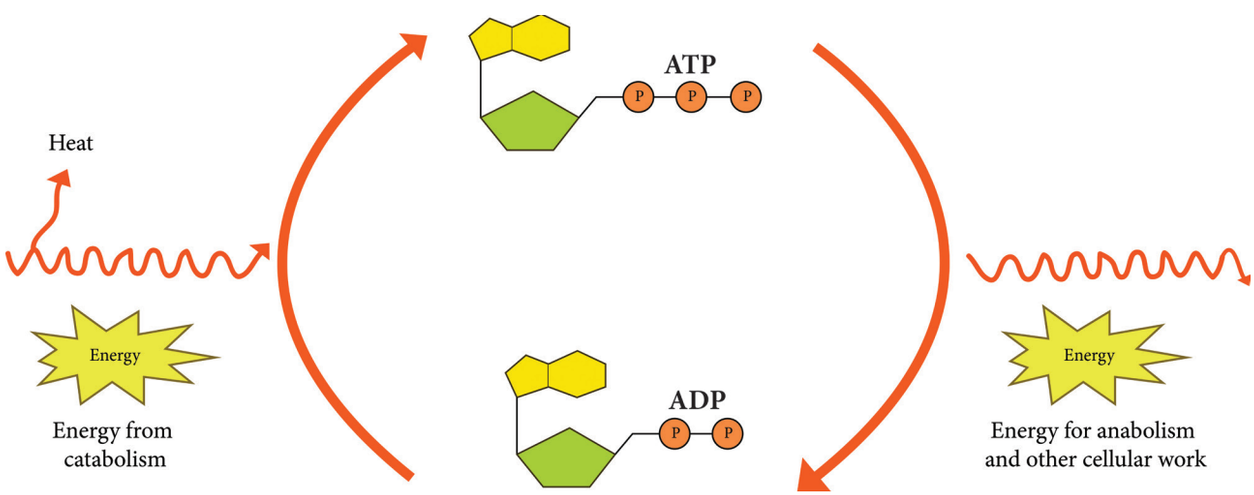

Figure 4.1: Catabolic and anabolic reactions

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Catabolic reactions furnish the energy needed to drive anabolic reactions. This coupling of energy requiring and energy releasing reactions is made possible through the molecule Adenosine tri-phosphate (ATP).

4.2 Energy of Chemical Reaction

Light energy is trapped by phototrophs during photosynthesis, in which it is absorbed by bacteriochlorophyll and other pigments and converted to chemical energy for cellular work. The energy is required by the bacterium for synthesis of cell wall or membrane, synthesis of enzymes, cellular components, repair mechanism, growth and reproduction.

Some change of energy occurs whenever bonds between atoms are formed or broken during chemical reactions. When a chemical bond is formed, energy is required. Such a chemical reaction which requires energy is called an endergonic reaction (energy is directed inward). When a bond is broken, energy is released. A chemical reaction that release energy is an exergonic reaction (energy is directed outward).

During chemical reaction energy is either released or absorbed and the quantum of energy liberated or taken up is useful energy and is referred to Free Energy Change (ΔG) of the reactions.

4.2.1 High Energy Phosphate

Adenosine Tri-Phosphate (ATP) is the principal energy carrying molecule of all cells and is indispensable to the life of the cell. It stores the energy released by some chemical reactions, and it provides the energy for reactions that require energy. ATP consists of an adenosine unit composed of adenine, ribose with three phosphate groups. In ATP and some other phosphorylated compounds, the outer two phosphate groups are joined by an **anhydride bond**.

Some of the other high energy nucleotides involved in biochemical processes are given in Table 4.1.

Table 4.1: High energy nucleotides involved in biosynthesis

Name of the Nucleotide	Biosynthesis
Uridine triphosphate (UTP)	Polysaccharide
Cytidine triphosphate (CTP)	Lipid
Guanidine triphosphate (GTP)	Protein

Nutrients are broken from highly reduced compounds to highly oxidized compounds within the cells. Much of the energy released during oxidation - reduction reactions is trapped within the cell by the formation of ATP. A phosphate group is added to ADP with the input of energy to form ATP.

$$ATP + H_2O \longrightarrow ADP + pi \ (\Delta G^\circ = -7.3 \text{ K Cal/mol})$$

$$ATP + H_2O \longrightarrow AMP + ppi \ (\Delta G^\circ = -10.9 \text{ K Cal/mol})$$

ATP is ideally suited for its role as an energy currency. It is formed in energy trapping and energy generating processes such as photosynthesis, fermentation, and aerobic respiration. In bacterial and archeal cells, most of the ATP is formed on the cell membrane, while in eukaryotes the reactions occur primarily in the mitochondria (Figure 4.2).

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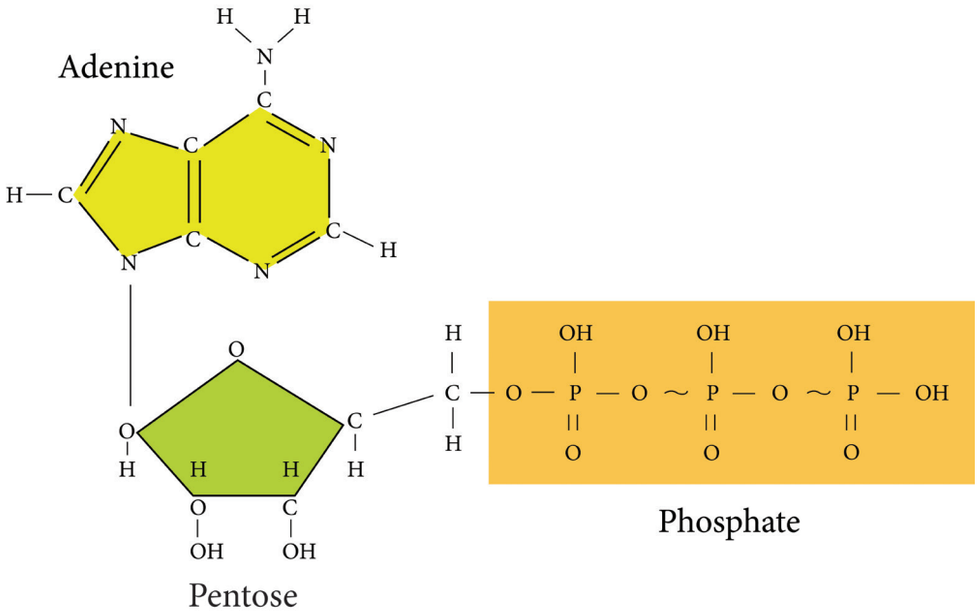

Figure 4.2: Structure of ATP

4.2.2 Oxidation – Reduction Reactions

Oxidation is the removal of electrons (e^-) from an atom or molecule and is often an energy producing reaction. Reduction of a substrate refers to its gain or addition of one or more electrons to an atom or molecule. Oxidations and reduction are always coupled. In other words, each time one substance is oxidized, another is simultaneously reduced.

$F_2 + 2e^- \longrightarrow 2F^-$

$H_2 + 2e^- \longrightarrow 2H^+ + 2e^-$

$NAD^+ + 2H^+ + 2e^- \rightleftharpoons NADH + H^+$

4.3 Generation of ATP

Much of energy released during oxidation reduction reaction is trapped within the cell by the formation of ATP. A phosphate group is added ADP with the input of energy to form ATP. The addition of a phosphate to a chemical compound is called phosphorylation.

Organism uses three different mechanisms of phosphorylation to generate ATP from ADP. They are

4.3.1 Substrate Level Phosphorylation

It is a metabolic reaction that results in the formation of ATP or GTP by the direct transfer of a phosphoryl group to ADP or GDP from another phosphorylated compound.



Alkaline phosphatase is a heat sensitive enzyme in milk which is used as an indicator in Pasteurization.

4.3.2 Oxidative Phosphorylation

In this reaction, electrons are transferred from organic compounds to molecules of Oxygen (O_2) or other inorganic molecules through a series of different electron carriers (Example: NAD^+ and FAD). Then the electrons are passed through a series of different electron carriers to oxygen. The process of oxidative phosphorylation occurs during electron transport chain (Figure 4.3).

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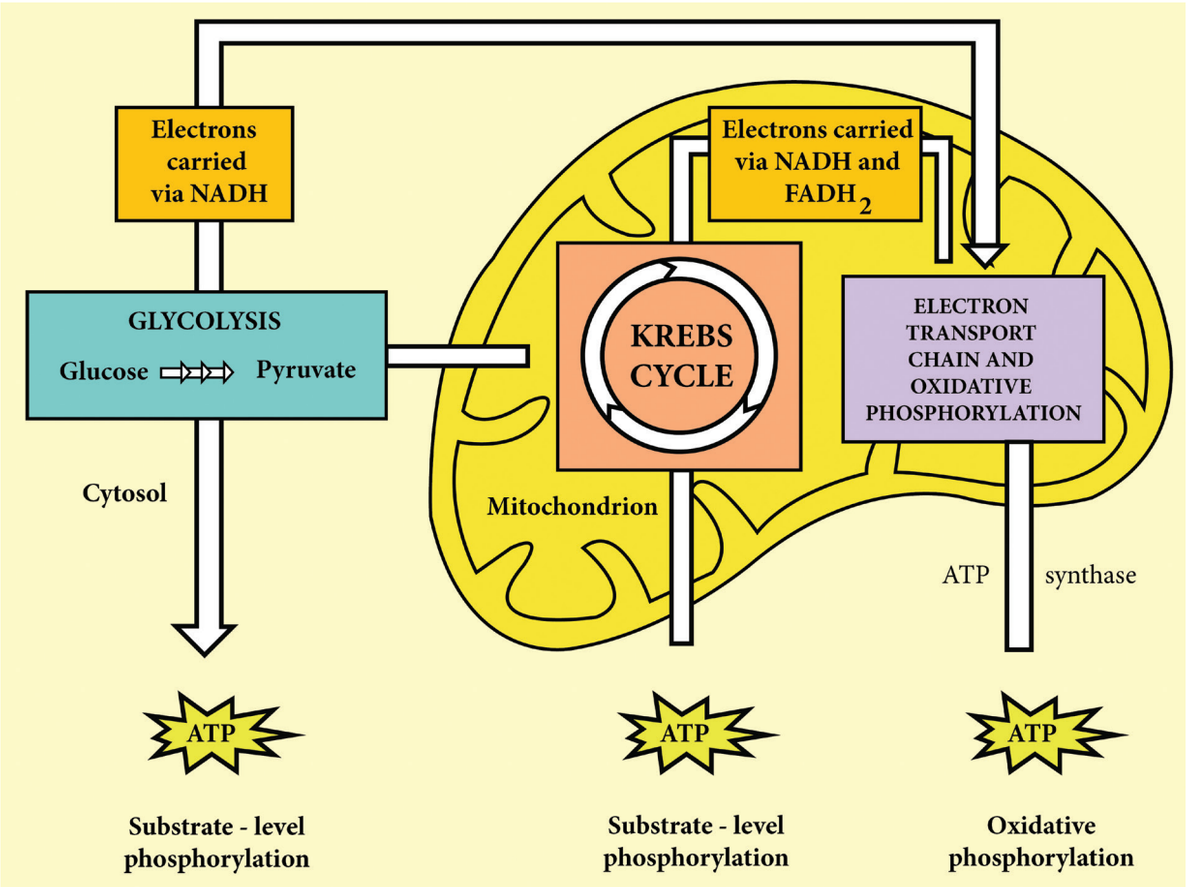

Figure 4.3: Phosphorylation

4.3.3 Photophosphorylation

It occurs only in photosynthetic cells which contain light trapping pigments. Example: In photosynthesis, photosynthetic pigment, Chlorophyll is involved in the synthesis of organic molecules especially sugars, with the energy of light from the energy poor building blocks like Carbon dioxide and water. In phototropic bacteria (purple, green sulphur bacteria, Cyanobacteria), photosynthetic pigments bateriochlorophylls are involved in ATP production.

4.4 Carbohydrate Catabolism

Most microorganisms oxidize carbohydrates as their primary source of cellular energy. Carbohydrate catabolism is the breakdown of carbohydrate molecule to produce energy and is therefore of great importance in cell metabolism. Glucose is the most common carbohydrate energy source used by cells.

To produce energy from glucose, microorganism use two general processes namely Respiration and Fermentation.

4.4.1 Cellular Respiration

Respiration is defined as an ATP generating process in which organic molecules are oxidized and the final electron acceptor is an inorganic compound. In aerobic respiration, the final electron acceptor is Oxygen and in anaerobic respiration the final electron acceptor is an inorganic molecule like NO_3, SO_4^{2-} other than Oxygen.

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The aerobic respiration of glucose typically occurs in three principal stages. They are

- ❖ Glycolysis
- ❖ Krebs cycle
- ❖ Electron transport chain

Glycolysis

Glycolysis is the process of splitting of sugar molecule, where the glucose is enzymatically degraded to produce ATP. Glycolysis is the oxidation of glucose to pyruvic acid with simultaneous production of some ATP and energy containing NADH. It takes place in the cytoplasm of both prokaryotic and eukaryotic cells. Glycolysis occurs in the extra mitochondrial part of the cell cytoplasm. Glycolysis was discovered by Emden, Meyerhof and Parnas. So, this cycle is shortly termed as EMP pathway, in honour of these pioneer workers. This cycle occurs in animals, plants and large number of microorganisms. Glycolysis does not require oxygen, it can occur under aerobic or anaerobic condition. Glycolysis is a sequence of ten enzyme catalyzed reactions.

Aerobic condition

$$\text{Glucose} \xrightarrow{\text{Glycolysis}} \text{Pyruvate} \xrightarrow[O_2]{\text{Respiration}} CO_2 + H_2O$$

Anaerobic condition

$$\text{Glucose} \xrightarrow{\text{Glycolysis}} \text{Pyruvate} \xrightarrow{\text{Fermentation}} \text{Fermented products}$$

$$C_6H_{12}O_6 + 2\ NAD + 2\ ADP + 2\ P \longrightarrow 2\ CH3\ COCOOH + 2\ ATP + 2\ NADH + 2\ H+$$

(Pyruvic acid)

Since glucose is a six carbon molecule and pyruvate is a three carbon molecule, two molecules of pyruvate are produced for each molecule of glucose that enters Glycolysis. Net energy production from each glucose molecule is two ATP molecules

The Glycolysis pathway consists of two phases. They are

1. The preparatory/Investment phase, where ATP is consumed
2. The pay off phase where ATP is produced (Figure 4.4).

1. In the preparatory stage, two molecules of ATP are utilized and then glucose is phosphorylated, restructured, and split into two 3 carbon compounds namely Glyceraldehyde-3-phosphate and Dihydroxyacetone phosphate.
2. In pay off phase or energy conserving stage, the two 3 carbon molecules are oxidized in several steps to 2 molecules of pyruvic acid and two molecules of NAD^+ are reduced to NADH, thus four molecules of ATP are formed by substrate level phosphorylation.

   Two molecules of ATP are needed to initiate Glycolysis and four molecules of ATP are generated at the end of the process. Therefore, the net gain of Glycolysis is two ATP for each molecule of glucose oxidized.

Alternatives to Glycolysis

Many bacteria have another pathway in addition to Glycolysis for the oxidation of glucose. Some of the common pathways that occur in most of the bacteria are

- Pentose phosphate pathway (PPP) or Hexose Mono Phosphate shunt
- Entner –Doudoroff Pathway

HOTS

Does Glycolysis require Oxygen?

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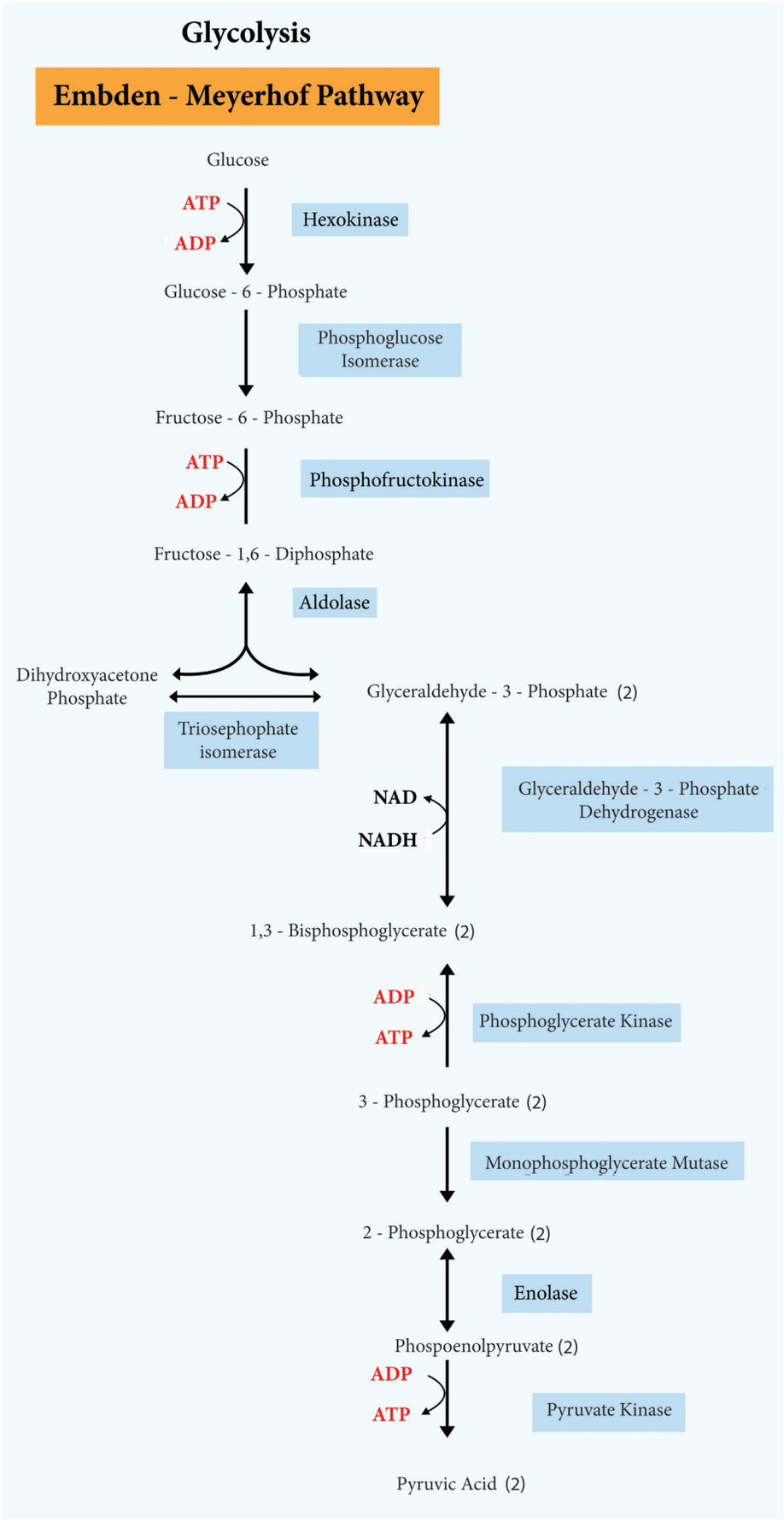

Figure 4.4: Glycolysis Pathway

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Strips used in Glucometer a chemical called glucose oxidase which reacts with the glucose in the blood sample and is converts it into an acid called gluconic acid.

4.5 Tricarboxylic Acid Cycle(TCA)

TCA cycle was first elucidated by Sir Hans Adolf Krebs, a German Biochemist in 1937. It is also known as Tricarboxylic acid cycle, Citric acid cycle or Amphibolic cycle. In prokaryotic cells, the citric acid cycle occurs in the cytoplasm; in eukaryotic cells it takes place in the matrix of the mitochondria.

The process oxidizes glucose derivatives, fatty acids, and amino acids to carbon dioxide (CO_2) through a series of enzyme controlled steps. The purpose of the Krebs cycle is to collect high energy electrons from these fuels by oxidizing them, which are transported by activated electron carriers such as NADH and $FADH_2$ to electron transport chain. The Krebs cycle is also the source for the precursor of many other molecules and is therefore an amphibolic pathway (both anabolic and catabolic reactions take place in this cycle) and therefore, it can be used for both the synthesis and degradation of bio molecules.

$$\text{Pyruvate} + \text{CoA-SH} + \text{NAD}^+ \xrightleftharpoons{\text{Pyruvate dehydrogenase complex}} \text{Acetyl CoA} + \text{Co}_2 + \text{NADH}$$

Pyruvate cannot enter the Krebs cycle directly. In a preparatory step, it must lose one molecule of CO_2 and becomes a two-carbon compound. This process is called decarboxylation. The two-carbon compound, called acetyl group, attaches to coenzyme A through a high-energy bond, the resulting is a complex known as acetyl coenzyme (acetyl CoA). During this reaction, pyruvic acid is also oxidized and NAD^+ is reduced to NADH by pyruvate dehydrogenase complex (PDHC). This multi enzyme complex is responsible for the conversion of pyruvate to acetyl-coA. Therefore PDHC contribute to linking the Glycolysis pathway to the citric acid pathway.



Pyruvate dehydrogenase deficiency is a common cause of lactic acidosis in new born and often present with poor feeding.

The Krebs cycle generates a pool of chemical energy (ATP, NADH, and $FADH_2$) from the oxidation of Pyruvic acid and it loses one carbon atom as CO_2 and reduces NAD^+ to NADH. The resulting two carbon acetyl molecule is joined to Co enzyme A. Acetyl CoA transfers its acetyl group to a 4C compound (oxaloactate) to make a 6C compound (Citrate) and the Coenzyme A is released which goes back to the link reaction to form another molecule of acetyl CoA. Oxaloacetate is both the first reactant and the product of the metabolic pathway (creating a loop).

After citrate has been formed, the cycle machinery continues through seven distinct enzyme catalyzed reactions that produce in order isocitrate, α – ketoglutarate, succinyl CoA, succinate, fumarate, malate and oxaloacetate.

At the end of Krebs cycle, each pyruvic acid produces 2 CO_2, 1 ATP (substrate

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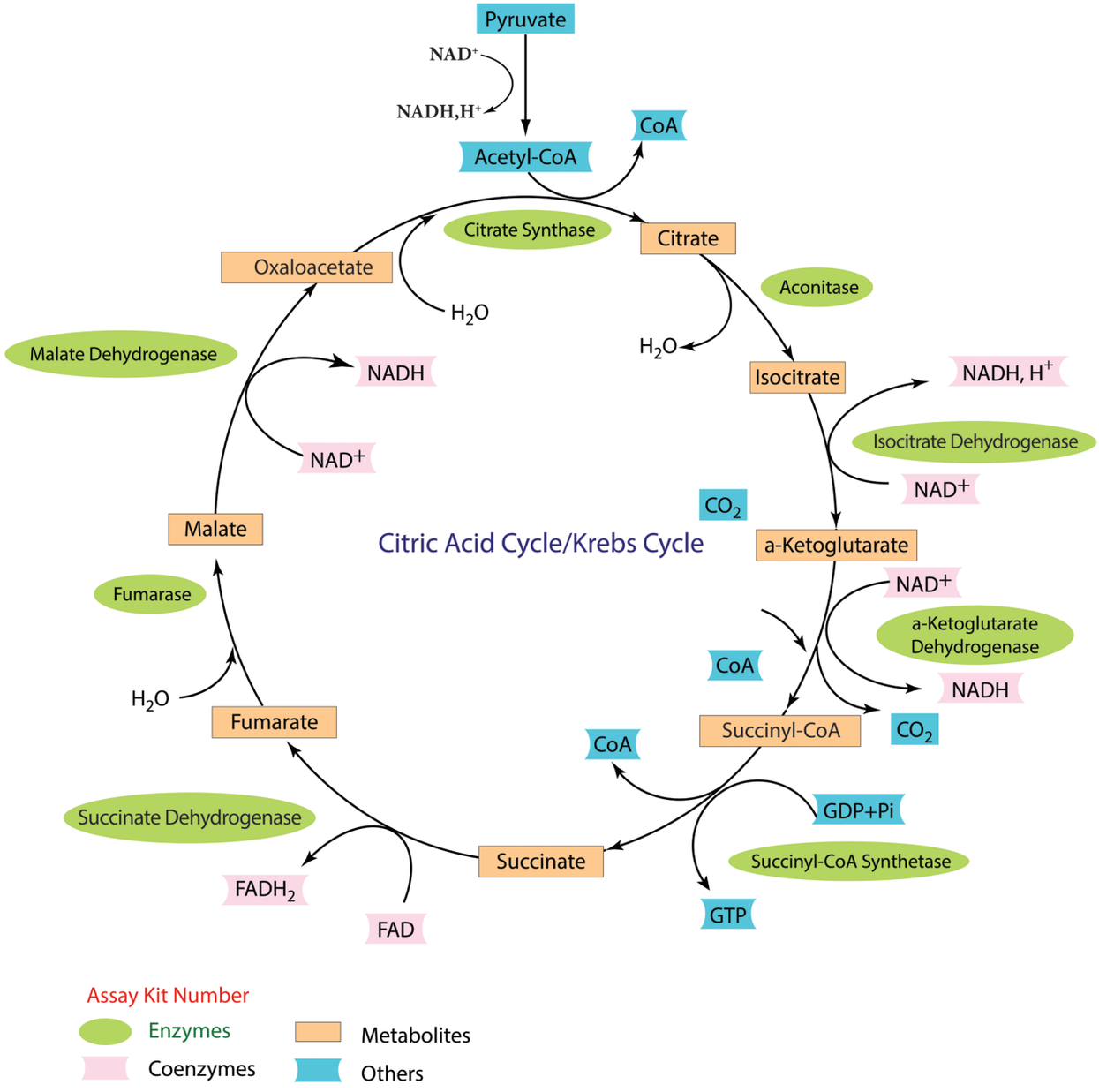

Figure 4.5: Krebs cycle

level phosphorylation), 3 NADH and 1 $FADH_2$. Then NADH and $FADH_2$ can be oxidized by electron transport chain to provide more ATPs.

4.6 Electron Transport Chain

An electron transport chain consists of a sequence of carrier molecules that are capable of oxidation and reduction. In Eukaryotic cell, the ETC is contained in the inner membrane of mitochondria or chloroplast membrane, whereas in prokaryotic cells, it is found in plasma membrane or cytoplasmic membrane.

The ETC is carried out through a series of electron transporters embedded in the inner mitochondrial membrane that transfer electrons from electron donors NADH

TN_GOVT_XII_Micro_Biology_CH04.indd 50 07-12-2021 15:09:07

and $FADH_2$ to acceptor such as molecular Oxygen. In the process, protons are pumped from the mitochondrial matrix to the inner membrane space, and eventually combine with O_2 and H^+ to form water (Figure 4.6).

HOTS

Why each NADH makes 3 ATPs and each $FADH_2$ makes 2 ATPs?

As the electrons flow through the chain, much of their free energy is conserved in the form of ATP. The process by which energy from electron transport is used to make ATP is called as oxidative phosphorylation. Respiratory chain is an electron transport chain where a pair of electrons or hydrogen atoms containing electron from the substrate oxidized is coupled to reduction of oxygen to water. The mitochondrial system is arranged into three complexes of electron carriers. They are

1. **Flavoproteins:** These proteins contain flavin, a coenzyme derived from riboflavin (Vit B_{12}). One important flavoprotein is flavin mono nucleotide.
2. **Ubiquinones (coenzyme Q):** These are small non protein carriers.
3. **Cytochromes:** These are proteins with iron containing group, capable of existing alternately as reduced (Fe^{2+}) and oxidized form (Fe^{3+}). Cytochromes involved in ETC include cyt (b),cyt c_1, cyt c, cyt a, cyt a_3.

The first step in electron transport chain is the transfer of high energy electrons from NADH to FMN. This transfer actually involves the passage of hydrogen atom with 2 e^- to FMN, which then picks up an additional H^+ from the surrounding aqueous medium.

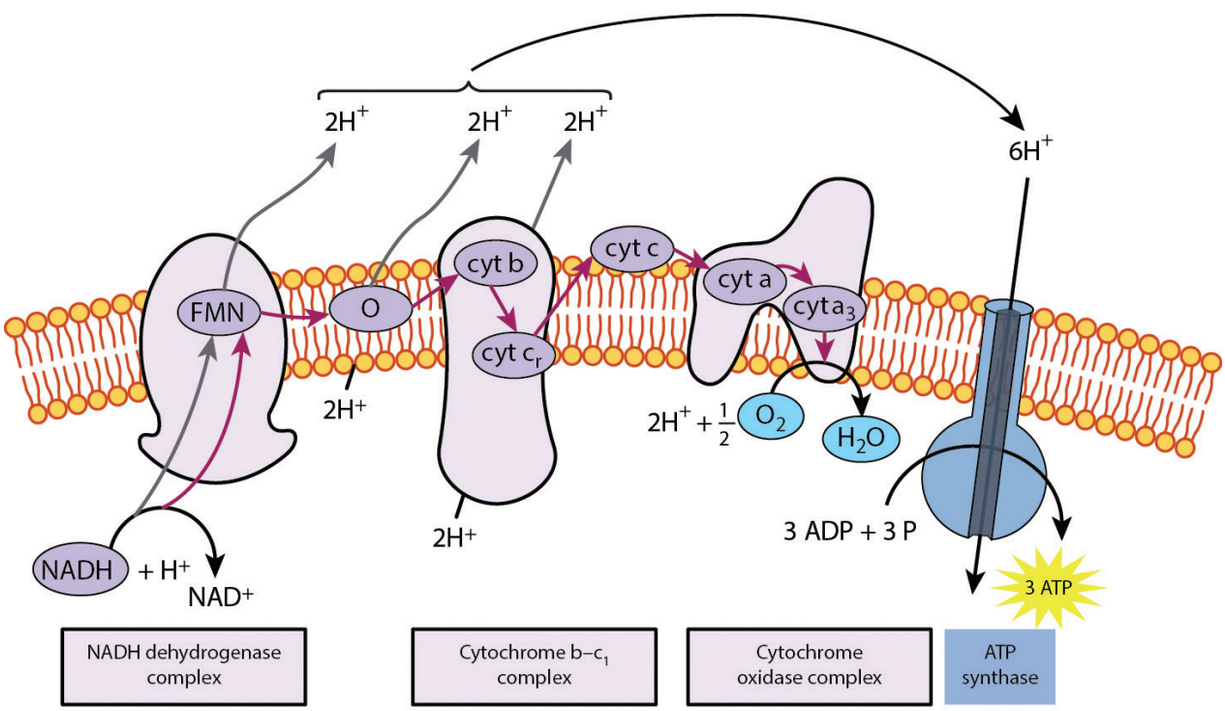

Figure 4.6: Electron Transport Chain and Chemiosmotic mechanism of ATP

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As a result of the first transfer, NADH is oxidized to NAD+, and FMN is reduced to $FMNH_2$.

In the second step, $FMNH_2$ passes 2 H+ to the other side of the mitochondrial membrane and passes 2 e^- to coenzyme Q. As a result, $FMNH_2$ is oxidized to FMN. Coenzyme Q also picks up additional 2H+ from the surrounding aqueous and releases to other side of the membrane.

In the next step, electrons are passed successively from coenzyme Q to cyt b_1, cyt c_1, cyt c, cyt a, cyt a_3. Each cytochrome in the chain is reduced, as it picks up electrons and is oxidized as it gives up electrons. The last cytochrome cyt a3 passes its electrons to molecular O_2 which picks up protons from the surrounding medium to form H_2O.

$FADH_2$ derived from the Krebs cycle is another source of electrons. Thus at the end of ETC, NADH pumps three protons (synthesizes 3ATPs) whereas $FADH_2$ pumps only two protons (synthesizes 2ATPs).

4.6.1 Chemiosmotic Mechanism of ATP

Chemiosmotic mechanism of ATP synthesis was first proposed by the Biochemist, Peter Mitchell in 1961. In ETC, when energetic electrons from NADH pass down the carriers, some of the carriers (proton pumps) in the chain pump [actively transport] protons across the membrane to inner membrane space. Thus in addition to a concentration gradient, an electrical charge gradient is created. The resulting electro chemical gradient has potential energy called proton motive force.

The proton diffuses across the membrane through protein channels that contain an enzyme called ATP synthase. When this flow occurs, energy is released and is used by the enzyme to synthesize ATP from ADP and phosphate.

At the end of the chain, electrons join with protons and O_2 in the matrix fluid to form H_2O. Thus O_2 is the final electron acceptor. ETC also operates in photophosphorylation and is located in thylakoid membrane of Cyanobacteria (BGA), and of eukaryotic chloroplasts.

Overview of Aerobic respiration (Figure 4.7):

- Electron transport chain regenerates NAD and FAD which can be used again in Glycolysis and Krebs cycle.
- Various electrons transfer in the electron transport chain generates about 34 ATP, (10 NADH = $10 \times 3 = 30$ + 2 $FADH_2 = 2 \times 2 = 4$).
- A total of 38 ATP molecules is generated from one molecule of glucose oxidized in prokaryotes, whereas in eukaryotes, 36 molecules of ATP is generated because in eukaryotes, some energy is lost when electrons are shuttled across the mitochondrial membranes that separate Glycolysis (in the cytoplasm) from the electron transport chain (Table 4.2). There is no such separation exists in prokaryotes.

$$C_6H_{12}O_6 + 6CO_2 + 38ADP + 38P_i \longrightarrow 6CO_2 + 6H_2O + 38\ ATP$$

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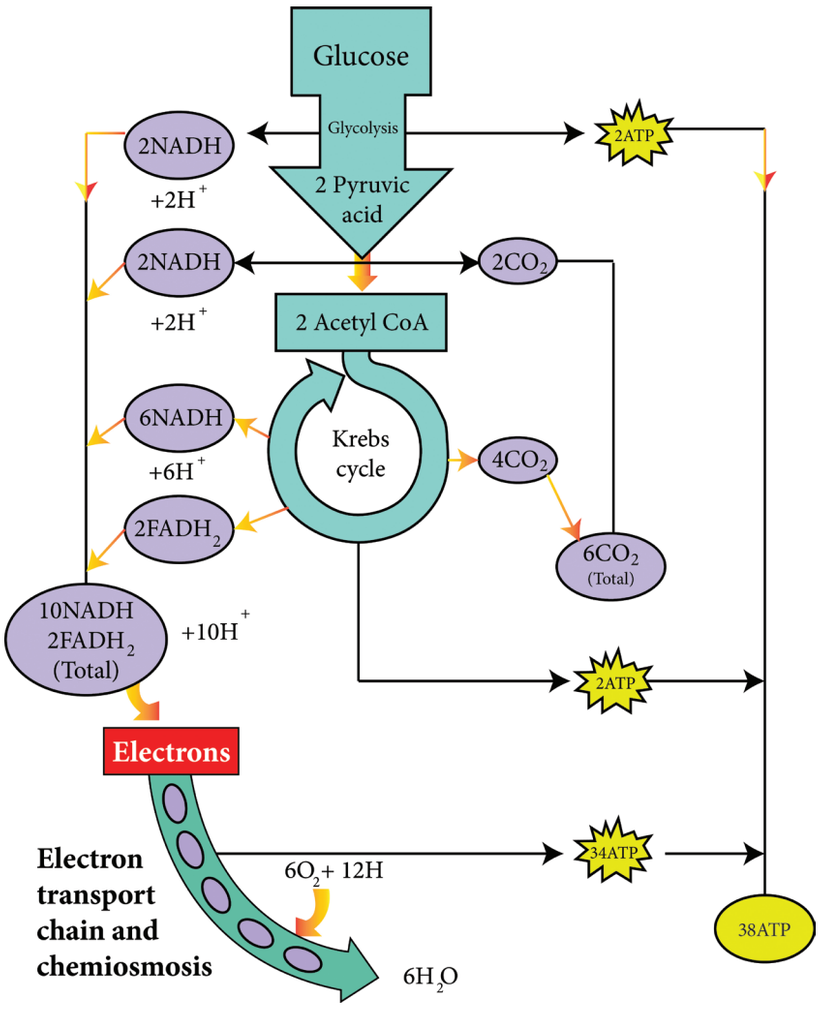

Figure 4.7: Overview of aerobic respiration

Table 4.2: Net gain of ATP produced during aerobic respiration of glucose in prokaryotes

Glycolysis 1. Oxidation of glucose to Pyruvic acid. 2. Production of 2 NADH	**Preparatory step** 2 ATP (substrate level phosphorylation) 6 ATP (Oxidative phosphorylation in ETC)
Preparatory step 1. Formation of acetyl CoA produces 2NADH	6 ATP (Oxidative phosphorylation in ETC)
Krebs cycle 1. Oxidation of succinyl CoA to succinic acid 2. Production of 6 NADH 3. Production of 2 FADH	2 ATP (Substrate level phosphorylation) 18 ATP (Oxidative phosphorylation in ETC) 4 ATP (Oxidative phosphorylation in ETC) **Total 38 ATP**

1 NADH = 3 ATPs and 1$FADH_2$ = 2 ATP

TN_GOVT_XII_Micro_Biology_CH04.indd 53 07-12-2021 15:09:08

4.7 Lipid Catabolism

Microorganisms frequently use lipids such as triglyceride or triacylglycerol (esters of glycerol and fatty acids) as common reserve energy sources. These can be hydrolyzed to glycerol and fatty acid by microbial lipases. The glycerol is then phosphorylated and oxidized to Dihydroxyacetone phosphate and then catabolized in the Glycolysis pathway. Fatty acids from triacylglycerols and other lipids are often oxidized in the β-oxidation pathway. In this pathway fatty acids are degraded to acetyl CoA (2C segment), then it enters into the TCA cycle.



Bubble Test: Bubbles are a positive result for the presence of catalase. If an organism can produce catalase, it will produce bubbles of oxygen when hydrogen peroxide is added to it.

Infobits

One of the major environmental problems today is hydrocarbon contamination resulting from the activities related to the petrochemical industry. Several bacteria can use hydrocarbon as a feed and reduce pollution.

Pseudomonas putida (Super Bug) *Alcanivorax borkumens, Mycobacterium, Brevibacterium, Aspergillus, Penicillium, Candida lipolytica* are the most active agents in petroleum degradation and they work as primary degraders in oil spilled environment. These organisms are mainly in involved in bioremediation which reduce environmental pollution

4.8 Protein Catabolism

Many microbes use protein as their source of carbon and energy. Pathogenic microorganisms secrete protease enzyme that hydrolyze proteins and polypeptides to amino acids which are then transported into the cell and catabolized. Protease (Peptidase or proteinase) helps in proteolysis (Figure 4.8). These proteolytic enzymes break the long chains of proteins into peptides and eventually into amino acids. The enzymes are classified based on the sites at which they catalyse the cleavage of proteins as exopeptidase and endopeptidase.

The protein catabolism involves two reactions namely,

- Deamination and
- Transamination

Deamination is the removal of the amino group from an amino acid. Transamination is the transferring of amino group from an amino acid to an amino acid acceptor.

The organic acid resulting from deamination can be converted to pyruvate, acetyl CoA or TCA cycle intermediates to release energy. Excess nitrogen from deamination may be excreted as ammonium ion.



Various metabolic processes such as blood coagulation, fibrinolysis, complement activation, phagocytosis and blood pressure control are regulated by proteases.

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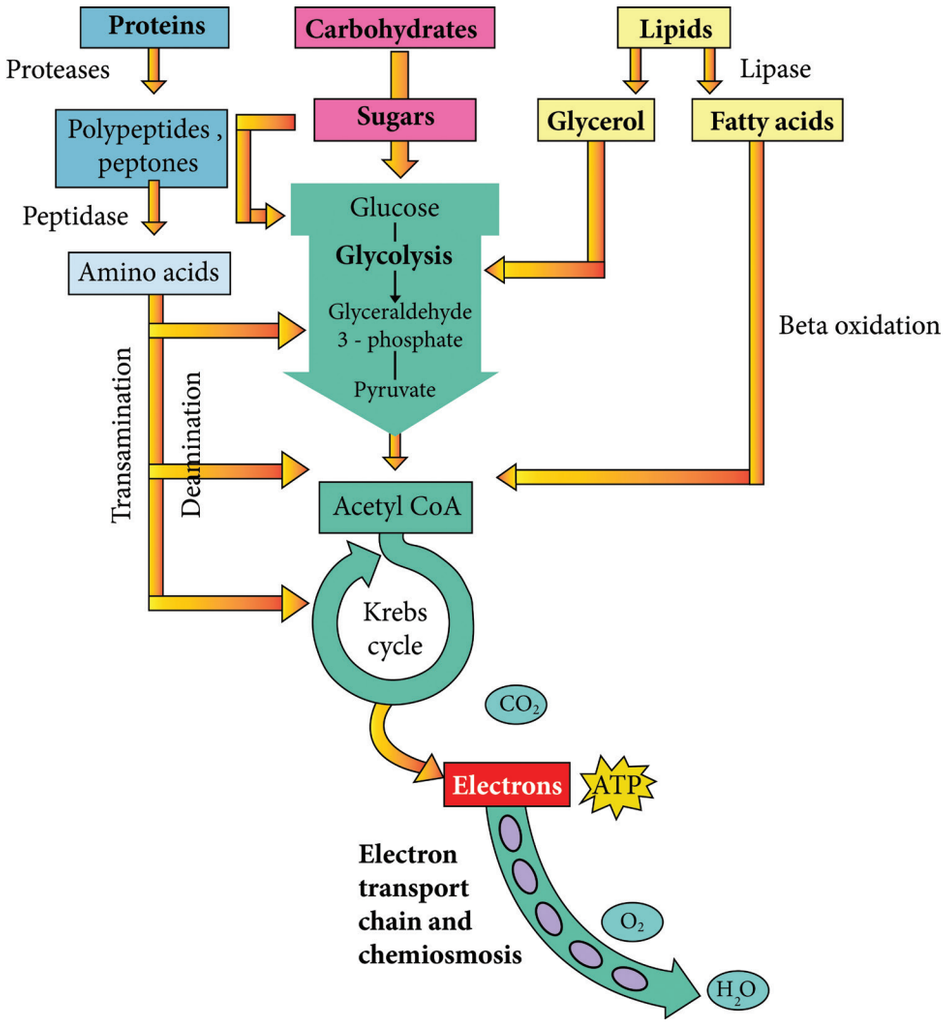

Figure 4.8: Overall Metabolism of Protein , Carbohydrates and Lipids

4.9 Fermentation

In 1856 fermentation, reaction was first demonstrated by Louis Pasteur in yeast. The study of fermentation and its practical uses is named as Zymology. Any energy releasing metabolic process that takes place only under anaerobic condition is called fermentation. It can also be defined as a metabolic process that release energy from a sugar or other organic molecule. It does not require oxygen or an electron transport system, and uses an organic molecule as the final electron acceptor. Fermentation reaction yields only a small amount of energy (2 ATP).

Anaerobes do not use an electron transport chain to oxidize NADH to NAD^+ and therefore use fermentation as alternative method to maintain a supply of NAD^+ for the proper function of normal metabolic pathways. Facultative anaerobes can use fermentation under anaerobic condition and carryout aerobic respiration when oxygen is present. Fermentation reoxidizes NADH to NAD^+ by converting pyruvic acid into various organic acids.

During fermentation, NADH is converted back into the coenzyme NAD^+ so that it can be used again for Glycolysis

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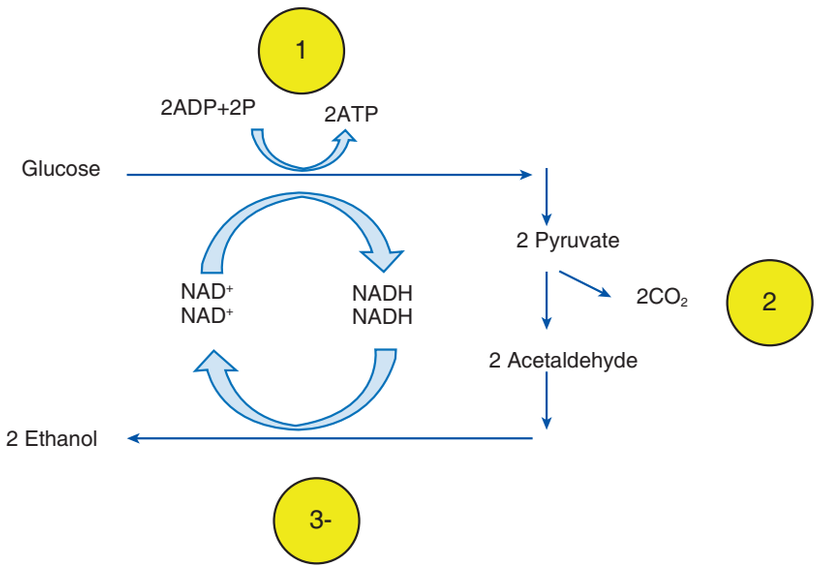

Figure 4.9: Biochemical exchange of NADH and NAD^+

(Figure 4.9). Organic electron acceptors such as pyruvate or acetaldehyde react with NADH to form NAD^+, producing CO_2 and organic solvent like ethanol. Fermentation can be classified as Lactic acid fermentation and alcohol fermentation.



Aquifex (water maker) of Aquificae is a diverse collection of bacteria that live in harsh environmental settings. These can produce water by oxidizing hydrogen.

4.9.1 Lactic acid fermentation

During Glycolysis, in the first step of lactic acid fermentation, a molecule of glucose is oxidized to 2 molecules of pyruvic acid and it generates the energy. In the next step pyruvic acid is reduced by NADH to form lactic acid. *Lactobacillus* and *Streptococcus* are some of the lactic acid producing genera (Figure 4.10).

Milk is converted into fermented products such as curd, yogurt and cheese. The fermentation of lactose in milk by these bacteria produces lactic acid which acts on milk protein to give yogurt its texture and characteristic tart flavour. Here lactase enzyme is produce by the bacteria which convert the lactose into lactic acid.



Builds up of Lactic acid in muscle cells causes muscle cramp.

Homolactic acid fermentation

In this type of fermentation, organism produces lactic acid alone. So it is referred to as homolactic fermentation.

Glucose + 2ADP + 2P ⟶ Lactic acid + 2 ATP

Heterolactic acid fermentation

In this type of fermentation, organism produces Lactic acid as well as other acids or alcohol. So it is known as hetero

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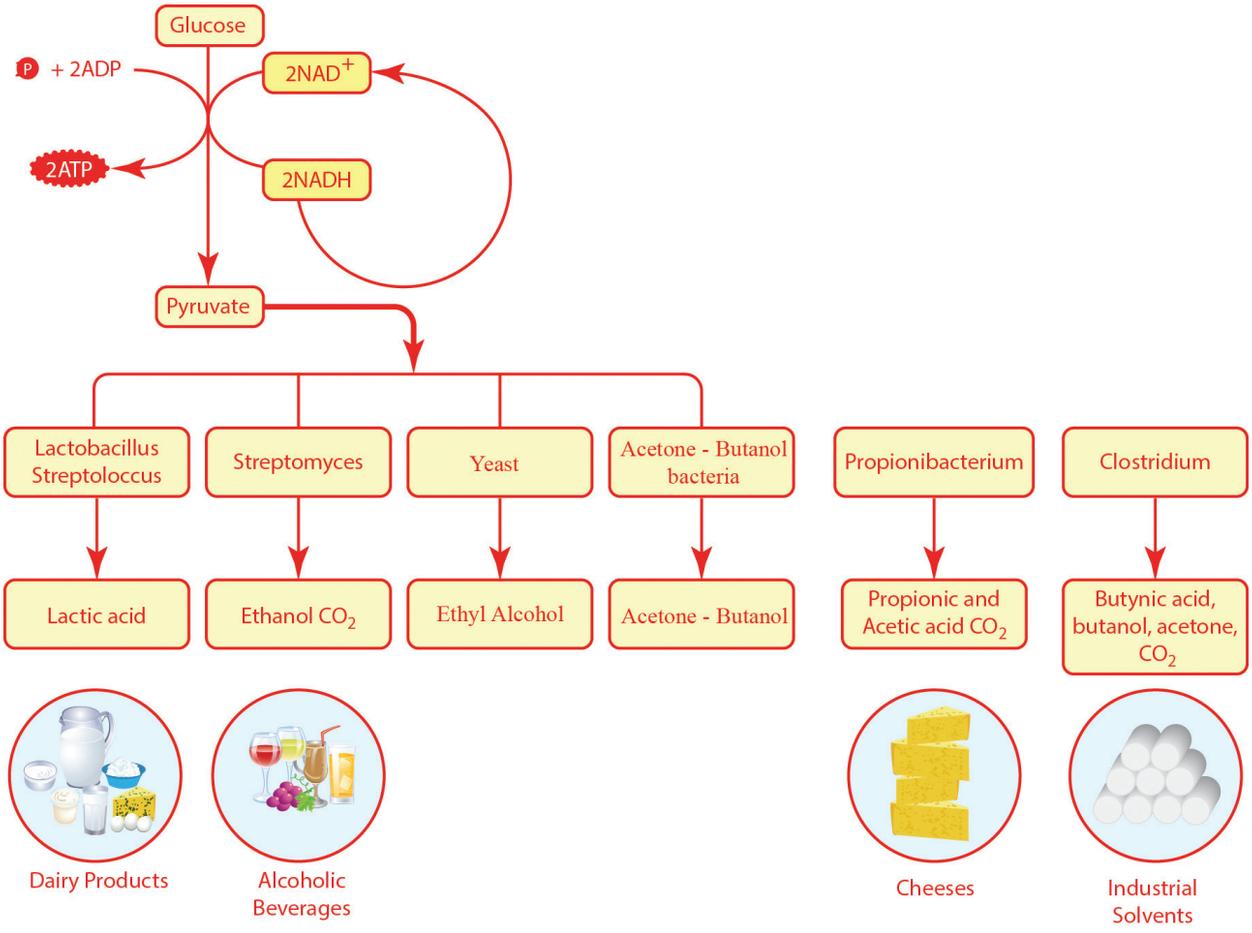


Figure 4.10: Varoius products of microbial fermentation

fermentation or heterolactic and often uses the pentose phosphate pathway.

$$G + ADP + (P) \longrightarrow \text{Lactic acid} + \text{ethanol} + CO_2 + ATP$$

HOTS

Why do cells need to ferment when they get 2ATPs from Glycolysis?

4.9.2 Alcohol Fermentation

Alcohol fermentation begins with the Glycolysis which yields two molecules of pyruvic acid and two molecules of ATPs. In the next step, the two molecules of pyruvic acid are converted to two molecules of acetaldehyde and two molecules of CO_2. The acetaldehydes are then reduced by NADH to form ethanol. The ethanol and CO_2 produced by the yeast Saccharomyces is used in alcoholic beverages and to raise bread dough respectively.

4.10 Enzymes

Life is an intricate meshwork involving a perfect coordination of a vast majority of chemical reactions. This is due to the presence of some catalysts synthesized inside the body of the organism. The term 'enzyme' was coined by Friedrich Wilhen Kuhne (1878) to designate these biological catalysts. The name 'enzyme' (en – in, zyme – yeast) literally means 'in yeast'. The name of enzyme usually ends in – ase. Example: Cytochrome

TN_GOVT_XII_Micro_Biology_CH04.indd 57 07-12-2021 15:09:10

dehydrogenase. The study of enzyme is called Enzymology.

Enzymes are proteins or large biomolecules that can catalyze certain biochemical reactions for metabolic process within the cell. The substances that can speed up a chemical reaction without being permanently altered itself are called catalysts. Enzymes accelerate the rate of chemical reactions. The molecule upon which enzyme may act are called substrate and the enzyme convert the substrate into different molecules known as products. The enzyme serves as biological catalyst (Table 4.3).



Tyrosinases are synthesized by *Agaricus bisporus,* which is involved in melanogenesis (pigmentation of skin and hair).

4.10.1 Characteristics of Enzymes

Enzymes

- are highly substrate specific
- are reused at several times
- synthesized within the cells are determined by genes
- speed up the chemical reaction
- decrease the activation energy needed to start
- act as a biocatalyst

Infobits

Proteins have four levels of structure (i) primary (sequence of amino acids), (ii) secondary (regular coils or pleats linked by peptide bonds), (iii) tertiary overall three dimensional structure of a polypeptide linked by disulphide bonds) and (iv) quatenary structure (two or more polypeptides chains). Like all proteins, enzymes are composed of one or more long chain of inter connected amino acids.

Table 4.3: Enzyme Classification Based on Type of Chemical Reaction

Class	Type of Chemical Reaction	Reactions	Examples
Oxido-reductase	Oxidation-reduction in which oxygen and hydrogen are gained or lost	$Xr_{ed} + Y_{ox} \longrightarrow X_{ox} + Y_{re}d$	Cytochrome oxidase, lactate dehydrogenase
Transferase	Transfer of functional groups, such as an amino group, acetyl group, or phosphate group	$X - P + Y \longrightarrow X + Y - P$	Acetate kinase, alanine deaminase, transaminase, phosphotransferase
Hydrolase	Hydrolysis (addition of water)	$X - Y + H_2O \longrightarrow X - H + Y - OH$	Lipase, sucrose
Lyase	Removal of groups of atoms without hydrolysis	$X - Y \longrightarrow X + Y$	Oxalate decarboxylase, isocitrate, lyase
Isomerase	Rearrangement of atoms within a molecule	$X - Y - Z \longrightarrow X - Z - Y$	Glucose-phosphate isomerase, alanine racemase
Ligase	Joining of two molecules (using energy usually derived from breakdown of ATP)	$X + Y + ATP \longrightarrow X - Y + ADP + pi$	Acetyl-CoA synthetase, DNA ligase

TN_GOVT_XII_Micro_Biology_CH04.indd 58 07-12-2021 15:09:10



Low level of catalase plays a major role in greying process of human hair.

4.10.2 Structure of Enzymes

Enzymes are generally globular proteins that range in molecular weight from about 10,000 to several million. Each enzyme possesses a unique sequence of amino acid that causes it to fold into a characteristic three dimensional shape with a specific surface configuration. This enables it to find the correct substrate from large number of diverse molecules in the cell.

A molecule acted upon by an enzyme is called a substrate. Enzymes are specific and act on specific substrates and each enzyme catalyzes only one reaction. Enzyme consists of a protein portion, named apoenzyme and a non protein component, named cofactor (Figure 4.11).

Apoenzyme (Protein portion) (Inactive form)	+	Cofactor (Non protein) (Inactive form)	→	Holoenzyme (Active enzyme)

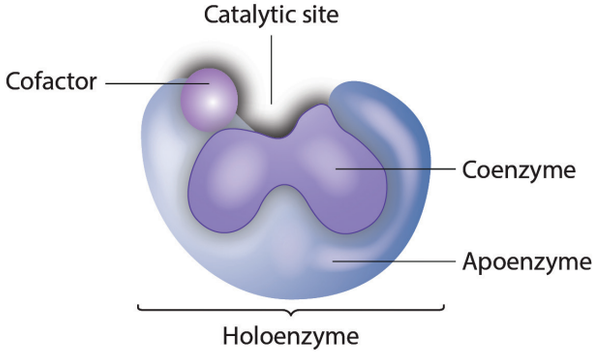


Figure 4.11: Structure of enzyme

The region of an enzyme where substrate molecules bind and undergo a chemical reaction is its active site. Each active site is specially designed in response to their substrate; as a result most enzymes have specificity and can only react with particular substances. After the formation of enzyme substrate complex (Figure 4.12), forces exerted on the substrate by the enzyme cause it to react and become the product of the intended reaction.

Example: Sucrase catalyses the hydrolysis of sucrose to glucose and fructose.

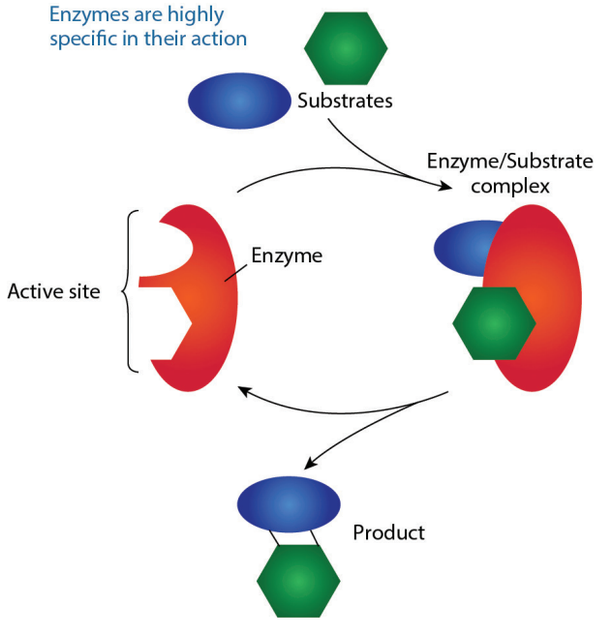


Figure 4.12: Mechanism of product formation from substrate through Enzyme

Apoenzyme is the inactive form of the enzyme which gets activated after binding with a cofactor. Coenzymes are small organic molecules that can be loosely bound to an apoenzyme and they transport chemical group from one enzyme to another.

Cofactor is a chemical compound or metallic ion that is required for enzyme activity. Example: NAD^+ is derived from

TN_GOVT_XII_Micro_Biology_CH04.indd 59 07-12-2021 15:09:10

vitamin B. Some cofactors are metal ions including iron (Fe), copper (Cu), magnesium (Mg), manganese (Mn), Zinc (Zn), calcium (Ca) and cobalt. If the cofactor is tightly or firmly attached to the apoenzyme it is called a prosthetic group. The prosthetic group may be organic [such as vitamin, sugar, and lipid] or inorganic [such as metal ion] but is not composed of amino acids.

The complete enzyme consisting of the apoenzyme and its cofactor is called the holoenzyme.

4.10.3 Microbial Enzymes

Many microbes synthesize and excrete large quantities of enzymes into the surrounding medium. Using this feature of these tiny organisms many enzymes like Amylase, Cellulase, Catalase, Protease, and Lipase are produced commercially.

Microbial enzymes are extensively used in food processing, preservation, washing powder preparation, leather industry, and paper industry and in scientific research (Table 4.4).

Table 4.4: Industrial application of microbial enzymes

Industries	Enzymes	Microbial Sources	Application
Pharmaceutical industry	• Glucose oxidase • Streptokinase • Protease • coagulase	• *Penicillium notatum* • *Streptococci* • *Clostridium spp* • *Staphylococcus aureus*	• To detect free glucose level in diabetic patients • Anti coagulants • Conversion of fibrinogen to fibrin
Dairy Industry	• Catalase • Lactase	• *Aspergillus niger* • *Lactobacillus spp*	• Remove Hydrogen peroxide in milk (detoxification) • Increase sweetness in milk
Baking Industry	• Amylase • Lipase	• *Bacillus subtilis* • *Candida Lipolytica*	• Increase bread shelf life • Enhances flour quality and dough stability
Polymer Industry	• Lipase • Peroxidase	• *Candida spp* • *Pseudomonas spp*	• Polyester preparation • Formation of cross links
Leather Industry	• Protease Lipase	• *Bacillus spp* • *Aspergillus spp*	• Unbarring of hides, degreasing and softening of leather
Textile Industry	• Cutinase • Collagenase • Laccase	• *Pseudomonas spp* • *Clostridium histolyticum*	• Cotton Scouring • Wool Finishing • Bleach termination • Fabric dyeing
Recombinant DNA technology	• DNase • Ligase	• *Escherichia coli* • *Actinomycetes*	• Nuclease enzyme that break phosphodiester bond of DNA or RNA • Joins the nick in DNA fragments

TN_GOVT_XII_Micro_Biology_CH04.indd 60 07-12-2021 15:09:10

Infobits

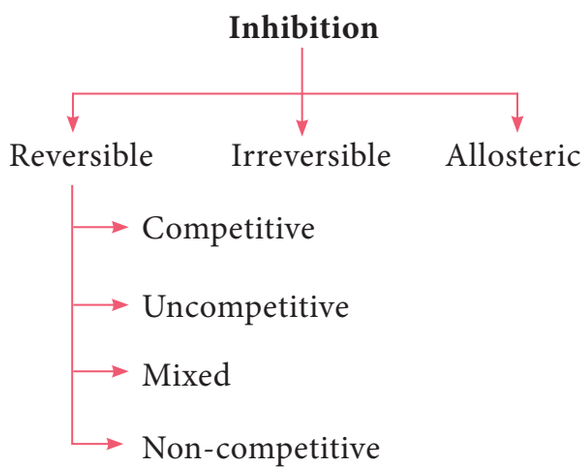
Idoenella sakaiensis is a bacterium capable of breaking down PET plastics. The bacterium first uses PETase to break down the PET plastic. This has potential importance in the recycling process of PET plastics.



Lipase is used in the determination of triglyceride and blood cholesterol level. Lipase producing microorganism have been found in industrial wastes, vegetable oil processing factories, diary plants and soil contaminated with oil.

4.10.4 Enzyme Regulation

Inhibitors: An enzyme inhibitor is a molecule that binds to an enzyme and decreases its activity (Flowchart 4.1). This adverse affect of inhibitors on the rate of enzymatically catalyzed reactions are called inhibition.



Flowchart 4.1: Types of Inhibition

An irreversible inhibitor inactivates an enzyme by binding covalently to a particular group at the active site. A reversible inhibitor inactivates an enzyme by non covalent, more easily reversible interactions. Competitive inhibitor is any compound that bears a structural resemblance to a particular substrate for binding at the active site of an enzyme. Non competitive inhibitors do not compete with the substrate for the enzyme's active site; instead, they interact with another part of the enzyme. Uncompetitive inhibitors bind only to the enzyme substrate complex without binding to the free enzyme (Figure 4.13).

a. Competitive Inhibition

b. Non-competitive Inhibition

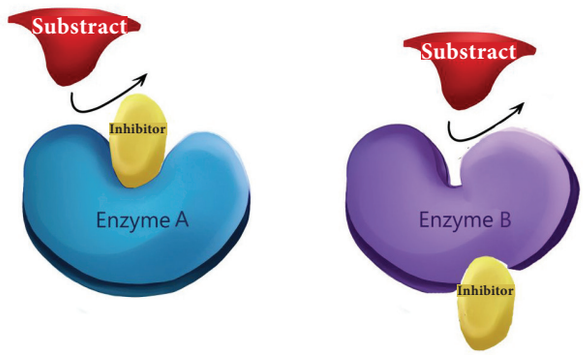


Figure 4.13: Competitive and non-competitive inhibition



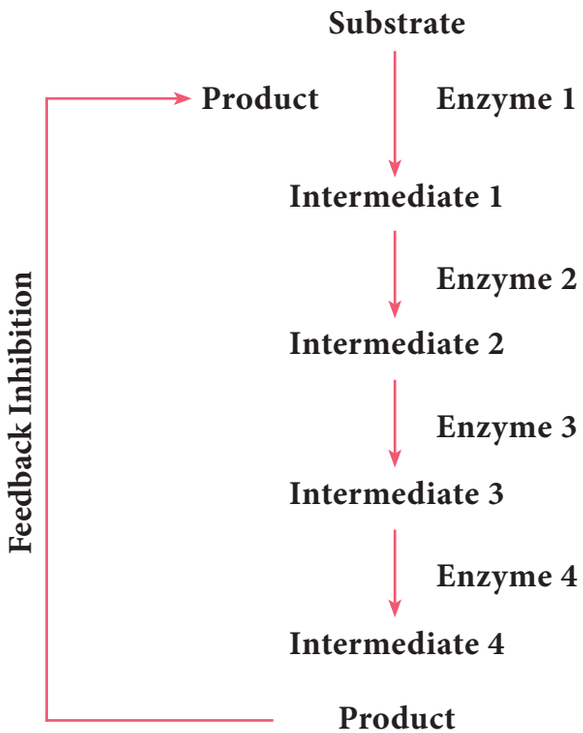
Administration of the enzyme DNase I to the lungs of cystic fibrosis patients decrease the viscosity of the mucus and the breathing is made easier.

Feedback inhibition

In Feedback inhibition, the final product allosterically inhibits the enzyme that catalyses the first stage in the series

TN_GOVT_XII_Micro_Biology_CH04.indd 61 07-12-2021 15:09:10

of reactions. This process is used to regulate the synthesis of amino acids (Flowchart 4.2). Example: Threonine deaminase is the first enzyme in the conversion of Threonine to Isoleucine. Isoleucine inhibits Threonine deaminase through feedback inhibition.



Flowchart 4.2: Feedback Inhibition

4.10.5 Uses of Microbial Enzymes

Microbial enzymes are

- helpful to save energy and prevent pollution
- highly specific
- be immobilized and reused
- inexpensive and more stable
- easily extracted and purified
- genetically manipulated to yield higher quality

Summary

The sum of all chemical reactions within a living organism is known as Metabolism. Biochemical pathway that functions in both anabolism and catabolism are called amphibolic pathways, meaning that they are dual purpose. The energy of catabolic reactions is used to drive anabolic reactions. The energy for chemical reactions is stored in ATP. The chemical reactions are catalyzed by different enzymes. Enzymes catalyze chemical reactions by lowering the activation energy. Most of the cells energy is produced from the oxidation of carbohydrates. During respiration organic molecules are oxidized. Energy is generated from the ETC. In aerobic respiration, O_2 function as the final election acceptor. In anacrobic respiration, the final electron acceptor is an inorganic molecule NO2-, SO42- other than O_2.

Complete oxidation of glucose molecule takes place in 3 sequential reactions.

- Glycolysis occurring in cytoplasm
- Krebs cycle occurring is mito chondrial matrix

ETC (Oxidative Phosphorylation) occurring is inner mitochondrial matrix. In aerobic prokaryotes, 38 ATP molecules can be produced from complete oxidation of a glucose molecule in glycolyins, krebscycle, and ETC. In eucaryotes 36 ATP molecules are produced from complete oxidation of a glucose molecule. In incomplete oxidation of glucose molecules will revolt in fermentation, O2 in anaerobic condition. Various commercial products are produced from pyruvic acid. Lipid can be catabolised by lipase which hydrolyze lipid into glycerol and fatty acid. Then fetly acids are catabolised by Beta oxidation. Proteins can be catabolised by Deamination and

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Transamination process into amino acids. Carbohydrate, Fat, Protons can all be the source of electrons and protons for respiration. Microbial enzymes are extensively used in food processing, preservation, paper industry and in scientific research.

Evaluation

Multiple choice questions

1. High energy transfer compounds are capable of
   a. Accepting large amount of free energy
   b. Transferring large amount of free energy
   c. Measuring free energy
   d. None of the above



2. In an aerobic respiration the terminal electron acceptor is
   a. oxygen
   b. nitrogen
   c. hydrogen
   d. nitrate
3. Utilizable energy or energy is available to do work is termed as
   a. free energy
   b. Utilisable energy
   c. Kinetic energy
   d. Thermal energy
4. The reactant in glycolynis is
   a. Pyruvic acid
   b. Citric acid
   c. glucose
   d. Glucose-6-phosphate
5. The correct sequence of anaerobic reactions in yeast is
   a. Glucose $\xrightarrow{\text{Cytoplasm}}$ pyruvate $\xrightarrow{\text{Mitochondria}}$ Ethanol + CO_2
   b. Glucose $\xrightarrow{\text{Cytoplasm}}$ pyruvate $\xrightarrow{\text{Cytoplasm}}$ Lactic acid
   c. Glucose $\xrightarrow{\text{Cytoplasm}}$ pyruvate + Energy $\xrightarrow{\text{Mitochondria}}$ $CO_2 + H_2O$
   d. Glucose $\xrightarrow{\text{Cytoplasm}}$ pyruvate $\xrightarrow{\text{Cytoplasm}}$ Ethanol + CO_2
6. For every one molecule of sugar glucose which is oxidized _____ molecule of pyruvic acid are produced.
   a. 1 b. 2 c. 3 d. 4
7. Assertion (A) : In substrate level phosphorylation ATP is generated when a high energy phosphate is directly transferred from a phosphorylated compound (substrate) to ADP.

   ADP ATP

   Reason (R) : Phosphoenol pyruvic acid ⟶ pyruvic acid
   a. A is true, A is supported by R
   b. A is false, but R is not supported by A
   c. Both A and R are false
   d. A is true, R is false
8. Which one of the following is correct
   a. Apoenzyme + Cofactor = Holoenzyme
   b. Holoenzyme + Coenzyme = Apoenzyme
   c. Apoenzyme + Holoenzyme = Coenzyme
   d. Coenzyme + Cofactor = Holoenzyme

TN_GOVT_XII_Micro_Biology_CH04.indd 63 07-12-2021 15:09:10

9. Identify the correct match.

a. Catalase	-	1. Detect Blood glucose level
b. Glucose oxidase	-	2. Break down of H_2O_2
c. Protease	-	3. Clot the plasma
d. Coagulase	-	4. Leather manufacture

10. Statement A: Oxidation of glucose to pyruvic acid yield only 4 ATP by substrate level phosphorylation.

    Statement B: The total ATP which is produced through TCA is 24.

    a. Statement A is true, B is false

    b. Both A and B are true

    c. A is false, B is true

    d. Both A and B are false

Answer the following

1. Define metabolism
2. Write the difference between catabolism and Anabolism.
3. ATP is an energy storage compound, where does it get this energy from?
4. What is holoenzyme?
5. What is Active site?
6. Explain the structure of ATP.
7. Write about the types of phosphorylation.
8. Mention the classification of enzymes based on chemical reaction.
9. Define fermentation.
10. Write about the types of fermentation with few examples.
11. Mention the importance of the enzymes.
12. Write short note on the component of the enzyme.
13. Explain the enzyme regulation mechanism.
14. Explain EMP pathway or glycolytic pathway.
15. Describe TCA cycle.
16. Explain electron transport chain.
17. These are three mechanism for the phosphorylation of ADP to ATP. Write the name of the mechanism in the following reaction given below.

1	An electron liberated from chlorophyll by light, is passed down an ETC	?
2	Cytochrome c passes two electrons to cyt a	?
3	Phosphoenol pyruvic acid ↓ pyruvic acid	?

18. Name the stages of aerobic respiration?

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Chapter 5

Food Microbiology

Learning Objectives

After studying this chapter the students will be able to,

- *Know the sources of microorganisms in food*
- *Understand the factors that influence growth of microorganisms in food*
- *Learn about the food spoilage*
- *Appreciate the food preservation methods*
- *Learn and compare food poisoning and food intoxication*
- *Classify the food borne diseases*
- *Understand the microbial standards and grading of milk*
- *Know the fermented milk products like cheese, yoghurt and curd*

Chapter Outline

5.1 Food Microbiology
5.2 Food Spoilage
5.3 Food Borne Disease
5.4 Food Preservation Methods
5.5 Dairy Microbiology
5.6 Cheese
5.7 Yogurt
5.8 Curd

5.1 Food Microbiology



The field of food microbiology is very broad, encompassing the study of microorganisms which have both beneficial and deleterious effects on the quality and safety of raw and processed foods. The primary tool of microbiologists is the ability to identify and quantitate food-borne microorganisms. Microorganisms in food include bacteria, molds, yeasts, algae, viruses, parasitic worms and protozoans.

Microorganisms are associated with the food we eat in a variety of ways. They may influence the quality of our food. Naturally occurring foods such as fruits and vegetables normally contain some microorganisms and may be contaminated with additional organisms during handling and processing. Food can serve as a medium for the growth of microorganism, and microbial growth may cause the food to undergo decomposition and spoilage. Food may also carry pathogenic microorganisms which when ingested can cause disease. When food with microorganisms that produce toxic substances is ingested, it results in food poisoning. Some microorganisms are used in the preparation and preservation of food products.

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FSSAI: Food Safety and Standards Authority of India (FSSAI) is an autonomous body established under the ministry of health and family welfare, Government of India. FSSAI maintains the food quality levels in order to ensure safety and provides satisfaction to every consumer.

5.1.1 Classification of Foods

Foods may be classified as

a. Fresh foods

These are foods which have not been preserved and not spoiled yet. For example; vegetables, fruits and meat spoil immediately after harvesting or slaughtering.

b. Preserved foods

Foods are preserved by adding salt, sugar, acetic acids and ascorbic acids. Example: Jam, Pickles. In this way their shelf life is improved.

c. Canned foods

In canning, food products are processed and sealed in the air tight containers. It provides longer shelf life ranging from one to five years. Example: Baked beans, Olives.

d. Processed foods

During food processing, original nature of food is changed or altered. It is done by Freezing, Canning, Baking and Drying. Example: Breakfast cereals, Cakes, Biscuits and Bread.

e. Fermented food products

These foods are subjected to fermentation by the action of microorganisms. Example: Kefir, Cheese.

5.1.2 Sources of Microorganism in Food

The primary sources of microorganisms in food include,

1. Soil and water
2. Plant and plant products
3. Food utensils
4. Intestinal tract of human and animals
5. Food handlers
6. Animal hides and skins
7. Air and dust

5.1.3 Factors that Influence Growth of Microorganisms in Food

Many factors influence the growth of the microorganisms in food. Some of the factors are intrinsic and some others are extrinsic.

1. Intrinsic factors

The intrinsic factors include pH, moisture content, oxidation – reduction potential, nutrient status, antimicrobial constituents and biological structures.

a. pH: Every microorganisms has a minimal or maximal, and an optimal pH for its growth. Microbial cells are significantly affected by the pH of food because they apparently have no mechanism for adjusting their internal pH. In general, yeasts and molds are more acid tolerant than bacteria. Foods with low pH values (below 4.5) are usually not readily spoiled by bacteria and are more susceptible to spoilage by yeast and molds. Most of the microorganisms grow best at pH value around 7.0.

b. Moisture content: The preservation of food by drying is a direct consequence of removal of moisture, without which

TN_GOVT_XII_Micro_Biology_CH05.indd 66 07-12-2021 15:13:28

microorganisms do not grow. The water requirement of microorganism is defined in terms of the water activity (aw) in the environment. Water activity is defined as the ratio of the water vapour pressure of food substrate to the vapour pressure of pure water at the same temperature. The water activity of most fresh food is above 0.99. The minimum value of aw for the growth of the microorganisms in foods should be around 0.86.

c. Oxidation reduction (O/R) potential

The oxygen tension or partial pressure of oxygen around a food and the O-R potential or reducing and oxidizing power of the food itself influence the type of organisms which can grow and the changes produced in the food. The O-R potential of the food is determined by,

i. The O-R potential of the original food.

ii. The poisoning capacity (the resistance of the food against change).

d. Nutrient Content

The kinds and proportions of nutrients in the food are all important in determining what organism is most likely to grow. Consideration must be given to (i) foods for energy (ii) foods for growth and (iii) accessory food substances or vitamins which may be necessary for energy or growth.

e. Antimicrobial constituents

The stability of foods against attack by microorganism is due to the presence of certain naturally occurring substances that have been shown to have antimicrobial activity. Some species contain essential oils that possess antimicrobial activity. Among these are allicin in garlic, eugenol in cloves and cinnamon.

2. Extrinsic factors

These include those properties of the storage environment that affect both the foods and microorganisms present in them. Storage temperature, pH, presence and concentration of gases in the environment are some of the extrinsic factors that affect the growth of microorganisms.



Food Corporation of India: FCI is an organization created and run by Government of India. FCI is a statutory body established through Food Corporation Act, 1964 to meet the following objectives of food policy. Effective price support operations for safeguarding the interests of the farmers.

5.2 Food Spoilage

Spoilage of food can be defined as any visible or invisible change which can make food or product derived from food unfit for human consumption. Spoilage of food not only causes health hazard to the consumer but also causes great economic losses. Spoilage leads to loss of nutrients from food and cause change in original flavor and texture. It is estimated that about 25% of total food produced is spoilt due to microbial activities despite a range of preservation methods available. Food spoilage is considered as a complex phenomenon where by a combination of microbial and bio-chemical activities take place. Due to such activities various types of metabolites are formed which aid in spoilage (Figure 5.1).

TN_GOVT_XII_Micro_Biology_CH05.indd 67 07-12-2021 15:13:28



Figure 5.1: Food spoilage

i. Perishable foods

These foods are readily spoiled; require special preservation and storage condition for use. This includes, foods such as dairy products, eggs, poultry, meat, fish, fruits and vegetable. These foods get spoiled easily by natural enzymes.

ii. Semi – perishable foods

This class of foods if properly stored can be used for a longer duration. These foods include processed cereals, pulses and their products like flour, semolina, parched rice and popcorn. Shelf life of these products depends on the storage temperature and moisture in the air. Foods like potato, onion, nuts, frozen foods and certain canned foods can be stored for a week to a couple of months at room temperature without any undesirable changes in the products.

iii. Non – perishable foods

These foods remain stable for long period unless handled improperly. Non-perishable foods include sugar, jaggery, hydrogenated fat, vegetable oil, ghee, whole grains, dhals, whole nuts and processed foods like dry salted fish/meat, papads, canned foods, jams and murabbas. These foods do not spoil unless they are handled carelessly.

5.2.1 Causes of Food Spoilage

Food and water may be infected by germs. Fly carries germs to food. There are various factors which are responsible for food spoilage such as.

- Microorganism
- Insects
- Rough handling
- Transport
- improper storage
- enzyme activity (Chemical reaction)
- unhygienic conditions
- physical changes, such as those caused by freezing, burning, drying pressure.

Signs of food spoilage include difference in appearance from the fresh food such as a change in colour, a change in texture and an unpleasant odour or taste.

HOTS

1. Why do concentrated citrus juices prevent spoilage problems?
2. Name a few organisms responsible for food spoilage.

TN_GOVT_XII_Micro_Biology_CH05.indd 68 07-12-2021 15:13:32

5.3 Food Borne Disease

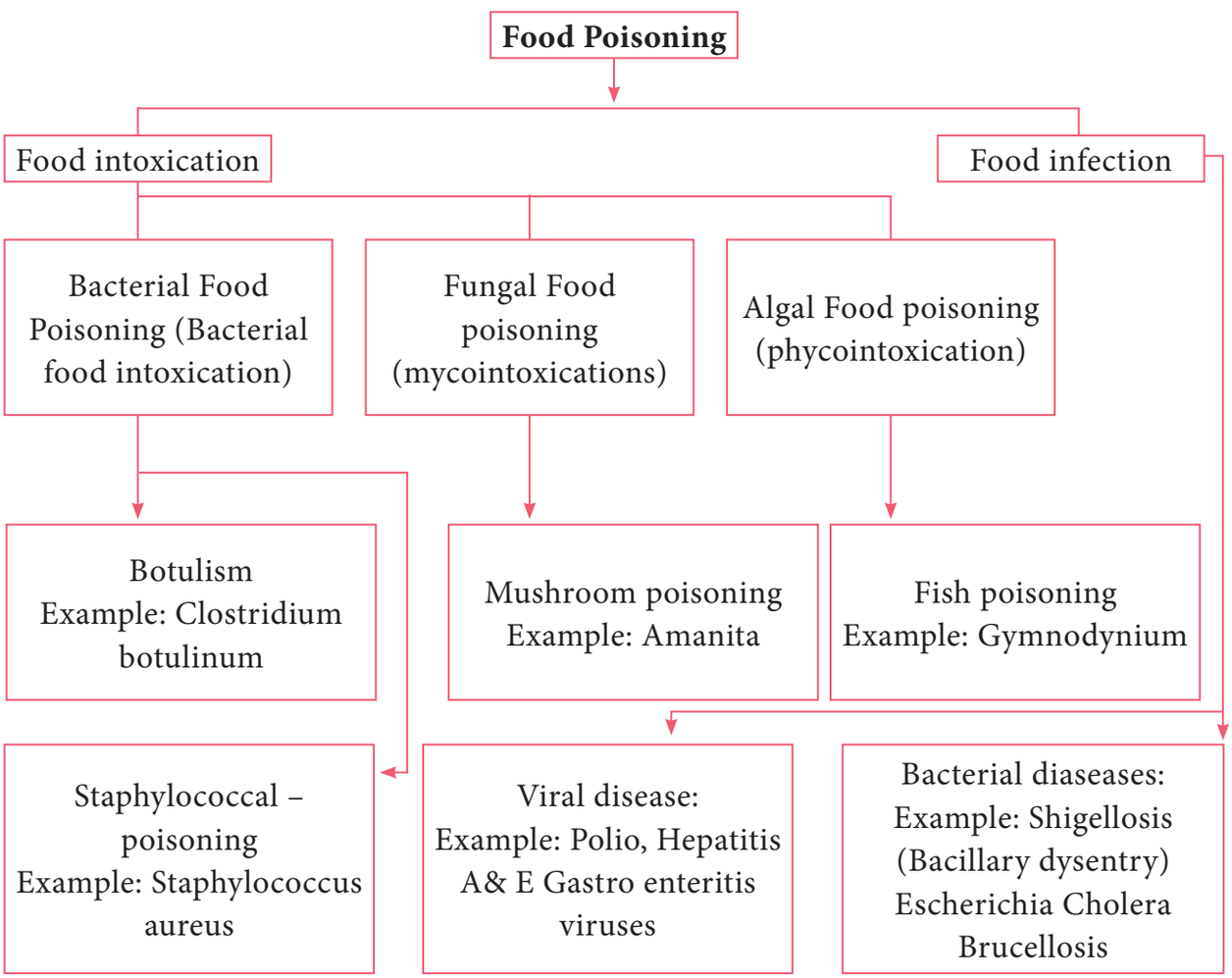
Food borne disease has been defined by the world health organization (WHO) as a disease of an infectious or toxic nature caused by or thought to be caused by the consumption of food or water. The term "food poisoning" as applied to diseases caused by microorganisms is used very loosely to include both illness caused by the ingestion of toxins elaborated by the organisms and those resulting from infection of the host through the intestinal tract. A further classification of food borne disease is shown in flowchart 5.1.

All these food – borne diseases are associated with poor hygienic practices. Whether by water or food transmission, the fecal – oral route is maintained, with the food providing the vital link between hosts. Fomites, such as sink faucets, drinking cups, and cutting boards, also play a role in the maintenance of fecal – oral route of contamination.

There are two primary types of food related diseases: food – borne infections and food intoxications or food poisoning.

HOTS

Name some of the uncooked foods that have been implicated in food borne disease transmission?



Flowchart 5.1: Types of Food – Poisoning

TN_GOVT_XII_Micro_Biology_CH05.indd 69 07-12-2021 15:13:32

5.3.1 Food Borne Infection

Food borne infection involves the ingestion of the pathogen followed by growth in the host, including tissue invasion and/or the release of toxins. The major diseases of this type are summarized in table (5.1).

5.3.2 Food Poisoning

Food borne intoxication (or) food poisonings is caused by ingesting food containing toxins formed by bacteria which resulted from the bacterial growth in the food item. Food poisoning refers to the toxicity introduced into food by microorganisms and their products.

Microbial growth in food products also can results in food intoxication. Intoxication produces symptoms shortly after the food is consumed because growth of the disease – causing microorganism is not required. Toxins produced in the food can be associated with microbial cells or can be released from the cells.

Food poisoning is caused by various factors as follows.

1. Microorganism of plant food products.
2. Microorganism of Animal food products.
3. Microorganism of processed food.
4. Standard chemicals added to the food.
5. Excess use of preservatives in food.
6. Presence of higher population of Microorganism in food.

Table 5.1: Major Food – Borne Infectious Diseases

S.No	Disease	Organism	Incubation period and characteristics	Major Foods Involved
1.	Salmonellosis	*Salmonella typhimurium* *S. enteritidis*	8–48 hr Enterotoxin and cytotoxins	Meat, poultry, Fish, eggs, dairy product.
2.	Campylobacteriosis	*Campylobacter jejuni*	Usually 2–10. Most toxin heat labile	Milk, or, poultry product, water.
3.	Listeriosis	*L. monocytogenes*	Varying periods. Related to meningitis and abortion newborns and the elderly.	Meat products, especially pork and milk.
4.	Diarrhea and colitis	*Escherichia coli*, includes serotype 0157:H7	24–72 hrs Entero toxigenic Positive and negative strains : hemorrhagic colitis	Cooked ground beef, raw milk
5.	Shigellosis	*Shigella sonnei*, S. *flexneri*	24–72 hrs	Egg products, puddings
6.	Acute gastroenteritis	*Vibrio parahaemolyticus*	16–48 hr	Sea food, shellfish

TN_GOVT_XII_Micro_Biology_CH05.indd 70 07-12-2021 15:13:32

7. Toxin produced by various types of Microorganism.

5.4 Food Preservation Methods

Foods can be preserved by a variety of methods. It is vital to eliminate or reduce the populations of spoilage and disease – causing microorganisms and to maintain the microbiological quality of a food with proper storage and packaging. Contamination often occurs after a package or can is opened and just before the food is served. This can proved an ideal opportunity for growth and transmission of pathogens, if care is not taken. Preservation of food is the process by which food is stored by special methods. Cooked or uncooked food can be preserved in different ways to be used later Table 5.2. Some methods of preservation are:

1. Freezing

Food kept in a refrigerator remains fresh for some day. Germs do not grow easily in cool places. We preserve food items, like milk, fruit, vegetable and cooked food by keeping them in a refrigerator.

2. Boiling

By this method, we can preserve food for a short period of time. Germs in milk are killed by pasteurization. It is done by boiling milk for sometimes and then cooling it quickly.

3. Salting

Add salt to preserve pickles and fish.

4. Sweetening

Sugar act as a preservative when added in large quantities. For example, food can be stored for a long time in the form of jams, jellies and murabbas (Figure 5.2) by adding sugar.



Figure 5.2: Murabbas

5. Drying

In this method, the food items are dried in sun to stop the growth of bacteria in them. Certain foods, like raw mangoes, fishes, potato chips and papads are preserved by this method.

6. Canning

In this method, food is processed and sealed in airtight cans. Food items like vegetables, seafood, and dairy product are preserved through this method.

Advantages of food preservation

- Germs do not grow easily in preserved food and make it safe to eat.
- Preservation enables us to enjoy seasonal fruits like strawberries and mangoes even during the off season.

Disadvantages

- Excess salt and sugar are used in the preservation of food which is not good for health.
- Some methods of food preservation may lead to loss of nutrients.

TN_GOVT_XII_Micro_Biology_CH05.indd 71 07-12-2021 15:13:32

Table 5.2: Basic Approaches to Food preservation

S.No	Approach	Examples of process
1.	Removal of microorganisms	Avoidance of microbial contamination, physical filtration, centrifugation.
2.	Low temperature	Refrigeration, Freezing
3.	High temperature	Partial or complete heat inactivation of microorganisms (pasteurization and canning
4.	Reduced water availability	Water removal, as with Lyophilization or freeze drying use of spray dryers or heating drums decreasing water availability by addition of solutes such as salt or sugar.
5.	Chemical – based preservation	Addition of specific inhibitory compounds (Example: organic acids, nitrates, sulfur dioxide)
6.	Radiation	Use of ionizing (gamma rays) and non ionizing (UV) radiation
7.	Microbial product – based inhibition	The addition of substances such as bacteriocins to foods to control food – borne pathogens

Infobits

"Typhoid Fever and Canned Meat"

Minor errors in canning have led to major typhoid outbreaks. In 1964 canned beef produced in South America was cooled, after sterilization with non chlorinated water. The vacuum created when the cans were cooled drew *Salmonella typhi* into some of the cans, which were not completely sealed. This contaminated product was later sliced in an Aberdeen, Scotland, Food store and the meat slicer became a continuing contamination source the result was a major epidemic that involved 400 people. The *Salmonella typhi* was a South American strain and eventually the contamination was traced to the contaminated water used to cool the cans. This emphasizes the importance of careful food processing and handling to control the spread of disease during food production and preparation.



Principles of Food preservation

In accomplishing the preservation of foods by the various methods, the following principles are involved.

1. Prevention or delay of microbial decomposition.
   a. By keeping out microorganism (asepsis)
   b. By removal of microorganism. Example: Filtration
   c. By hindering the growth and activity of microorganism Example: Low temperature, drying, anaerobic conditions or chemicals.
   d. By killing the microorganism Example: Heat or radiation
2. Prevention or delay of self – decomposition of the food.
   a. By destruction or inactivation of food enzymes Example: Blanching
   b. By prevention or delay of purely chemical reactions Example: Prevention of oxidation by means of antioxidants.

TN_GOVT_XII_Micro_Biology_CH05.indd 72 07-12-2021 15:13:33

3. Prevention of damage because of insects, animals, mechanical causes, etc.

5.5 Diary Microbiology

The area of dairy microbiology is large and diverse. The bacteria in dairy products may cause disease or spoilage. Some bacteria may be specifically added to milk for fermentation to produce products like yoghurts and cheese (Figure 5.3).



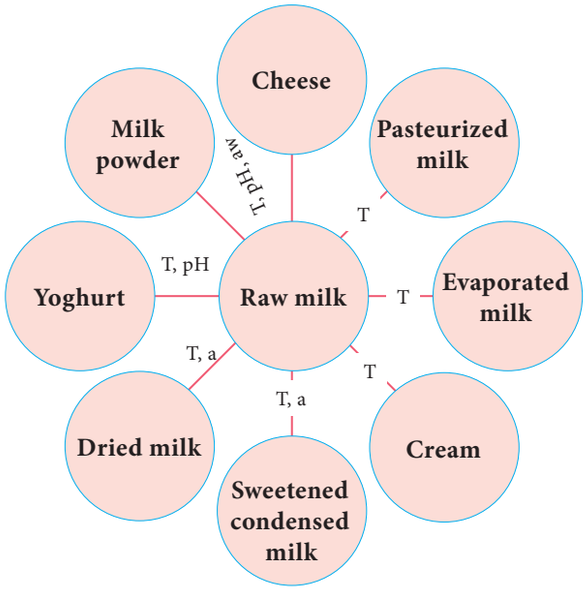
Figure 5.3: Yoghurts and cheese

5.5.1 MILK

Milk is the fluid, secreted by mammals for the nourishment of their young ones. It is in liquid form without having any colostrum. The milk contains water, fat, protein and lactose. About 80–85% of the protein is casein. Due to moderate pH (6.4–6.6), good quantity of nutrients and high water content, milk an excellent nutrient for the microbial growth. (Flowchart 5.2).

5.5.2 Composition and Properties

Milk is considered to be the "Most nearly perfect" food for man and hence is one of the most important ingredients of the



pH–Hydrogen ion concentration

T–Elevated temperature

H–Reduced water pressure

aw–water activity

Flowchart 5.2: Various products obtained from raw milk

diet. It is an extremely complex mixture and usually contains (Table 5.3).

5.5.3 Sources of Microorganisms in Milk

- Three sources contribute to the microorganism found in milk the udder interior, the teat exterior and its immediate surroundings, and the milking and milk handling equipment.
- Bacteria that get on to the outside of the teat may be able to invade the opening and hence the udder interior. The organisms most commonly isolated are *Micrococcus, Streptococci* and the diptheroid *Corynebacterium bovis*. Aseptically taken milk from a healthy cow normally contains low number of organisms, typically fewer than 10^2–10^3 cfu ml^{-1}

TN_GOVT_XII_Micro_Biology_CH05.indd 73 07-12-2021 15:13:34

Table 5.3: Complex mixture

S.No	Composition	Approximate percentage
1.	Liquid (Water)	87%
2.	Solids	13%
3.	Fat	4%
4.	Protein	3.3%
5.	Lactose (Milk Sugar)	5%
6.	Ash content (Vitamins and minerals)	0.7%

- The udder exterior and its immediate environment can be contaminated with organisms from the cow's general environment. Heavily contaminated teats have been reported to contribute up to 10^5 cfu ml^{-1} in the milk. Contamination from bedding and manure can be source of human pathogens such as *E.Coli, Campylobacter, Salmonella, Bacillus spp.* and *Clostridia spp.*
- Milk – handling equipment such as teat cups, pipe work, milk holders and storage tanks is the principal source of the microorganisms found in raw milk. *Micrococcus* and *Enterococcus*.

5.5.4 Microbiological Standard and Grading of Milk

In India, raw milk is graded by Bureau of Indian standards (BIS) 1977. The Indian standard institute (ISI) has prescribed microbiological standard for quality of milk.

- Coliforms count in raw milk is satisfactory if, coliforms are absent in 1:100 dilution.
- Coliforms count in pasteurized milk is satisfactory is coliforms are absent in 1: 10 dilution (Table 5.4).

Grading of milk

The quality of milk is judged by certain standards and it is known as grading milk. Grading of milk is based upon regulations pertaining to production, processing and distribution. This includes sanitation, pasteurization, holding conditions and microbiological standards. The U.S public health secrine publication "Milk ordinance and code" shows the following chemical, bacteriological and temperature standards for grade A milk and milk products.

Table 5.4: Microbiological Standard and Grading of Milk

S.No	Product	Temperature	Bacterial count/ml	Chemical and others
1.	Grade A raw milk for pasteurization	Cooled to 50°F and maintained there at until processed	Individual producer milk should not exceed 100,000/ml prior to combining with other produce of milk	Antibiotics should be less than 0.05 unit/ml
2.	Grade A pasteurized milk products	Cooled to 45°F or less	Milk and Milk products 20,000/ml coliforms limit not exceeding 10/ml	Phosphates less than 1mg/ml

TN_GOVT_XII_Micro_Biology_CH05.indd 74 07-12-2021 15:13:34

5.5.5 Methylene Blue dye Reduction Test (MBRT)

Methylene blue dye reduction test commonly known as MBRT test is used as a quick method to access the microbiological quality of raw and pasteurized milk. This test is based on the fact that the blue colour of the dye solution added to the milk get decolorized when the oxygen present in the milk get exhausted due to microbial activity. The sooner the de colorization, more inferior is the bacteriological quality of milk assumed to be MBRT test may be utilized for grading of milk which may be useful for the milk processor to take a decision on further processing of milk.

Procedure

The test has to be done under sterile conditions. Take 10ml milk sample in sterile MBRT test tube. Add 1 ml Methylene Blue dye solution (dye concentration 0.005%). Stopper the tubes with sterilized rubber stopper and carefully place them in a test tube stand dipped in a serological water bath maintained at 37°C, records this time as the beginning of the incubation period. Decolourization is considered complete when only a faint blue ring (about 5mm) persists at the top (Figure 5.4).

Recording of Results – During incubation, observe colour changes as follows:

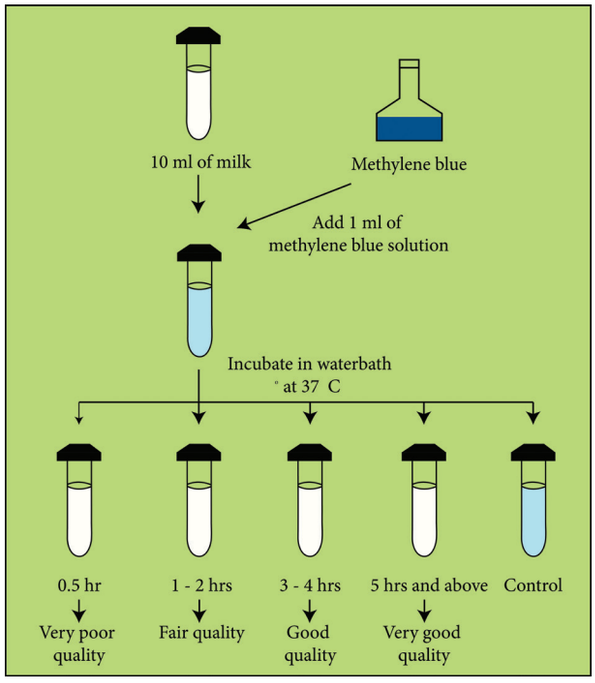


Figure 5.4: Methylene Blue dye Reduction Test

a. If any sample is decolourized on incubation for 30 minutes, record the reduction time as MBRT 30 minutes.

b. Record such readings as, reduction times in whole hours. For example, if the colour disappears between 0.5 and 1.5 hour readings, record the result as MBRT 1 hour, similarly, if between 1.5 and 2.5 hours as MBRT-2 hour and so on.

c. Immediately after each, reading, remove and record all the decolourized samples and then gently invert the remaining tubes if the decolourization has not yet begun (Table 5.5).

Table 5.5: Microbiological Quality of Milk

S.No	Grade	Methylene blue reductase test in hrs	Total plate count/ml
1.	Very good	5 and above	Not exceeding 0.2 million
2.	Good	3–4	Between 0.2 to1.0 million
3.	Fair	1–2	Between 1–5 million
4.	Very poor	0.5	over 5 million

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Infobits

Major categories and Examples of Fermented Milk Products

Category	Typical Examples
i. Lactic Fermentations	Buttermilk cultured buttermilk Langofil, Tetmjolkymer
Mesophilic	Yogurt, Laban, Zabadi Bulgarian buttermilk
Thermophilic	Bifighurt, Acid ophilur milk, Yakult
Therapeutic	
ii. Yeast-lactic fermentations	Kefir, Koumiss, Acidophilus-yeast milk
iii. Mold – lactic fermentations	Villi

5.6 Cheese

There are about 2000 varieties of cheese made from mammalian milk. Cheese is thought to have originated in south western Asia some 8000 years ago. The Romans encouraged technical improvements and stimulated the development of new varieties during their invasion in Europe between 60 B.C and A.D. 300. The cheese name is derived from Latin name *caseus* (Figure 5.5).



Figure 5.5: Cheese

They are two groups of cheese, fresh cheese and ripened cheese. The fresh cheese are made up of milk coagulated by acid or high heat. Example: cottage cheese. Ripened cheese are made through lactic acid bacterial fermentation and coagulated by an enzyme preparation. The curd is removed and salted and whey is separated. The salted curd is held in controlled environment. During this process, various physical and chemical changes occur to give a characteristic flavour and texture. So the mammalian origin of milk influences the flavour and aroma of a natural ripened cheese.

Microbiology of cheese

A large number of microorganisms plays a role in the ripening process. On the first day of cheese making process, the microbial number in the starting material ranges from one to two billion. Therefore, the production declines because of insufficient oxygen, high acidity and the presence of inhibitory compounds that are produced as the cheese ripens. It is mainly the action of their cellular enzymes on lactose, fat and proteins that creates the ripened cheese flavour. The gas forming culture of *Propionibacterium shermanii* is essential

TN_GOVT_XII_Micro_Biology_CH05.indd 76 07-12-2021 15:13:36



Figure 5.6: Swiss cheese

for giving swiss cheese its eye, or holes and flavour (Figure 5.6).

The specificity of cheese depends upon the varieties of microorganisms used. The process of cheese making, involves nine steps:

a. Preparing the milk
b. Forming a curd.
c. Cutting
d. Cooking
e. Separating the whey
f. Salting the residue
g. Applying microbes
h. Pressing the curd
i. Ripening the young cheese

Types of Cheese

Cheese can be divided among different categories or types, according to their firmness. There are various system for classifying cheese and there are variations within each system (Table 5.6).

5.7 Yoghurt or Bulgarian Milk

Yoghurt is derived from a Turkish word 'Jugurt' which is the most popular fermented milk in the world now – a – days. It is made from milk, skimmed milk or flavoured milk. For the preparation of yoghurt, the milk should be free from contamination. The solid content (not fat should be between 11–15% which can be obtained by adding skin or whole milk powder in fresh

Table 5.6: Types of Cheese

Soft cheese	
	Soft, Smooth and creamy texture. Soft cheese is not pressed or cooked during the manufacturing process. Example: Camembert
Semi-soft cheese	
	A little more firm and compact than soft cheese, the semi-soft category contains the largest variety of cheese. Example: Havarti
Firm cheese	
	Cheese in the category is considered to be an "all purpose" cheese. Cheese is pressed to remove as much whey as possible after the curdling process which creates a firm cheese. Example: Cheddar

TN_GOVT_XII_Micro_Biology_CH05.indd 77 07-12-2021 15:13:37

Hard cheese



Hard cheese has a moisture content of less than 50% due to the cheese being the cheese to lose some of its moisture content and have a stronger flavour. Example: Romano

Blue cheese



Cow, sheep or goats milk with a blue or green-blue mold. The mold is derived from spores from Penicillium roqueforti, Penicillium glaucum or other being injected into the cheese curds. People who are allergic to penicillin are not advised to eat blue cheese. Example: Roquefort

Fresh, un ripened or infant cheese



Fresh cheese is not ripened, aged or fermented during the manufacturing process or at any point during the lifespan of the cheese. Fresh cheese has a very short shelf life. Example: Cottage cheese, Cream cheese.

light or lite cheese



Light cheese is made by reducing the amount of butterfat which makes the cheese rubbery in texture and much less flavourful than full fat versions of cheese. Light cheese has a high moisture content which makes it have a shorter shelf life. Example: Cheese with 7% Milk Fat, Cheddar which 19% Milk fat.

Processed cheese



This cheese is created by melting together blend of grated cheese, milk, milk solids or water, food colouring and seasonings. Example: Processed cheese shies, cheese spreads "swokies".

milk that normally contains 8% solids. the product can be further improved by adding small amount of modified gums which bind water and impart thickening to the product. At this stage the size of the fat particles in the milk should be around 2µm because this improves the milk's viscosity, product's stability. The milk is then heated at 80–90°C for 30 min., starter culture is added to it. Heating improves the milk by inactivating immunoglobulins, remove excessive oxygen to produce micro aerophilic environment which support the growth of starter culture. Besides, heating also induce the interactions between whey or serum proteins and casein which increase yoghurt viscosity. The milk is now cooled to 40–43°C so as to allow fermentation using starter organisms such as *Streptococcus salivarius* sub *sp. thermophilus* and *Lactobacillus delbruckii* sub *sp*. bulgaricus together at a level of 2% by volume (10^6–10^7 cfu/ml). It is to be carried out for about 4h during which lactose is converted into lactic acid, pH decreases to a level of 6.3 – 6.5 to 4.6 – 4.7. The flavour in yoghurt is due to acetaldehyde which should be present at 23 – 41 mg/kg (Figure 5.7).

TN_GOVT_XII_Micro_Biology_CH05.indd 78 07-12-2021 15:13:37



Figure 5.7: Yoghurt



Kefir: Kefir is in fact, fermented milk, produced by a mixed lactic acid bacteria and alcoholic yeast. The microflora responsible is not spread uniformly throughout the milk but is supplemented as discrete kefir grains. The Kefiran i.e. large layers of polysaccharide material folds upon to produce a cauliflower like Florets produce Kefir. The capsular homo fermentative *Lactobacillus kefiranolaciens* produces Kefiran. Lactobacillus Kefir contributes the required effervescence in the product.



5.8 Curd

Curd is a dairy product obtained by curdling or coagulating milk with rennet or an edible acidity substance such as lemon juice or vinegar and then draining off the liquid portion called whey milk that has been left to sour (raw) milk alone or pasteurized milk with added lactic acid bacteria or yeast (Example: *Lactobacillus*



Figure 5.8: Curd

acidophilus) will also naturally produce curds and sour milk cheese is produced this way. The increased acidity causes the milk protein (casein) to tangle into solid masses or curds in cow's milk, 80% of the protein and caseins (Figure 5.8).

Uses

- Enhances healthy digestion
- improves immunity
- For stronger bones and teeth
- Helps to lose weight
- Beauty benefits of curd – for healthy and Radiant skin, prevent premature wrinkles remove dark spots and dandruff.

Summary

Micro organisms are associated, in a variety of ways with all of the food we eat. They may influence the quality, availability and quantity of our food naturally occurring foods such as fruits and vegetables normally contain same micro organisms and may be contaminated with additional organisms during handling. Many factors that influence the growth of the micro

TN_GOVT_XII_Micro_Biology_CH05.indd 79 07-12-2021 15:13:39

organisms in food some of the factors are intrinsic and some others are extrinsic factors. Food poisoning refers to the toxicity introduced into food by micro organism and their product. Food intoxication or food poisoning results from ingestion of foods containing performed microbial toxins. Foods can be preserved by a variety of methods. It is vital to eliminate or reduce the populations of spoilage and disease causing micro organisms and to maintain the micro biological quality of a food with proper storage and packaging.

Food borne disease has been defined by the world health organization (WHO) as a disease of an infectious or toxic nature caused by or thought to be caused by the consumption of food or water. Food borne infection involves the ingestion of the pathogen followed by growth in the host, including tissue invasion the release of toxins. The bacteria in diary products may cause disease or spoilage. Some bacteria may be specifically added to milk for fermentation to produce products like yoghurt and cheese.

Evaluation

Multiple choice questions

1. The primary sources of micro organisms in food include_____

   i. Soil and water

   ii. Food utensils

   iii. Food handlers

   iv. Air and dust

   a. (i) and (ii)

   b. (ii), (iii)

   c. (i), (ii), (iii) and (iv)

   d. None of the above

2. The micro organisms grow best at PH value around _____

   a. 4.0 b. 7.0 c. 3.4 d. 9.2

3. The aw of most fresh food is above _____

   a. 0.99 b. 0.88 c. 0.77 d. 0.66

   O6Z0X

4. The minimum value of aw for the growth of the micro organisms in foods should be around _____

   a. 0.99 b. 0.86 c. 0.78 d. 0.50

5. Choose mismatched pair:

   Asepsis – Keeping out of Micro organisms

   Filteration – Removal of Micro organisms

   Heat (or) Radiation – Killing the Micro organisms

   Prevention of Damage – Blanching

6. Milk is contain _____% of the case in protein.

   a. 90–95% b. 80–85%

   c. 60–65% d. 50–100%

Answer the following

1. What is food spoilage?
2. What is perishable food?
3. Define food poisoning.
4. Define food intoxication.
5. What is food borne infection?
6. List out the sources of micro organism in food.
7. Tabulate the major causes of food spoilage.
8. Explain food poisoning.

TN_GOVT_XII_Micro_Biology_CH05.indd 80 07-12-2021 15:13:39

9. What are the advantage and disadvantages of food spoilage.
10. Write about the bacterial food infection.
11. Explain – Milk.
12. Tabulate – the micro biological quality of milk.
13. What are the factors that influence growth of micro organisms in food?
14. Write about the causes of food spoilage.
15. Classify the Food intoxication?
16. List out the methods of food preservation?
17. What are the principles of food preservation?
18. Classify the food poisoning?
19. Describe the Food-Borne intoxications.
20. Write about bacterial food borne disease?
21. Write about the composition of milk.
22. What are the sources of milk.
23. Write about MBRT.

Student Activity

To study the growth of fungus

Take a piece of bread. Make it moist and keep it in a warm corner of the room for 3–4 days observe it after 3–4 days. Record your observation.

To understand the principle of food preservation

Take two apples. Keep one apple in the fridge and one outside for 2–3 days. Record your observation.

TN_GOVT_XII_Micro_Biology_CH05.indd 81 07-12-2021 15:13:39

Chapter 6

Industrial Microbiology

Learning Objectives

After studying this chapter the students will be able to,

- *Know the concepts involved in industrial microbiology and the production of industrially important products by microorganisms.*
- *Understand the primary and secondary screening process.*
- *Gain knowledge in the field of strain improvement of microorganisms.*
- *Describe the structure components and function of a fermentor.*
- *Known the principles behind fermentation medium, fermentation process, upstream processing and downstream processing.*
- *Know the values of microorganism used in the production of penicillin citric acid, wine and single cell protein.*
- *Analyze the basics behind immobilization of microorganisms*

Chapter Outline

6.1 Industrially Important Microorganisms and their Products

6.2 Screening of Industrially Important Microorganism

6.3 Strain Improvement

6.4 Preservation of Industrially Important Microorganisms

6.5 Fermentors

6.6 Industrial Production of Penicillin

6.7 Industrial Production of Wine

6.8 Industrial Production of Single Cell Protein

6.9 Industrial Production of Citric Acid

6.10 Immobilization

Industrial microbiology is a branch of science that deals with the study and uses of various microorganisms that are responsible for the production of many products which has industrial and economic applications. Man has been using many microorganisms for the production of foods, (bread, cheese, yogurt, pickles)–beverages (beer, wine) for many centuries. The birth of industrial microbiology largely began with the studies of Pasteur on fermentation. The term Fermentation originates from a Latin verb "Fervere" which literally means to boil. In alcohol production, CO_2 (gas bubbles) Figure 6.1 are formed during boiling of liquid.

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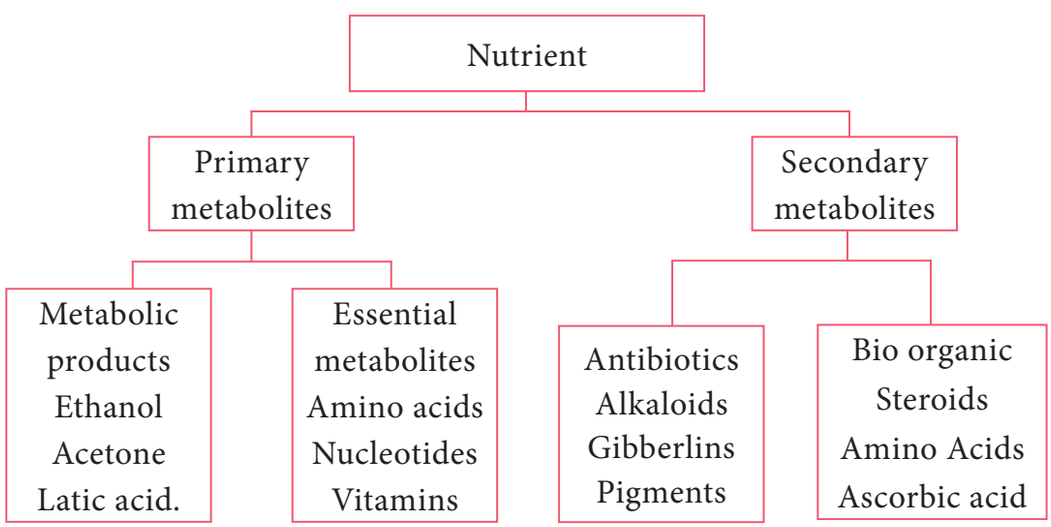
Figure 6.1: Bubble formation in grape juice fermentation

6.1 Industrially Important Microbes and their Products

Microorganisms have the powerful capacity to produce numerous products, during their life cycle. Flowchart 6.1 shows the production of valuable metabolic products during the growth of microorganisms on a suitable medium under controlled environmental conditions. Microbial products are often classified as primary and secondary metabolites.

Primary metabolites consist of compounds related to the synthesis by microbial cells in the growth phase. Primary metabolites such as amino acids, vitamins, enzymes, organic acids and nitrogenous bases are produced by wide variety of microorganisms. These primary metabolites are essential for the growth of microorganisms and they are produced during Logarithmic phase. Secondary metabolites do not play a role in development, growth and reproduction of microorganisms. They are produced at the end of growth phase near stationary phase. They usually accumulate during the period of nutrient limitation or waste product accumulation that follows the exponential phase. These compounds have no direct relationship to the synthesis of cell materials and normal growth. They are the end products of the primary metabolism. Products such as steroids, alkaloids, antibiotics are secondary metabolites.

Excessive production of the primary and secondary metabolites produced by the microorganisms are useful in the large scale in industrial production. Unlike primary metabolites, secondary metabolites are produced in small quantities and their extraction is difficult (Figure 6.2).



Flowchart 6.1: Various metabolites produced in Industrial fermentation

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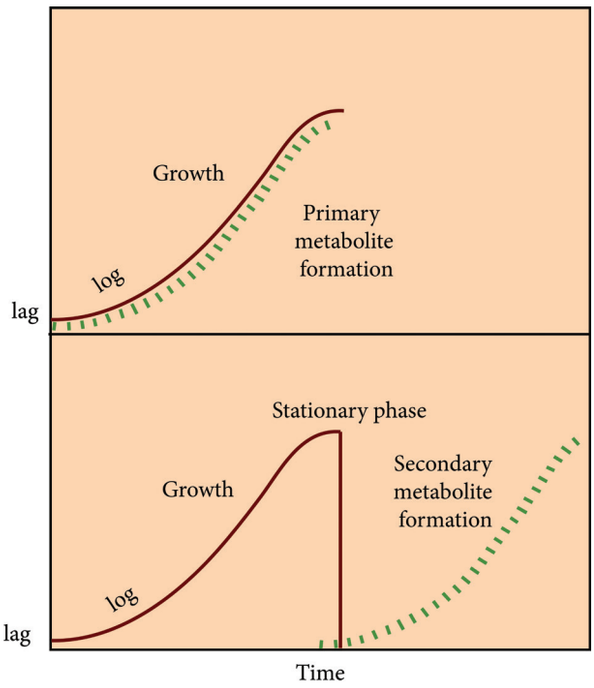


Figure 6.2: Production of primary and secondary metabolites in the growth cycle of microorganism

Some industrially important products are,

- microbial cells (living or dead), microbial biomass and components of microbial cells
- microbial metabolites
- intracellular or extracellular enzymes
- modified compounds that has been microbiologically transformed, and
- recombinant products through the DNA recombinant technology. (Table 6.1 shows some industrially important microorganisms)



The nutritional yeast is called food yeast. The yeast cells are killed during manufacturing, and not alive in the final product. It is used in cooking; it has a cheesy, nutty or savory flavour. Yeast S. *cerevisiae* is used as food yeast. It is a vegan food, available in both fortified (with some vitamins) and unfortified form.

The industrial production of commercial products is carried out by fermentation process. The term fermentation is defined scientifically in a strict sense as a biological process that occurs in the absence of oxygen (anaerobic). In industrial sense any process mediated by or involving microorganisms in which a product of economic value is obtained is called fermentation. The term Industrial fermentation also means large scale cultivation of microorganisms even though most of them are aerobic.

Table 6.1: Industrially important microorganisms

Product	Microorganisms	Uses
Vitamin B12	*Streptomyces*	Vitamin supplements
Lactic acid	*Lactobacillus delbrueckii*	Chemical reagents
Citric acid	*Aspergillus niger*	Food preservative
Acetic acid	*Acetobacter*	Vinegar, solvent
Ethanol	*Saccharomyces*	Chemical reagents drinks
Penicillin	*Pencillium chrysogenum*	Antibiotic

TN_GOVT_XII_Micro_Biology_CH06.indd 84 07-12-2021 15:16:14

There are many microbiological processes that occur in the presence of air (aerobically) yielding incomplete oxidation products. Examples: i) the formation of acetic acid (vinegar) from alcohol by vinegar bacteria ii) citric acid from sugar by certain molds such as Aspergillus niger. These microbial processes are often referred to as fermentations, although they do not decompose in the absence of air.

Infobits

The German Eduard Buchner, winner of the 1907 Nobel Prize in chemistry, determined that fermentation was actually caused by a yeast secretion that he termed zymase. The experiment for which Buchner won the Nobel Prize consisted of producing a cell–free extract of yeast cells and showing that this "press juice" could ferment sugar. This finding dealt yet another blow to vitalism by demonstrating for the first time that fermentation could occur outside living cells.

6.2 Screening of Industrially Important Microorganism

Isolation of industrially important microorganisms

Success of fermentation depends upon the isolation of microorganism. The microorganisms are isolated from their natural habitats like soil, lakes, river mud or even in unusual habitats or environments such as extreme cold, high altitude, deserts, and deep sea and petroleum fields and are tested directly for the product formation and isolated or it can be genetically modified. Different types of microorganisms are isolated by different methods.

Different microbes with desired activity are isolated using various culture techniques. The next step after isolation of microorganisms is the selection or screening. For the successful fermentation process, selection of microorganisms is the prime important step. Screening includes primary screening and secondary screening.

Primary screening: The elementary steps that are performed to select the desired organisms and eliminate the undesirable organisms are termed as primary screening. Methods such as crowded plate technique, auxanography and enrichment culture technique are some of the techniques used in primary screening. For screening of antibiotic producing organisms crowded plate technique is described here,

Crowded plate technique

1. Soil is serially diluted
2. The serially diluted sample is spread on the nutrient agar plates
3. The plates are incubated and the agar plate having 300 to 400 colonies are observed for antibiotic producing activity
4. The ability of a colony to exhibit antibiotic activity is indicated by the presence of a zone of inhibition surrounding the colony
5. The technique is improved by using test organism
6. The antibiotic produced by the organisms in the soil may inhibit the growth of test organism

TN_GOVT_XII_Micro_Biology_CH06.indd 85 07-12-2021 15:16:14

7. The formation of inhibitory zones around certain colonies indicates their antibiotic sensitivity
8. The diameter of the zones of inhibition is measured in millimeters. Crowded plate technique is depicted in the diagram (Figure 6.3).

Enrichment isolation

The process of enrichment provides a suitable condition to support the growth of microorganisms. It allows the growth of the specific microbe while inhibiting the other non-target microbe. The growth of target microorganisms is enriched by providing sole carbon source. For screening microorganisms degrading the compound, different inhibitors are employed which have the ability to block a specific metabolic pathway of the non–target microbe. pH and temperature are also adjusted favoring the growth of desired microorganisms. Soil Calcium carbonate enrichment technique is used for isolation of secondary metabolite producing microorganisms (actinomycetes).

Secondary screening

It is very useful in sorting out microorganisms that have real commercial value from many isolates obtained during primary screening.

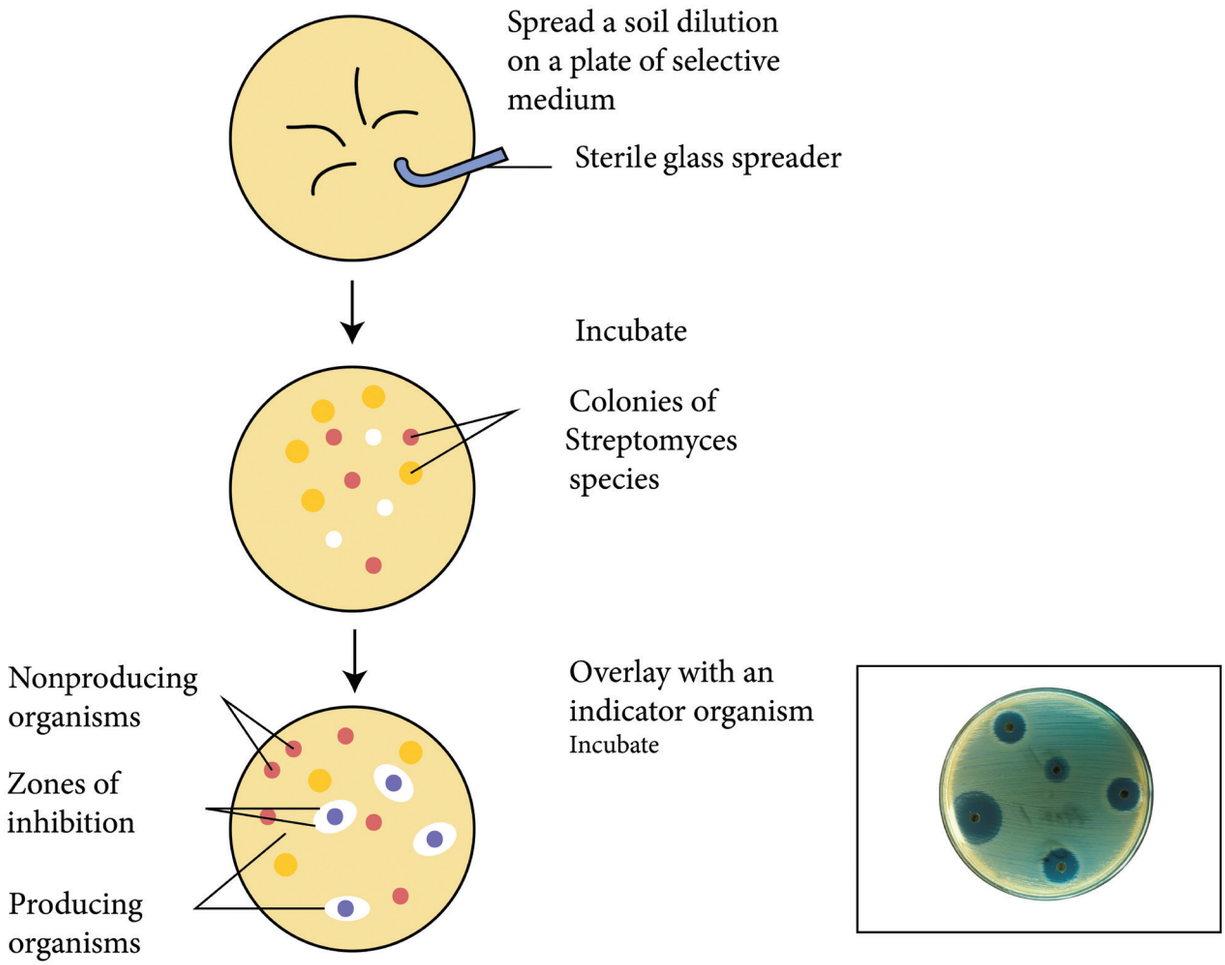


Figure 6.3: Crowded plate technique

TN_GOVT_XII_Micro_Biology_CH06.indd 86 07-12-2021 15:16:16

HOTS

Why microorganisms are exploited more than plant and animal cells for production of commercial products?

1. As primary screening allows the detection and isolation of microorganisms which posses, potentially interesting industrial applications. It is further followed by secondary screening, to check the capabilities and gain information about these organisms.
2. Through primary screening only few or many microorganism that produce a industrially important product, are isolated. The information about the product formed is very less. So, through secondary screening, further sorting out is performed. In this method, only microorganisms with real commercial value are selected and those that lack the potential are discarded.
3. Secondary screening should yield the types of information which are needed in order to evaluate the true potential of a microorganisms industrially usage.
4. Secondary screening may be qualitative and quantitative in its approach.
5. It is done by using paper, thin layer or other chromatographic techniques.
6. The product's physical, clinical, and biological properties are determined.
7. It detects gross genetic instability in microbial cultures.
8. It gives information about the number of products produced in a single fermentation.
9. It determines the optimum conditions for growth or accumulation of a product associated with particular culture.
10. It gives information about the different components of the fermentation medium.
11. It helps in providing information regarding the product yield potential of different isolates.
12. It reveals whether microorganisms are capable of a chemical change or it destroys their fermentation product.

There are various methods employed for secondary screening which includes test conducting on petridish containing solid media or by using flasks or small fermentors containing liquid media, giant colony technique, and filtration method liquid medium method (using Erlenmeyer flask). Here giant colony technique is explained in detail.

Giant Colony Technique

The Streptomyces culture is inoculated onto the central areas of petriplates containing a nutritious agar medium or they are streaked in a narrow band across the centre of plates. The plates are then incubated until growth and possibly, sporulation have occurred. Strains of micro organisms to be tested for possible sensitivity to the antibiotics (the test organisms) are then streaked from the edges of the plates up to but not touching the *Streptomycete* growth. The plates are further incubated to allow the growth of the test organism. The growth of the

TN_GOVT_XII_Micro_Biology_CH06.indd 87 07-12-2021 15:16:16

test organism inhibited by antibiotic in the vicinity of the *Streptomycete* is then measured in millimeters. These *Streptomycetes* that have produced antibiotics with observable microbial inhibition spectrum are retained for further testing (Figure 6.4).

The microbes used in the industrial microbiology should have following characters.

1. The strain should be a high–yielding strain.
2. The strain should have stable biochemical and genetical characteristics.
3. It should not produce undesirable substances.
4. It should be easily cultivated on large scale.

The strain should be in pure culture, free from other microorganisms including Bacteriophages. These characters are screened for the production of desirable products from microorganisms.

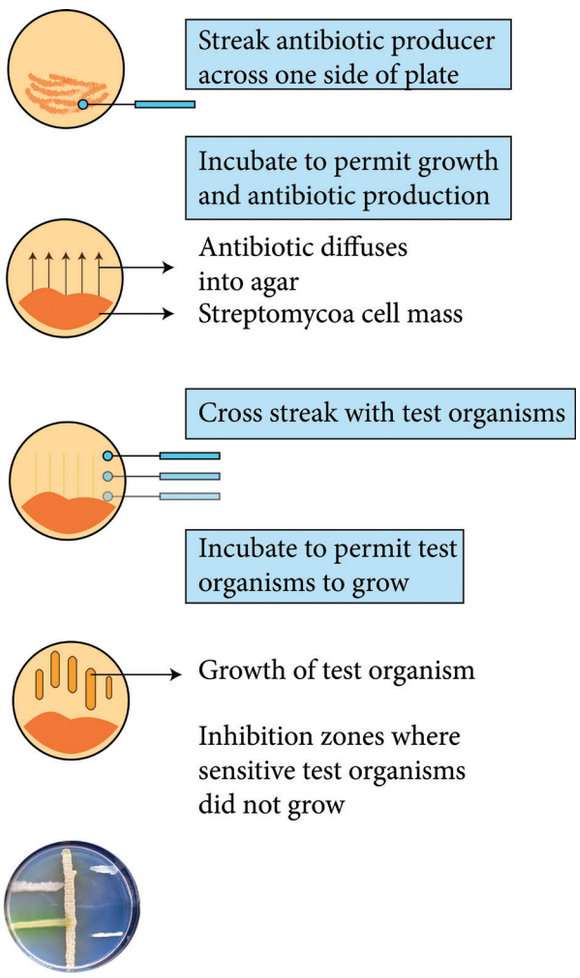


Figure 6.4: Giant Colony Technique

6.3 Strain Improvement

Improvement of the production strain(s) offers the great opportunities for cost reduction without significant capital outlay in industries. Moreover, success in making and keeping a fermentation industry competitive depends greatly on continuous improvement of the production strain(s). Improvement usually resides in increased yields of the desired metabolite. The science and technology of manipulating and improving microbial strains, in order to enhance their metabolic capacities for biotechnological applications, are referred to as strain improvement.

Need for strain improvement

Microbes exist in the nature produce certain compounds of biological interest. However the industrial application of producing those compounds by natural strains is not an economical one so, wild strains are changed by the changing their gene pattern or by regulating their enzymes production. As a result, the specific product is produced in excess.

Knowledge of the function of enzymes, rate limiting steps in pathways, and environmental factors controlling synthesis further helps in designing screening strategies.

TN_GOVT_XII_Micro_Biology_CH06.indd 88 07-12-2021 15:16:18

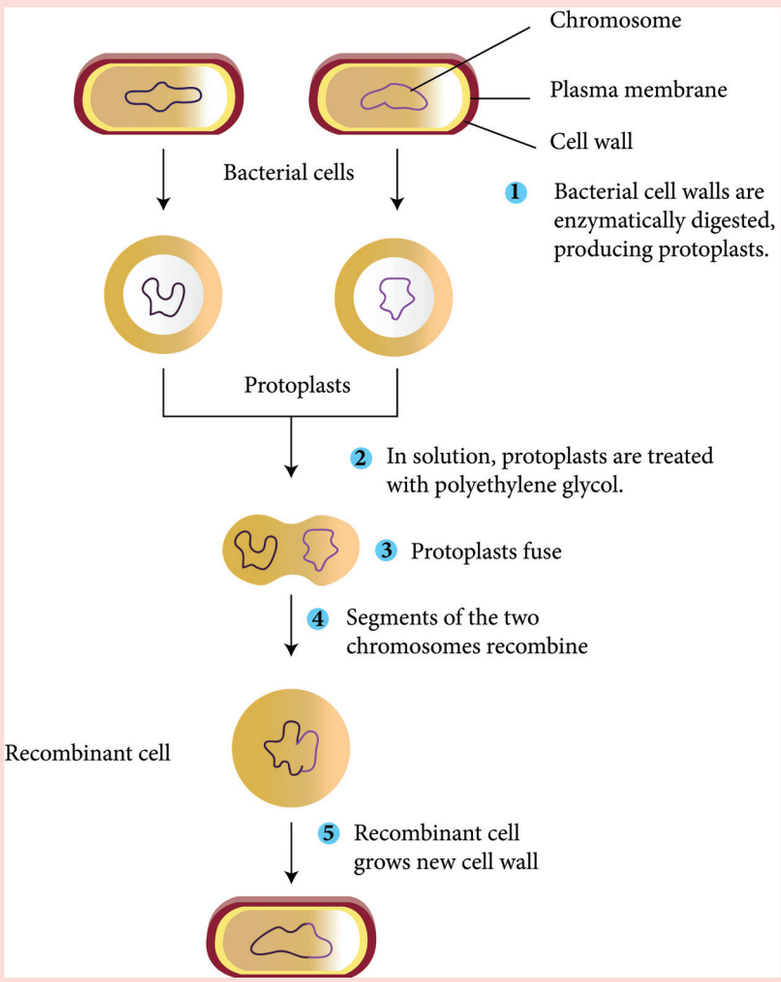
Attributes of Improved strains

1. Assimilate inexpensive and complex raw materials efficiently.
2. Alter product ratios and eliminate impurities or by products in downstream processing.
3. Reduce demand on utilities during fermentation (air, cooling water, or power).
4. Provide cellular morphology in a form suitable for product separation.
5. Create tolerance to high product concentration.
6. Shorten fermentation times.
7. Overproduce natural products or bioactive molecules not synthesized naturally for example insulin.
8. Excrete the product to facilitate product recovery.

Generally wild strains of microorganisms produce low quantities of commercially important metabolites. So, genetic improvements have to be made and new strains need to be developed for any substantial increase in the product formation in a cost effective manner.



Protoplast fusion is defined as fusion of two different protoplasts. A cell wall less nature of plant, bacteria, fungal is called protoplast. It is re moved by either mechanical or enzymatic means. Protoplast has nucleus other protoplasmic contents which are surrounded by cytoplasmic membrane.



TN_GOVT_XII_Micro_Biology_CH06.indd 89 07-12-2021 15:16:21

HOTS

An organism is isolated from soil, which is a very low yielding one. How will you enhance the production activity?

The following techniques at practical genomic level help to improve the microbial strain. They are:

1. Selection of mutants
2. Recombination
3. Regulation
4. Genetic engineering
5. Protoplast fusion

6.4 Preservation of Industrially Important Micro Organisms

The selected microorganism of industrial interest must be preserved in its original form for any further use and research. There are different methods for microbial preservation. Suitable methods are selected based on the:

a. Type of micro-organism

b. Effect of the preservation method on the viability of micro-organism

c. Frequency at which the cultures are withdrawn

d. Size of the microbial population to be preserved

e. Availability of resources

f. Cost of the preservation method. Followings are some of the methods of microbial preservation:

a. Desiccation

This involves removal of water from the culture. Desiccation is used to preserve actinomycetes (a form of fungi-like bacteria) for very long period of time. The microorganisms can be preserved by desiccating on sand, silica gel, or paper strips.

b. Agar Slopes

Microorganisms are grown on agar slopes in test tubes and stored at 5 to –20 °C for six months. If the surface area for growth is covered with mineral oil the micro-organisms can be stored for one year.

c. Liquid Nitrogen

This is the most commonly used technique to store micro-organisms for a long period. Storage takes place at temperatures of less than -196 °C and even less in vapour phase. Microorganisms are made stationary and suspended in a cryoprotective agent before storing in liquid nitrogen.

d. Drying

This method is especially used for sporulating microorganisms (organisms that produce spores). They are sterilized, inoculated, and incubated to allow microbial growth, then dried at room temperature. The resultant dry soil is stored at 4° to 5 °C.

e. Lyophilization

This process is also known as freeze-drying. The microbial culture is first filled in ampoules (glass vessels) and frozen, then dried under vacuum. This is a most convenient technique, since it is cheap to store and easy to ship. The disadvantage is that it is difficult to open the freeze dried ampoules; also, several subcultures have to be done to restore the original characteristics of the microorganisms.

TN_GOVT_XII_Micro_Biology_CH06.indd 90 07-12-2021 15:16:21

6.5 Fermentors

The main function of a fermenter is to provide a suitable environment in which an organism can efficiently produce a target product. Most of them are designed to maintain high biomass concentrations, which are essential for many fermentation processes. Fermentor design, quality of construction, mode of operation and the level of sophistication largely depend upon the production organism, the optimal operating conditions required for target product formation, product value and the scale of production. The performance of any fermenter depends on many factors, but the key physical and chemical parameters that must be controlled are agitation rate, oxygen transfer, pH, and temperature and foam production.

HOTS

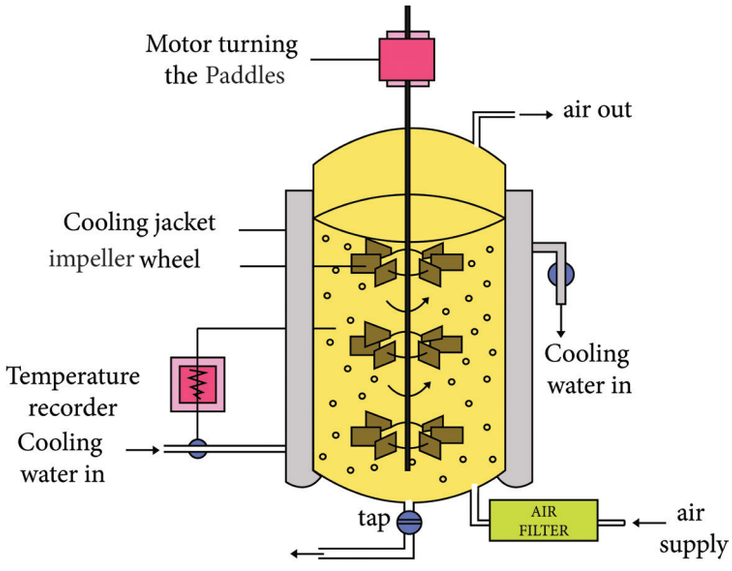
What will happen if antifoam agents are not used in the Fermentation process?

6.5.1 Basic Design of a Fermenter

The materials used for construction of fermenter withstand repeated steam sterilization and are nontoxic. The reaction vessel is designed to withstand vacuum or else it may collapse while cooling. The internal surface is smooth and corrosion resistant. Either stainless steel or glass is used for construction.

Conventional bioreactors are cylindrical vessels with dome top and bottom (Figure 6.5).

It is surrounded by a jacket and sparger at the bottom through which air is introduced. The agitator (for mixing of cells and medium) shaft is connected to a motor at the bottom. It has ports for pH, temperature, dissolved Oxygen sensors for regulation. Antifoam agents like animal vegetable oil, lard oil, corn oil and soya bean oil are used to control the foam. Modern fermentors are usually integrated with computers for efficient process monitoring and data acquisition. Parts of the fermenter and their functions are given in Table 6.2.



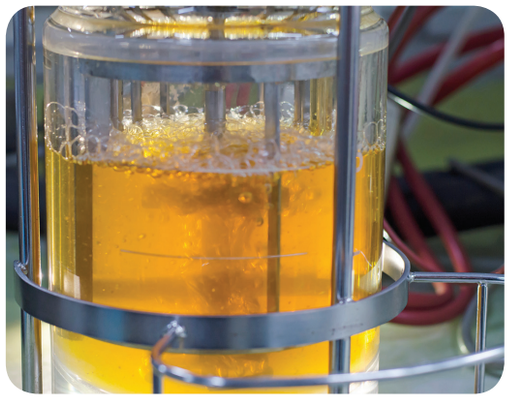


Figure 6.5: Design of a fermenter

TN_GOVT_XII_Micro_Biology_CH06.indd 91 07-12-2021 15:16:22

Table 6.2: Components of fermenter and their uses:

S. no	Parts of fermenter	Functions
1	Impeller (agitator)	To stir the media continuously and hence prevent cells from settling down and distribute oxygen throughout the medium. Impeller speed decreases as the size of the fermenter increases
2	Sparger (aerator)	Introduce sterile oxygen to the media in case of aerobic fermentation process
3	Baffles (vortex breaker)	Disrupt vortex and provide better mixing
4	Inlet Air filter	Filter air before it enter the fermenter
5	Exhaust Air filter	Trap and prevent contaminants from escaping
6	Rota meter	Measure flow rate of Air or liquid
7	Pressure gauge	Measure pressure inside the fermenter
8	Temperature probe	Measure and monitor change in temperature of the medium during the process
9	Cooling jacket	To maintain the temperature of the medium throughout the process
10	pH probe	Measure and monitor pH of the medium
11	Dissolved oxygen probe	Measure dissolve oxygen in the fermenter
12	Level probe	Measure the level of medium
13	Foam probe	Detect the presence of the foam
14	Sampling point	To obtain samples during the process
15	Valves	Regulates and controls the flow of liquids and gases



There are different types of fermentor used in industrially micro biology which includes.

1. Stirred tank bioreactor
2. Tower bioreactors
3. Air lift bioreactors
4. Packed–bed bioreactors
5. Fluidized bed bioreactors
6. Photo bioreactors

6.5.2 Media Used in the Industrial Productions

Fermentation Medium

Most fermentation requires liquid media, often referred to as broth, although some solid-substrate fermentations are also operated. Fermentation media must satisfy all the nutritional requirements of the microorganism and fulfill the technical objectives of the process. Animal fats and plant oils are also incorporated into some media, often as supplements to the main carbon source.

Medium used for large scale production should have the following characteristics.

1. It should be cheap and easily available.
2. It should maximize the growth of the microorganism productivity and the rate of formation of the desired product.
3. It should minimize the formation of undesired products.

TN_GOVT_XII_Micro_Biology_CH06.indd 92 07-12-2021 15:16:22

It should contain carbon source, nitrogen source, energy source, micro nutrients required for the industrial production. Table 6.3 shows common substances used in the industrial fermentation process.

Waste products from other industrial processes such as molasses, ligno cellulosic waste, and corn steep liquor are generally used as substrates for industrial fermentation.

Apart from carbon and nitrogen sources, some other components like minerals, vitamins, growth factors are also used in Industrial fermentations.

Minerals

Normally, sufficient quantities of cobalt, copper, iron, manganese, molybdenum, and zinc are present in the water supplies, and as impurities in other media ingredients. For example, corn steep liquor contains a wide range of minerals that will usually satisfy the minor and trace mineral needs.

Vitamins and growth factors

Many bacteria can synthesize all necessary vitamins from basic elements. For other

Table 6.3: Some common substrates used in the industrial fermentation process:

Carbon source	
Molasses	It is a byproduct of sugar industry. It is a cheap source of carbohydrates It also contains nitrogenous substances, vitamins, trace elements. (Example:) sugar cane, beetroot molasses
Malt extract	It is an aqueous extract of malted barley
starch, dextrin cellulose	They can be metabolized by microorganism. They used for the industrial production of alcohol
Whey	It is a byproduct of dairy industry used in the production of alcohol, SCP, vitamin B12, lactic acid, gibberllic acid
Methanol ethanol	Methanol in the cheapest substrate. It is utilized only by few bacteria yeast. Methanol is used for SCP. Ethanol is used for acetic acid production
Hydro molasses	It is a byproduct in glucose production from corn
Sulphate waste liquor	It is a spent sulfite liquor form the paper pulping industry. It is used in the production of ethanol by *Saccharomyces cerevisiae*, and in the growth of *Torula utilis* as a feed
Nitrogen sources	
Inorganic: Ammonium salts and ammonia	It is a cheap source of nitrogen
Urea (Organic)	It is a good and cheap source of organic source
Corn steep liquor (Organic)	It is formed during starch production from corn. It is rich in several amino acids
Yeast extract	It is rich in amino acids, peptides vitamins
Soy meal	It is a left out residue on preparing soybean oil from soybean seeds. It is used in antibiotic production
Peptones	The proteins hydrolysates are called as peptones. The source of peptones includes meat, cotton seeds and sunflower seeds

TN_GOVT_XII_Micro_Biology_CH06.indd 93 07-12-2021 15:16:22

bacteria, filamentous fungi and yeasts, they must be added as supplements to the fermentation medium. Most natural carbon and nitrogen sources also contain at least some of the required vitamins as minor contaminants. Other necessary growth factors, amino acids, nucleotides, fatty acids and sterols, are added either in pure form or, for economic reasons, as less expensive plant and animal extracts.

Precursors

Some fermentation must be supplemented with specific precursors, notably for secondary metabolite production. When required, they are often added in controlled quantities and in a relatively pure form. Examples: Phenyl acetic acid or phenylacetamide added as side chain precursors in penicillin production.

6.5.3 Large Scale Production

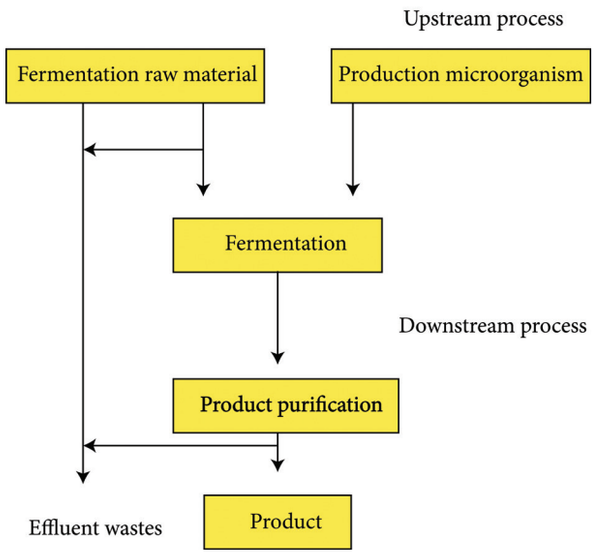


Figure 6.6: An overview of Fermentation process

Basic Steps of Industrial Fermentation

Successful development of a fermentation process and fermentors requires major contributions from a wide range of other disciplines, particularly biochemistry, genetics, molecular biology, chemistry, chemical engineering and process engineering, mathematics and computer technology. A typical operation involves both upstream processing (USP) and downstream processing (DSP) stages (Figure 6.6).

6.5.4 Upstream Processing

It is the first step in which biomolecules like bacteria or other cells are grown in a fermentor. Upstream processing involves inoculation development, scale up, medium preparation and sterilization of media and fermentation process.

Inoculum development

It is a preparation of a population of micro organisms from a stock dormant culture to a state useful for inoculating a final production fermentor.

It is a critical stage in fermentation process.

It is a stepwise sequence employing increasing volume of media.

Inoculum media is usually balanced for rapid cell growth and not for product formation.

Inoculum scale up

It is the preparations of the seed culture in amounts sufficient to be used in the larger fermenter vessel. It involves growing the microorganisms obtained from the pure stock culture in several consecutive fermenter. By doing this, the time required for the growth of microbes in the fermenter is cut down, so that the rate of productivity is increased. The seed culture obtained is then used for inoculation in

TN_GOVT_XII_Micro_Biology_CH06.indd 94 07-12-2021 15:16:23

fermentation medium. The size of the inoculums is generally 1–10% of the total volume of the medium.

In general, fermentation/ bioprocess techniques are developed in stages starting from a laboratory and finally leading to an industry. The phenomenon of developing industrial fermentation process in stages is referred to as scale–up. Scale–up is necessary for implementing new fermentation technique developed using mutant organisms.

The very purpose of scale–up is to develop optimal environmental and operating conditions at different levels for a successful fermentation industry where conditions like substrate concentration agitation and mixing, aeration, power consumption and rate of Oxygen transfer are studied. In a conventional scale–up, a fermentation technique is developed in 3–4 stages. The initial stage involves a screening process using Petri dishes or Erlenmeyer flasks followed by a pilot project to determine the optimal operating conditions for a fermentation process with a capacity of 5–200 litres. The final stage involves the transfer of technology developed in the laboratory to industry. (Figure 6.7)

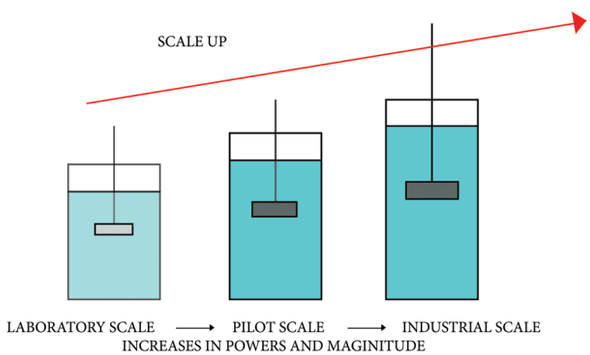


Figure 6.7: Scale up in industrial fermentation

It has to be continuously noted that a fermentation process that works well at the laboratory scale may work poorly or may not work at all on industrial scale. Therefore it is not always possible to blindly apply the laboratory conditions of a fermentation technique developed to industry.

At the laboratory scale, one is interested in the maximum yield of the product for unit time. At the industry level, besides the product yield, minimal operating cost is another important factor for consideration.

Preparation and sterilization of media

According to the specific industrial production basic components needed to carry out fermentation are selected as per the required volume.

Medium components should be free from contamination. So all the medium components employed in the fermentation process are sterilized. Sterilization is mostly carried out by applying heat and to lesser extent other physical methods, chemical methods (disinfectants) and radiation (using UV rays, γ rays). Batch Sterilization is carried out at 121°C (20 to 60 mins) where as continuous sterilization is done at 140°C for (30 to 120 secs). Much energy is wasted on batch sterilization on compared with continuous sterilization nearly 80 to 90% of energy saved during this process. Air and heat sensitive components are sterilizied by membrane filters.

Fermentation Process

It involves the propagation of the microorganism and the production of the desired product. Fermentation process is divided depending on the feeding strategy of the culture and medium as follows.

i. Batch Fermentation

TN_GOVT_XII_Micro_Biology_CH06.indd 95 07-12-2021 15:16:24

ii. Continuous Fermentation

iii. Fed batch Fermentation

i. Batch Fermentation

The medium and culture are initially fed into the vessel and it is then closed. After that, no components are added apart from Oxygen. The pH is adjusted during the course of process by adding either acid or alkali. The fermentation is allowed to run for a predetermined period of time and the product is harvested at the end. Foaming is controlled by adding antifoam agents such as palm oil or soybeans oil. Heat generated is regulated by providing water circulation system around the vessel for heat exchange.

ii. Continuous fermentation

This is an open system. It involves the removal of culture medium continuously and replacement of them with a fresh sterile medium in a bioreactor.

In this method, homogenously mixing reactors which include chemo stat and turbid stat bioreactors are used. Examples: production of antibiotics, organic solvents, beer, ethanol and SCP.

iii. Fed batch system

It is a combination of both batch and continuous systems. In this, additional nutrients are added to the fermentors as the fermentation is in progress. This extends the time of operation, but the products are harvested at the end of the production cycle as in batch fermenter.

HOTS

Why does industry prefer continuous culture?

Followed by the fermentation, production, products are harvested or separated by downstream processing.

6.5.5 Downstream Processing

The various processes used for the actual recovery of useful products from fermentation or any other industrial processes are called downstream processing. The cost of downstream processing (DSP) is often more than 50% of the manufacturing cost, and there is product loss at each step of DSP. Therefore, the DSP should be efficient, involve as few steps as possible and be cost-effective. Methods involved in the downstream processing are outlined in the flowchart (6.2). Table 6.4 shows Difference between upstream and downstream processing.

Table 6.4: Difference between upstream (usp) and downstream (dsp) processing

USP	DSP
USP overall makes the procurement and maintenance of inoculum	DSP depends upon selection of cost–effective media
USP involves in strain improvement to enhance and yield	DSP concentrates on media optimization for maximum productivity yield and profit
It is a continuous development of selected strains to increase the economic yield	For DSP, fermentation conditions are optimized for the growth of micro organism or the production of a desired product

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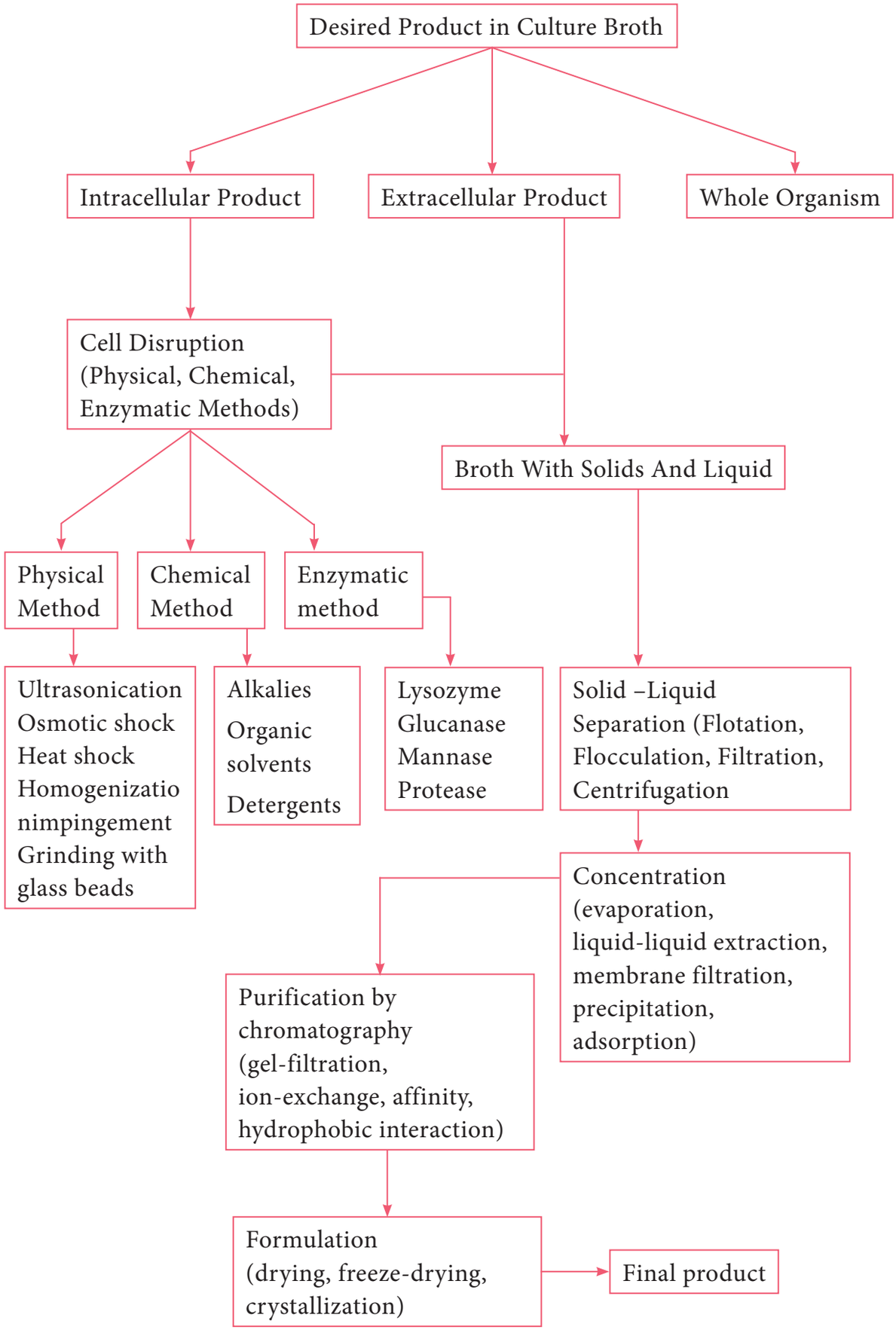

Flowchart 6.2: Downstream processing methods

TN_GOVT_XII_Micro_Biology_CH06.indd 97 07-12-2021 15:16:24

6.6 Penicillin Production

Penicillin is a broad spectrum antibiotic. Penicillin is first obtained from the mould, *Penicillium notatum* (Figure 6.8).



Figure 6.8: Structure of *Penicillium notatum*

Penicillium chrysogenum is a high yielding strain, used for the commercial production of penicillin. This strain is highly unstable, so the spore suspensions are maintained in a dormant state to prevent contamination. Most penicillin form filamentous broth and hence is difficult to mix and it hinders oxygen transfer due to their high viscosity. This is avoided by using bubble columns air lift reactors which agitates the medium providing even oxygen distribution.

Penicillin has a basic structure 6-amino penicillanic acid 6-(APA). It consists of a thiazolidine ring with a condensed β-lactum ring. It carries a variable side chain in position 6. Natural penicillins are produced in a fermentation process without adding any side chain precursors. If a side chain precursor is added to the broth, desired penicillin is produced and it is called bio-synthetic penicillin.

Semi synthetic penicillin is one in which, both fermentation and chemical approach are used to produce useful pencillins. It can be taken orally and active against gram negative bacteria. (eg) Amphicilin. Nowadays, semi synthetic pencillins makeup the bulk of the penicillin market.

Infobits

In later (1939) using (Flemings' work) Howard Florey and Ernst Chain managed to purify penicillin in a powdered form. In 1941, they successfully treated a human. In 1943, they produced penicillin on a large scale. This helped immensely to treat causalities during the 2nd World War ww1 that had bacterial inflations due to their wounds.



The main objective of producing semi synthetic penicillin is to generate compounds with improved properties. (eg) acid stability, Resistance to enzymic degradation, broader spectrum of activity. Here side chain is removed to form (6- APA) via immobilization in a column of penicillin acylase. Penicilln G is converted to (6-APA) and phenyl acetic. Then it is chemically to acylated produce Semi Synthetic Pencillin.

New kinds of synthetic penicillin can also be produced which are readily absorbed by the intestine compared to natural penicillin. Example: Phenithicilin.

The initial strain of Penicillium chrysogenum (NRRL, 1951) was low yielding strain and so it is was treated with mutagenic agents such as X-rays, UV right and some other repeated methods to get a high yielding strain Q-176.

TN_GOVT_XII_Micro_Biology_CH06.indd 98 07-12-2021 15:16:24

Production methods

Penicillin production is done by one of the following.

1. Surface culture
2. Submerged fermentation process

Inoculum Production

Inoculation methods

To inoculate fermentation medium one of the following methods can be employed.

1. Using dry spores to seed the fermentation medium.
2. Making suspension along with non toxic wetting agent like Sodium lauryl sulphate and inoculating germinated organism
3. Using pellet inocula obtained by the germination of spores

The lyophilized spores (or) spores in well sporulated frozen agar slant are suspended in water or in a dilute solution of a nontoxic wetting agent.

(1:10,000 sodium lauryl sulphonate)

↓

Spores are then added to a bottles containing wheat bran solution
It is incubated for 5-7 days at 24°C for heavy sporulation.

↓

The resulting spores are then transferred to production tank

↓

The micro organism in the inoculum tank is checked for contamination.

Production process

The production tanks are inoculated with a mycelial growth.

↓

Production medium contains following medium components.

Carbon source as Lactose, Nitrogen source as Ammonium sulphate, Acetate or Lactate (Corn steep liquor is the cheap and easy source of nitrogen)

Mineral sources as K, P (Potassium di hydrogen phosphate), Mg, S (Magnesium sulphate), Zn, Cu(Copper sulphate) (Corn steep liquor supply some of these minerals)

Precursor (Example: phenyl acetic acid) is added to the medium

↓

Antifoam agent (Example: corn or soybean oil) is added before sterilization

The sufficient aeration and agitation is given and are incubated at 25°C to 26°C for 3 to 5 days at PH range of 7 to 7.5

Penicillin Production

Process of penicillin production occurs in three phases:

First phase: Growth of mycelium occurs in this phase where the yield of antibiotic is low. The pH increases due to the release of NH_3.

Second phase: In this phase, intense synthesis of penicillin occurs due to rapid consumption of Lactose and Ammonium nitrogen. The mycelial mass increases and the pH remain unchanged (Figure 6.10).

Third phase: In this phase, the concentration of antibiotics decreases in the medium. Autolysis of mycelium starts, liberating Ammonia leading to slight rise in pH.

Recovery

After penicillin fermentation, the broth is filtered on rotary vacuum filter

↓

Mycelium is separated

TN_GOVT_XII_Micro_Biology_CH06.indd 99 07-12-2021 15:16:25

↓

To the broth sulphuric acid or phosphoric acid is added

↓

Penicillin is converted into anionic form

↓

It is extracted in counter current solvent extractor, by using organic solvent, amyl acetate, methyl isobutyl (ketone)

↓

It is then back extracted with water from the organic solvent by adding potassium or sodium hydroxide

↓

Shifts between water and solvent aid in the purification of penicillin

↓

The resulting sodium or potassium penicillin is then crystallized

↓

Then it is washed, dried and used for commercial purpose

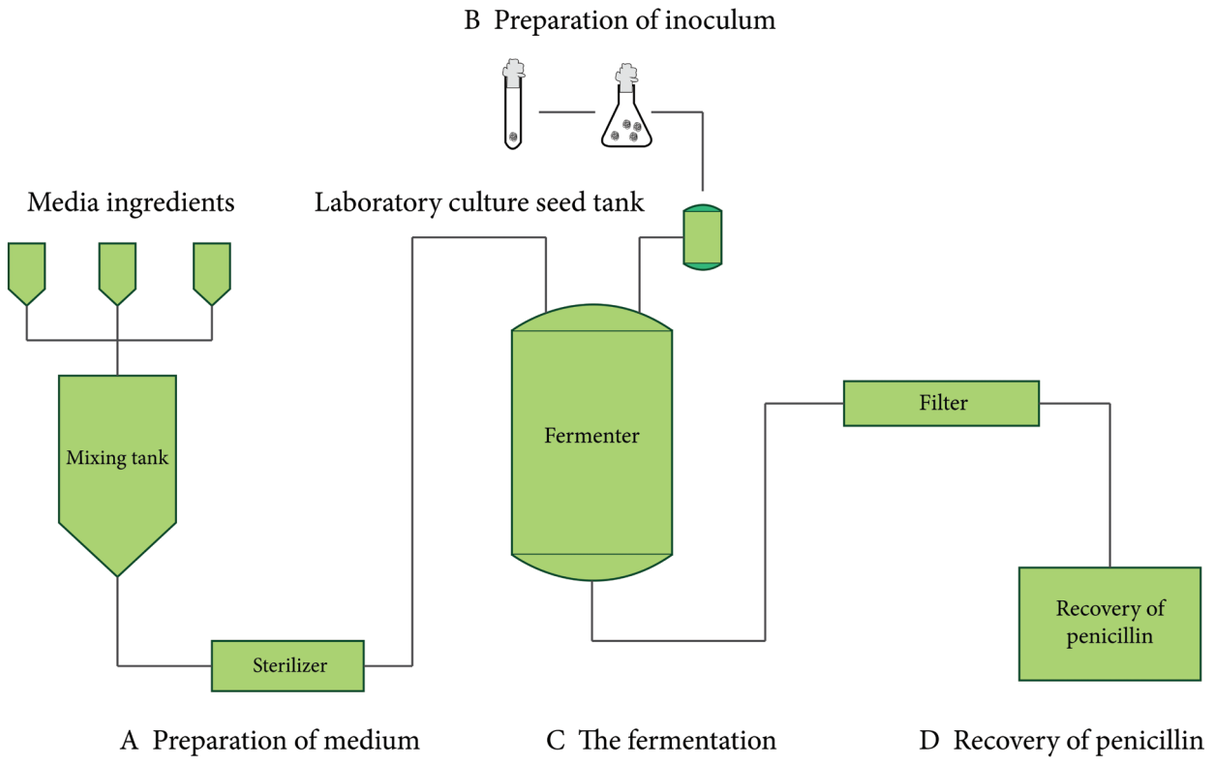

Figure 6.9: Production of Penicillin

6.7 Industrial Production of Wine

An alcoholic distilled beverage is produced by concentrating alcohol from fermentation by distillation. Beer or ale is produced by the fermentation of malted grains. Wine is prepared from grapes belonging to species *Vitis vinefera*. It is also produced from other fruits like peach, pear, dandelion and honey. Generally wine contains 16% of alcohol. Wine production from crushed grapes is called enology. The various forms of wine are listed below in the table 6.5.

Red wine is extracted from the skin of red grapes containing red pigment (anthocyanin). During the preparation of red wine, all the anthocyanin pigments are solubilized by the extract. Pink wine

TN_GOVT_XII_Micro_Biology_CH06.indd 100 07-12-2021 15:16:27

is obtained from either pink grapes or red grapes in which fermentation last for only 12 to 36 hour and only less amount of anthocyanin pigments are solubilized. White wine is prepared from the white grapes or from the red grapes in which pigment involved in colouring is removed.

Generally yeasts are the natural microbiota of grapes

Both wild yeast and cultivated yeast are involved in the wine fermentation. Natural yeast is not potable because they do not produce much wine and are less alcohol tolerant and produce undesirable compounds, affecting the quality of the wine.

The cultivated wine yeast, *Saccharomyces ellipsoideus*, is used for commercial production. Figure 6.10 shows steps involved in wine production

Table 6.5: Shows different varieties of wine

Red wine	It has red pigments
White wine	It does not contain red pigments
Rose wine	It has less red pigments
Dry wine	It has more alcohol content
Sweet wine	It has more sugar content
Fortified wine	It is fortified with other alcoholic beverage
Sparkling wine	It has considerable amount of CO_2
Still wine	It does not contain carbon dioxide
Distilled wine	Brandy (alcohol content 21%)
Table wine	It has low alcohol and sugar content



Aqu-aori is the concept that oceans and other bodies of water, might impart unique characteristics on the aging process of submerged wine in water. The ocean provides a unique environment with cold temperatures, constant pressure, and little to no light and constant motion.

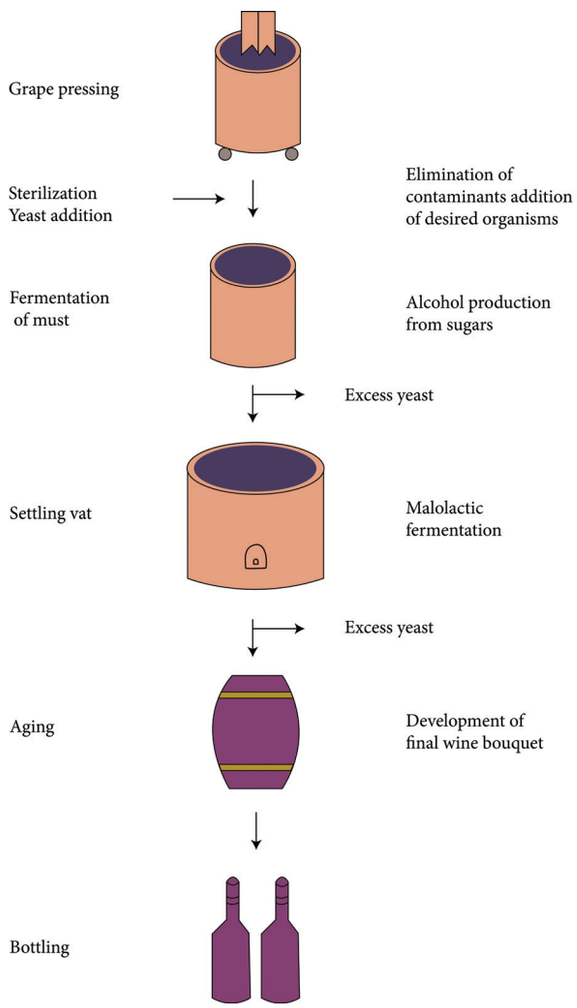

Figure 6.10: Steps involved in wine production

Steps involved in Wine production

Grapes are stemmed, cleaned and crushed

↓

TN_GOVT_XII_Micro_Biology_CH06.indd 101 07-12-2021 15:16:31

Sodium or Potassium Meta – bisulphate is added to check the undesirable microorganism

↓

Must (crushed grapes) is treated with Sulfur dioxide to kill the wild yeasts and bacteria or sometimes pasteurized to destroy the natural microbiota

↓

Must is inoculated with *Saccharomyces ellipsoideus* (2.5%) and selected fermentation is carried from 50 to 50000 gallons at 20 to 24°C

↓

Oak, cement, stone glass lined metal are used as fermentor

↓

Temperature and time required for fermentation White wine: 10–21°C, 7–12 days; Red wine: 24–27°C, 3–5 days

↓

In red wine production, after three to five days of fermentation, sufficient tanin and colour is extracted from the pomace and the wine is drawn off for further fermentation

↓

Racking improves flavour and aroma, where wine is separated from the sediment containing yeast cells as precipitate form

↓

The wine is subjected to aging at lower temperature. Ageing process is typically much longer for red wine than white wine

↓

Wines are clarified in a process called fining. Fining is done by filtration through casein, tannin, diatomaceous earth or bentonite clay, asbestos, membrane filters or centrifugation

↓

The wine produced is placed in casks, tank and bottles

Infobits

Saccharomyces is called Brewing Yeasts, or Baker's Yeast. The brewing strains can be classified into two groups. The ale strains (*Saccharomyces cerevisiae*) and the lager strains (*Saccharomyces pastorianus* or *Saccharomyces carlsbergenris*). The ale strains are top fermenting strains. Lager strains are hybrid strains of *Saccharomyces cerevisiae* and *Saccharomyces eubayanus* and are often referred to as bottom fermenting. (*Saccharomyces* yeasts can form symbiotic matrices with bacteria and are used to produce Kombucha, Kefir, and Ginger beer).

After wine production, cork should be used for preventing the entry of air into the bottles. The presence of air allows the growth of vinegar bacteria that convert the ethanol to acetic acid. The final alcohol content of wine varies depending upon the sugar content of the grapes, length of the fermentation and type of strain used.

6.8 Industrial Production of Single Cell Protein

Single cell protein refers to the microbial cells or total protein extracted from pure microbial cell culture (monoculture) which can be used as protein supplement for humans or animals. During ancient times, the tribes in the Central African Republic used a spiral shaped Cyanobacterium named *Spirulina platensis* as food. They collected it as mats from the bottom of seasonally dried up ponds and shallow waters around Lake Chad and dried them in the sun and made small cakes called "Dihe".

TN_GOVT_XII_Micro_Biology_CH06.indd 102 07-12-2021 15:16:31

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Zymology:

Zymology also known as "Zymurgy" (from the Greek–means, the workings of fermentation) is an applied science which studies the biochemical process of fermentation and its practical uses. It includes selection of fermenting yeast and bacteria, species and their use in brewing, wine making, fermenting milk and other fermented foods. The wine yeast Zymurgist one who studies or practices zymurgy; a knowledgeable brewer.

During the World war II, when there were shortage in proteins and vitamins in the diet, the Germans produced yeasts and a mould named *Geotrichum candidum* was used as food.

The term Single Cell Protein was coined by C.L Wilson (1966) at Massachusetts Institute of Technology (MIT), to represent the cells of algae, bacteria, yeasts and fungi, grown for their protein contents. The name was introduced by Prof. Scrimshow of MIT in 1967. The organisms like *Pseudomonas facilis*, P. *flava, Chlorella, Anabaena, Spirulina, Chlamydomonas*, and *Agaricus* are commonly used for SCP production. Large scale production of SCP is shown in the Figure 6.11

There are several methods available for SCP production. In the Japanese method, flat tray is used with artificial sunlight algae are cultivated in shallow ponds with mechanical stirrers or in deeper ponds (not more than 20–30 cm deep) with circulation pumps. Optimum, light is an important parameter for maximum growth of SCP. *Scenedesmus* sp. grows 20 times faster in optimum light than in natural conditions. Optimum temperature and optimum pH is varied according to the strain and intensity of light. Example: *Spirulina* is cultivated at 25–35°C with pH 9.5. Table 6.6 shows different types of microorganisms and substrates used for SCP production.

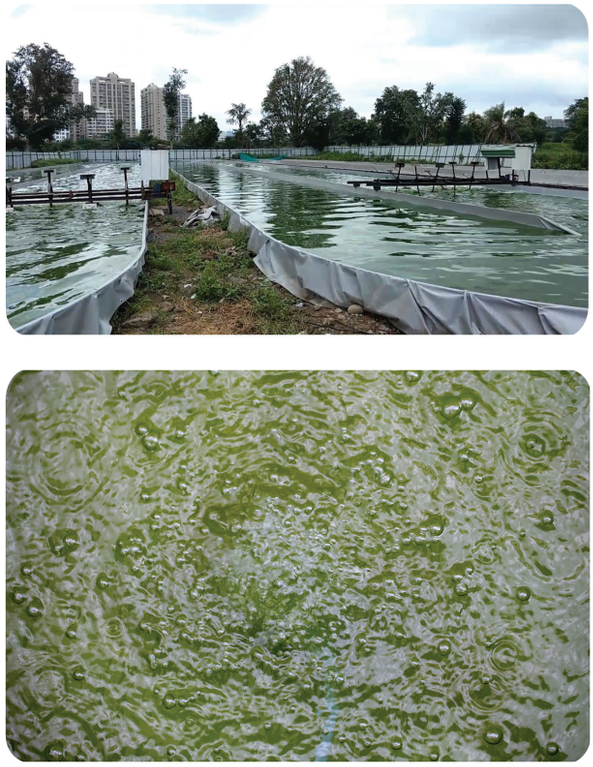

Figure 6.11: Large scale production of SCP

Steps involved in SCP production

Provision of carbon source with added nitrogen, CO_2, ammonia, trace minerals for growth

↓

Prevention of contamination by using sterilized medium and fermentation equipments

↓

Selected microorganism is inoculated in a pure form

TN_GOVT_XII_Micro_Biology_CH06.indd 103 07-12-2021 15:16:32

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Some Commercial Products of Yeast

Sl. No.	Product	Micro Organism	Uses
1.	Baker's yeast, beer, wine, ale, bread	*Saccharomyces cerevisiae*	Baking industry brewing industry
2.	Soy sauce	*Saccharomyces rouxii*	Food Condiment
3.	Sour French bread	*Candida milleri*	Baking
4.	Commercial alcohol (ethanol)	*Saccharomyces cerevisiae* *Kluyveromyces fragilis*	Fuel, Solvent
5.	Riboflavin	*Eremotherium ashbyi*	Vitamin supplement
6.	Microbial protein	*Candida utilis* *Saccharomyces lipolytica*	Microbial protein from petroleum products. Animal food supplement (single cell protein) from paper-pulp waste

↓

Adequate aeration and cooling is provided

↓

Microbial biomass is harvested and recovered by flocculation or centrifugation flocculants

↓

Harvested algae are dewatered and dried on open sand beds

↓

Processing biomass and enhancing it for use and storage

Advantages of using microorganisms for SCP production:

1. Microorganisms grow at a very rapid rate under optimal culture conditions.
2. The quality and quantity of protein content in microorganisms is better compared to higher plants and animals.



Pruteen was the 1st commercial SCP used as animal feed additive with 72% of protein. Pruteen was produced from bacteria named *Methylophilus mehylotrophus* cultured on methanol.

In India, National Botanical Research Institute (NBRI) and the Central Food Technological Research Institute (CFTRI) are involved in the production of SCP.

In CPFTRI, SCP is produced from algae cultured on sewage.

3. A wide range of raw materials which are otherwise wasted, can be fruitfully used for SCP production
4. The culture conditions and the fermentation processes are very simple.
5. Microorganisms can be easily handled and subjected to genetic manipulations.

TN_GOVT_XII_Micro_Biology_CH06.indd 104 07-12-2021 15:16:32

Table 6.6: List of microorganisms and substrates used for SCP production

Microorganisms	Substrates
Bacteria	
Pseudomonas sp.	Alkanes
Methylomonas sp.	Methanol
Yeast	
Candida utilis	Sulfite liquor
Lactobacillus bulgaricus	Whey
Fungi	
Aspergillus niger	Molasses
Trichoderma viridae	Straw, starch
Algae	
Spirulina maxima	Carbon di oxide
Scenedesmus acutus	Carbon di oxide
Actinomycetes	
Nocardia	Alkanes
Mushroom	
Agaricus bisporus	Compost, rice straw
Volvariella volvacea	Cotton straw

During the cultivation of SCP, care must be taken to prevent and control the contamination by other micro organisms, which produce mycoxins or cyanotoxins. This is controlled by using the fungus *Scytaliclium acidophilum* which grows at a low PH. It allows the hydrolysation of paper wastes to a sugar medium and also creates aseptic condition at low cost.

6.9 Industrial Production of Citric Acid

Citric acid is obtained from citrus fruits; pineapple etc., and after the development of microbial fermentation, citric acid production becomes very cheap, easy and cost effective. 70% of citric acid produced is used in food and beverage industry. Many microbial strains such as fungi *Aspergillus flavus, Aspergillus niger* and *Trichoderma viridae*, yeast *Hansenulla polymorpha* and *Candida lipolytica* are generally are involved in the production of citric acid.

Citric acid production can be carried out in the following three methods.

a. Koji process or solid state fermentation

b. Liquid surface culture

c. Submerged fermentation

Media used in citric acid production

Citric acid production is carried out by using carbohydrates and n–alkenes. Generally beet molasses, cane molasses, sucrose, commercial glucose and starch hydrolysate are used as carbohydrate sources. The carbohydrate material is diluted and mixed with a nitrogen source (ammonium salts or urea) and the pH and temperature are adjusted according to the process.

Inoculum development

Fungal strains that are used for production are stored in soil or silica gel in the form of spores. Spores are suspended in a freshly prepared sterile water containing Tween 80 and after a period of growth, it can be used as inoculum for large scale production.

Steps involved in citric acid production

Production Medium

Sucrose, beet molasses, used as carbon source need pretreatment, as it contains excessive amount of trace metals. So ferrocyanide or ferricyanide is added to the production medium before sterilization. Inorganic salts, carbon, hydrogen, oxygen trace metals. Nitrogen, potassium, phosphorus, sulphur and magnesium are taken in Aluminum or stainless steel shallow pans or tray (5–20 cm deep).

↓

Inoculated with spores of A. *niger* by blowing over the strains of *Aspergillus niger* for fermentation

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↓

The medium is kept at 28–30°C with relative humidity 40–60% and aerated with purified air for 8–12 days

↓

Citric acid produced is determined by checking the pH or the total acid content of the broth.

↓

Fermented liquid is drained off and processed further for the recovery of citric acid

Infobits

Influence of trace metals in citric acid production:

Citric acid production is highly influenced by the trace metals. Particularly, iron and manganese in excess amount affect the citric acid production. They affect the cellular morphology and change pellets to filamentous growth (i.e.,) from productive form to unproductive form.

Recovery

The mycelial mat is pressed.

↓

Milk of lime (calcium carbonate) is added so calcium citrate is formed.

↓

Again sulphuric acid is added, so calcium sulphate is formed.

↓

The remaining citric acid solution is filtered and washed. Finally the impure solution of citric acid subjected to treatment with activated carbon and finally pure form of citric acid is collected.

Uses

It is used as a Acidulant in food, (Jams, Preserved fruits, Fruit drinks) and pharmaceutical industries.

1. It is mainly used in food and beverage industry (Jams, preserved fruits, fruit drinks)
2. It is used is pharmaceuticals, and other industrial processes
3. Citrate and citrate esters are used as plasticizers
4. It is used as a chelating and sequestering agent (Tanning of animal skins)

Generally citric acid obtained from citrus fruits, pineapple etc., After the development of microbial fermentation, citric acid production becomes very cheap and easy cost effective.

6.10 Immobilization

It is technique used for the physical or chemical fixation of plant, animal cells, organelles, enzymes or other proteins (monoclonal antibodies) onto a solid matrix or retained by a membrane, in order to increase their stability and make possible their repeated or continued use.

The immobilized enzyme is defined as the enzyme physically confined or localized in a certain defined region of space with retention of its catalytic activity which can be used repeatedly and continuously.

The selection of appropriate carrier and immobilization procedure is very essential procedure is very essential for the immobilization technique.

TN_GOVT_XII_Micro_Biology_CH06.indd 106 07-12-2021 15:16:32

Various types of materials like cellulose, dextran, agarose, gelatin, albumin polystyrene, Calcium alginate polyacrylamide, collagen carrageenan and polyurethane, inorganic materials (brick, rand, glass, and ceramics, magnetic) are used for immobilization.

The linkage is mediated by ionic bonds, physical absorption or bio specific binding.

The immobilization methods can be classified into four categories

i. Carrier–binding

ii. Cross–linking

iii. Entrapping

iv. Combining

Among all these methods entrapping is discussed in brief.

Entrapping

The enzymes, cells are not directly attached to the support surface, but simply trapped inside the polymer matrix. Entrapping is carried out by mixing the biocatalyst into a monomer solution followed by a polymerization. It is done by change in temperature or by chemical reactions.

Advantages of immobilization

1. Immobilized growing cells serve as self proliferating and self regenerating bio catalyst
2. They are stable
3. They are used either repeatedly in a series of batch wise reactions or continuously in flow systems.

HOTS

The technique of immobilized enzymes may increase the use of enzymes in industry for product modification. Why? Give reason.

Summary

Industrial microbiology is a branch, of microbiology that deals with the study and uses of various microorganisms. The birth of industrial microbiology largely began with the studies of Pasteur on fermentation. Various products of both primary and secondary metabolites are produced by different microorganisms. Both primary and secondary screening is involved in the isolation of industrially important microorganisms. The isolated strains are modified for higher yield through various procedures (example) protoplast fusion. Thus strain improvement improves the fermentation efficiency. Fermentation is carried under a suitable parameters, in a controlled environment is called Fermentor. Fermentation process involves both cup stream processing and downstream processing. In cup stream processing, inoculum preparation scale up, preparation of medium and sterilization of media, are carried out.

Evaluation

Multiple choice questions

LS5C3

1. The term fermentation originates from a Latin verb _____

a. Wear

b. Fervere

c. Severe

d. Cheer

TN_GOVT_XII_Micro_Biology_CH06.indd 107 07-12-2021 15:16:32

2. _____ metabolites are produced in small quantities during industrial production.

   a. Secondary metabolites

   b. Primary metabolites

   c. Tertiary metabolites

   d. Neutral metabolites

3. The microbes used in the industrial microbiology has these qualities

   Statement A: The strain should have stable biochemical and genetical characteristics.

   Statement B: It is a high yielding strain.

   a. Statement A alone is true

   b. Statement B alone is true

   c. Statement A and B are true

   d. Both A and B are false

4. Match the following:

A. Lactic acid	1. Penicillium chrysogenum
B. Citric acid	2. Lactobacillus delbrukeii
C. Penicillin	3. Saccharomyces
D. Ethanol	4. Aspergillus niger

   a. A4 B2 C1 D3
   b. A2 B4 C1 D3
   c. A1 B3 C4 D2
   d. A2 B4 C3 D1

5. _____ is an example for primary screening.

   a. Photography

   b. Cinematography

   c. Auxanography

   d. Telegraphy

6. Strain improvement is the technology of (Assertion) Manipulating and improving microbial strains. (Reason) It is done by Recombination and Protoplast fusion.

   a. Statement (A) is not supported by (R)

   b. Statement A is supported by (R)

   c. Statement (A) alone correct

   d. Statement (B) alone is correct

7. Match the following:

A. Penicillin	1. Aspergillus niger
B. Wine	2. Penicillium chrysogenum
C. Citric acid	3. Scenedesmus
D. Single cell protein	4. Saccharomyces cerevisiae

   a. A4 B1 C2 D3
   b. A3 B2 C1 D4
   c. A2 B4 C1 D3
   d. A2 B3 C4 D1

8. _____ are produced at the end of the growth phase or stationary phase.

   a. Tertiary b. Secondary

   c. Primary d. All the above

Answer the following

1. Define Fermentation?
2. What is bioreactor?
3. What is racking?
4. Define fining?
5. What are primary metabolites? Give one example?
6. What are secondary metabolites? Give example?
7. What are the characteristics of microbes in industrial microbiology?
8. Define primary screening with example.

TN_GOVT_XII_Micro_Biology_CH06.indd 108 07-12-2021 15:16:32

9. Define secondary screening with example.
10. Crowded plate technique. Explain.
11. Giant colony technique–Explain in detail.
12. Write salient features of secondary screening.
13. What is strain development? What are the attributes of improved strains?
14. List any five methods of preservation of micro organisms.
15. Explain the components of fermentor.
16. What is cup stream processing?
17. Define downstream processing?
18. Difference between upstream and downstream processing.
19. Write the steps in downstream processing?
20. Write the components of fermentor and its function.
21. Explain the various types of fermentation median components used in Industrial microbiology?
22. Explain penicillin production any two process.
23. Define semi synthetic penicillin?
24. What are the different types of wine?
25. How will you prepare white wine from red grapes?
26. Explain the steps in wine production?
27. List the steps involved in SCP production.
28. What are the disadvantages of SCP?
29. Explain the steps involved in citric acid production.
30. What are the uses of citric acid.

Student Activity

1. Ask the students to prepare wine by using the grapes available in the super market.
2. Fermentor design and their components.
3. Wine production

TN_GOVT_XII_Micro_Biology_CH06.indd 109 07-12-2021 15:16:32

Chapter 7

Medical Bacteriology

Learning Objectives

After studying this chapter the students will be able to,

- *Understand the importance of Medical bacteriology.*
- *Describe the pathogenesis of various bacterial diseases such as skin, respiratory infection, food poisoning, diarrhea and dysentery diseases, STD and zoonotic diseases.*
- *Know the collection of appropriate clinical specimens and laboratory diagnosis of various bacterial infections.*
- *Specify how bacterial infections are prevented and treated with appropriate antibiotics.*

Chapter Outline

7.1 Pathogenic Attributes

7.2 Routes of Entry

7.3 *Staphylococcus aureus*

7.4 *Streptococcus pyogenes*

7.5 *Neisseria meningitidis*

7.6 *Corynebacterium diphtheriae*

7.7 *Clostridium tetani*

7.8 *Shigella dysenteriae*

7.9 *Salmonella typhi*

7.10 *Vibrio cholerae*

7.11 *Mycobacterium tuberculosis*

7.12 *Treponema pallidum*

7.13 *Leptospira interrogans*

CPX78

Medical Bacteriology is the subset of Medical microbiology, which deals with the study of bacterial pathogens. It includes the pathogenesis, diagnosis, treatment and prevention of various bacterial diseases. **Robert Koch** is considered as the **Father of Bacteriology.**

7.1 Pathogenic Attributes

The host-parasite relationship is determined by the interaction between host factors and the infecting pathogens. Pathogenicity refers to the ability of a pathogen to produce disease. Virulence is the ability of the pathogen to cause disease.

Adhesion, invasiveness (Streptococcal infections), Bacterial toxins (endotoxins and exotoxins), capsule enzymes (proteases, collagenase, coagulase and other enzymes). These are already explained in the XI Standard text book.

TN_GOVT_XII_Micro_Biology_CH07.indd 110 07-12-2021 15:18:50

7.2 Route of Entry

To establish an infection, pathogen must first enter the host. Normal defense mechanisms and barriers (For example Skin, mucus, ciliated epithelium, lysozyme) make it difficult for the pathogen to enter the body.

Sometimes these barriers are break through for example cut in the skin, wound, tumor, ulcer which provides portal of entry for the bacteria. Some bacterial pathogens have the means to overcome the barriers through various virulence factors and invade the body.

Certain bacteria are infective when introduced through optimal route. The various route of entry of pathogens, which are cut or abrasion or wound (skin), Ingestion, Inhalation, arthropod bite, sexual transmission and congenital transmission. These are already explained in the XI Standard text book. The various bacterial pathogens, its pathogenesis clinical symptoms, laboratory diagnosis, control, prophylaxis and treatment with appropriate antibiotics are discussed below.

7.3 *Staphylococcus Aureus* (Pyogenic Cocci)

The genus *Staphylococcus* is included in the family Micrococcaceae. *Staphylococcus* is a normal flora of skin and mucous membranes, but it accounts for human infections, which is known as staph infection. The name *Staphylococcus* was derived from a Greek word, **'staphyle'** means **bunch of grapes** and **'kokkos'** means **berry**. *Staphylococcus aureus* is a pathogenic species that causes pyogenic infections in human.

7.3.1 Morphology

- *Staphylococci* are gram positive spherical cocci, (0.8µm–1.0µm in diameter) arranged characteristically in grape like clusters (Figure 7.1).
- They are non-motile and non-sporing and few strains are capsulated.



The grape like cluster formation in *Staphylococcus aureus* is due to cell division occurring in three perpendicular planes, with daughter cells tending to be remaining in close proximity.

7.3.2 Cultural Characteristics

- They are aerobes and facultative anaerobes, optimal temperature is 37°C and optimum pH is 7.4–7.6.
- They grow on the following media and shows the characteristic colony morphology (Table 7.1 & Figure 7.2).

Table 7.1: Staphylococci aureus colony morphology on various media

Media	Colony Morphology
Nutrient Agar	Colonies are circular, smooth, convex, opaque and produces golden yellow pigment (most strains).
Blood Agar	Beta haemolysis
Mannitol salt Agar (MSA)	It is a selective medium for S. *aureus* produces yellow colored colonies due to fermentation of mannitol

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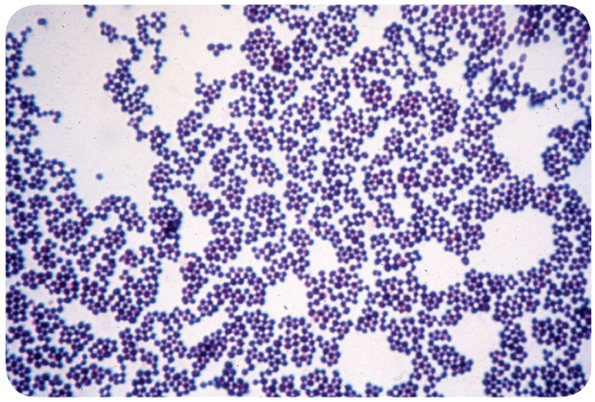

Figure 7.1: Gram staining of *Staphylococcus aureus*

7.3.3 Virulence Factors

1. Peptidoglycan → It is a polysaccharide polymer. It activates complement and induces the release of inflammatory cytokines.
2. Teichoic acid → it facilitates adhesion of cocci to the host cell surface.
3. Protein A → It is chemotactic, antiphagocytic, anticomplementary and induce platelet injury.
4. **Toxins:**
   a. Hemolysins – It is an exotoxin, those lysis red blood cells. They are of four types namely α-lysin, β-lysin, γ-lysin and delta lysin.
   b. Leucocidin – It damages PMNL (polymorphonuclear leucocytes) and macrophages.
   c. Enterotoxin – It is responsible for manifestations of Staphylococcus food poisoning.
   d. Exfoliative toxin – This toxin causes epidermal splitting resulting in blistering diseases.
   e. Toxic shock syndrome toxin – TSST is responsible for toxic shock syndrome.

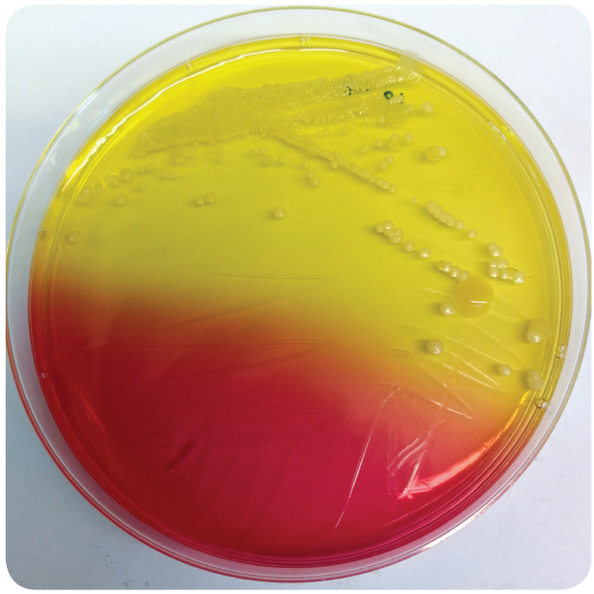

Figure 7.2: Colony morphology of *Staphylococcus aureus* on MSA

5. **Enzymes:** *S. aureus* produces several enzymes, which are related to virulence of the bacteria.
   a. Coagulase – It clots human plasma and converts fibrinogen into fibrin.
   b. Staphylokinase – It has fibrinolytic activity.
   c. Hyaluronidase – It hydrolyzes hyaluronic acid of connective tissue, thus facilitates the spread of the pathogens to adjacent cells.
   d. Other enzymes – S. *aureus* also produces lipase, nucleases and proteases

7.3.4 Pathogenicity

S. *aureus* is an opportunistic pathogen which causes infection most commonly at sites of lowered host resistance. (Example: damaged skin)

Mode of Transmission: *Staphylococcus* infections are transmitted by the following ways.

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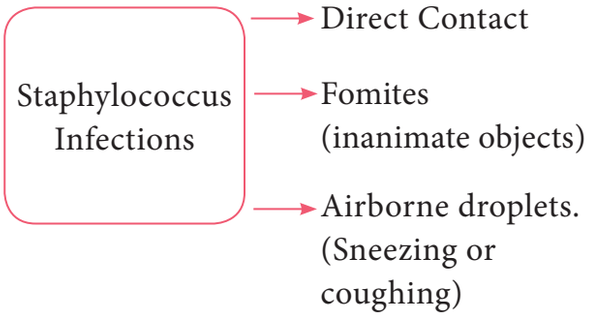
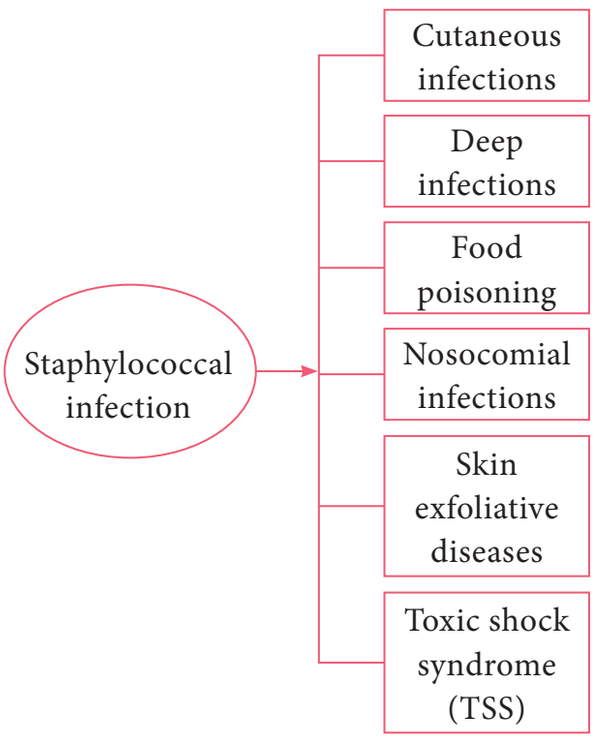
HOTS

Why many hospitalized patients are at increased risk for opportunistic infection?



Staphylococcal diseases may be classified as



It includes the following infections, which are as follows:

Cutaneous infections: Wound (injury), burn infections (tissue injury caused by heat), pustules (A small elevated skin lesions containing pus), furuncles (boil forms around a hair follicle and contains pus), styes (a painful swelling of hair follicle at eyelids), carbuncles (painful cluster of boils of the skin), Impetigo (skin infection with vesicles, pustules which ruptures), pemphigus neonatorum (an auto immune diseases that affect skin and mucous membranes)

Deep infections: It includes Osteomyelitis (inflammation of bones), tonsillitis (inflammation of tonsils), pharyngitis (inflammation of pharynx) sinusitis (inflammation of sinuses), periostitis (inflammation of membrane covering bones), bronchopneumonia (inflammation of lungs), empyema (collection of pus in the body cavity), septicemia (blood poisoning caused by bacteria and its toxins), meningitis (inflammation of meninge), endocarditis (inflammation of endocardium), breast and renal abscess.

Food Poisoning: Staphylococcal food poisoning may follow 2–6 hours after the ingestion of contaminated food (preformed enterotoxin). It leads to nausea, vomiting and diarrhea.

Nosocomial infection: S. *aureus* is a leading cause of hospital acquired infections. It is the primary cause of lower respiratory tract (LRT) infections and surgical site infections and the second leading cause of nosocomial bacteremia, pneumonia, and Cardiovascular infections.

Exfoliative diseases: These diseases are produced due to the production of epidermolytic toxin. The toxin separates the outer layer of epidermis from the underlying tissues leading to blistering disease. The most dramatic manifestation

TN_GOVT_XII_Micro_Biology_CH07.indd 113 07-12-2021 15:18:52

of this toxin is scalded skin syndrome. The patient develops painful rash which slough off and skin surface resembles scalding.

HOTS

Why most infections acquired through the skin are non-communicable diseases?

Toxic shock syndrome toxin: It is caused by TSST-1 and characterized by high fever, hypotension (low blood pressure), vomiting, diarrhea and erythematous rash. TSS became widely known in association with the use of vaginal tampons by menstruating women but it occurs in other situations also.

7.3.5 Laboratory Diagnosis

Specimens: The clinical specimens are collected according to the nature of Staphylococcal infections, which is given in the (Table 7.2).

Table 7.2: Clinical specimen collected for Staphylococcal infections

Infections	Clinical Specimens
Suppurative lesions	Pus
Respiratory infections	Sputum
Septicemia	Blood
Meningitis	CSF
Food poisoning	Faeces, food or vomitus

Specimens should be transported immediately to the laboratory and processed.

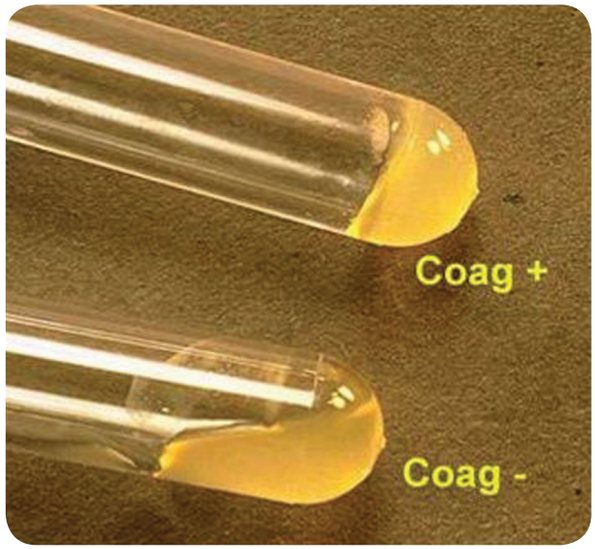


Figure 7.3: Skin infections caused by *Staphylococcus aureus*

Direct Microscopy: Gram stained smears of clinical specimens is done, where gram positive cocci in clusters were observed.

Culture: The collected specimen is inoculated on selective media-MSA and the media incubated at 37°C for 18–24 hours. Next day culture plates are examined for bacterial colonies, which are identified by gram staining, colony morphology and biochemical tests such as

a. Catalase test: The genus *Staphylococci* are catalase positive. This test distinguishes *Staphylococcus* from *Streptococcus* (catalase negative).

b. Coagulase test: This test helps in differentiating a pathogenic strain from non-pathogenic strain. *S. aureus* is coagulasepositive (Figure 7.3).

7.3.6 Treatment

Benzyl penicillin is the most effective antibiotic. Cloxacillin is used against beta lactamase. Producing strains (β-lactamase

TN_GOVT_XII_Micro_Biology_CH07.indd 114 07-12-2021 15:18:52

is produced by few strains of S. *aureus* which cleaves β – lactam ring of penicillin). Vancomycin is used against MRSA (Methicillin Resistant *Staphylococcus aureus*) strains.

Topical applications: For mild superficial lesions, topical applications of bacitracin or chlorhexidine is recommended.

Control measures: Proper sterilization of medical instruments must be done. Intake of antibiotics must be taken under proper medical advice. The detection of source & carriers among hospital staff, their isolation and treatment should be practiced.

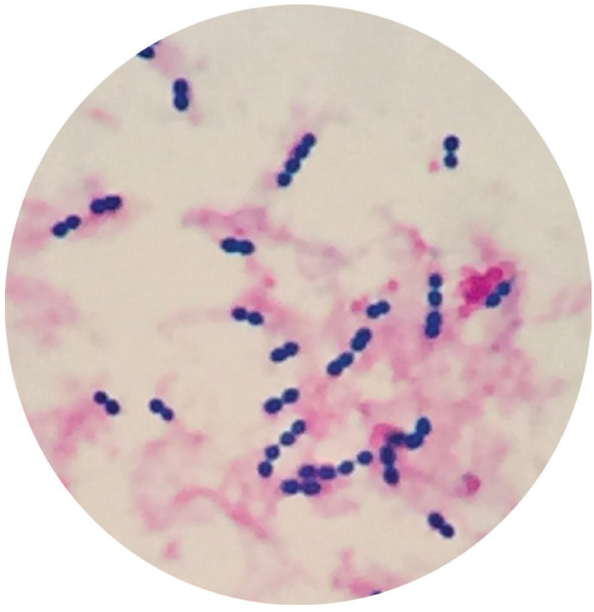


Figure 7.4: Gram staining of *Streptococcus pyogenes*

7.4 *Streptococcus Pyogenes* (Flesh eating Bacteria)

The genus *Streptococcus* includes a large and varied group of bacteria. They inhabit various sites, notably the upper respiratory tract. However, some species of which *Streptococcus* pyogenes is the most important and are highly pathogenic. The name *Streptococcus* is derived from **Greek** word **'Streptos'** which means twisted or coiled.

7.4.1 Morphology

- They are Gram positive, spherical or oval cocci and arranged in chains (0.6µm–1µm)
- They are non – motile, non – sporing. Some strains are capsulated (Figure 7.4).

7.4.2 Cultural Characteristics

- They are aerobe and facultative anaerobe. Optimum temperature is 37°C and pH is 7.4 to 7.6
- They grow only in media enriched with blood or serum. It is cultivated on blood agar. On blood agar, the colonies are small, circular, semitransparent, low convex, with an area of clear hemolysis around colonies (Figure 7.5).
- Crystal violet blood agar – a selective medium for *Streptococcus pyogenes*.



Year	Scientist	Contributions
1874	The odor Billroth	*Streptococci* were discovered
1884	Friedrich Julius Rosenbach	The cocci were isolated from human lesions and gave the name *Streptococcus pyogenes*

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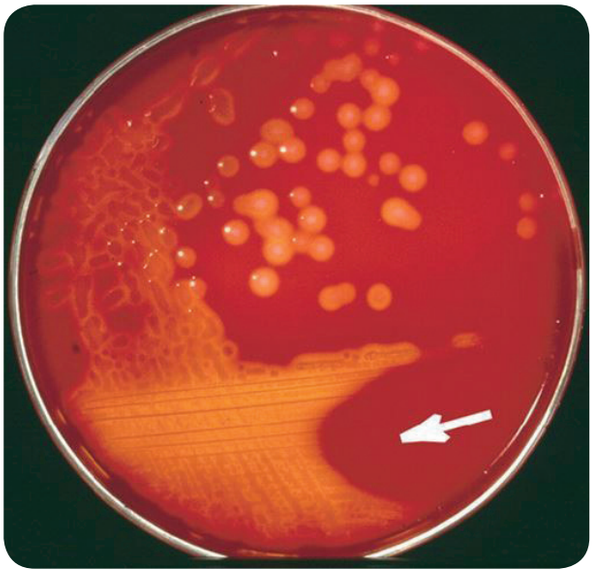


Figure 7.5: Colony morphology of *Streptococcus pyogenes* on blood agar

7.4.3 Antigenic Structure

Capsule: It inhibits phagocytosis

Cell wall: The outer layer of cell wall consists of protein and lipoteichoic acid which helps in attachment to the host cell. Middle layer of cell wall consists of Group Specific C – Carbohydrate that is used for Lancefield grouping. Inner layer of cell wall is made up of peptidoglycan which has pyrogenic and thrombolytic activity.

Toxins and Enzymes: *Streptococcus pyogenes* produces several exotoxins and enzymes which contribute to its virulence.

Toxins and Hemolysins: *Streptococci* produces two types of hemolysins which are Streptolysin O and Streptolysin S.

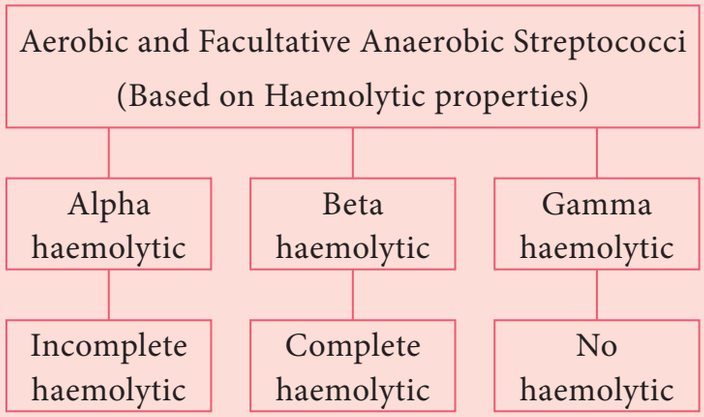
Erythrogenic toxin: (Pyrogenic exotoxin) – The induction of fever is the primary effect of this toxin and it is responsible for the rash of scarlet fever.

Enzymes: The various enzyme of *Streptococcus pyogenes*which exhibits virulence activity are listed in Table 7.3.

Infobits

Classification

The aerobic and facultative anaerobic *Streptococcus* are classified based on haemolytic properties. Three types of haemolytic reactions are observed on blood agar medium, which are:



Based on serological grouping of carbohydrate C antigen of Beta haemolytic organisms, they are classified into 20 Lancefield groups (A to H & K to V)

Streptococcus pyogenes is beta haemolytic organism which is included in Group A.

TN_GOVT_XII_Micro_Biology_CH07.indd 116 07-12-2021 15:18:53

Table 7.3: Enzymes of *Streptococcus pyogenes* and its virulence nature

Enzymes	Virulence nature
streptokinase (fibrinolysin)	It promotes the lysis of human fibrin clot by catalyzing the conversion of plasminogen into plasmin. It facilitates the spread of infection by breaking down the fibrin barrier around the lesions.
Deoxyribonucleases	It liquefy the highly viscous DNA that accumulate in thick pusand responsible for thin serous character of streptococcal exudates
Hyaluronidase	It breaks down hyaluronic acid of the tissues and favors spread of streptococcal lesion along intercellular spaces.
Other enzymes	NADase, lipase, amylase, esterase, phosphates and other enzymes

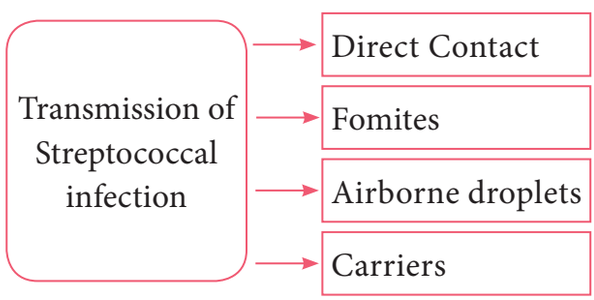


Streptokinase: It is given intravenously for the treatment of early myocardial infarction and other thromboembolic disorders. *Streptococcus equisimilis* is the source of streptokinase used for thrombolytic therapy in patients.

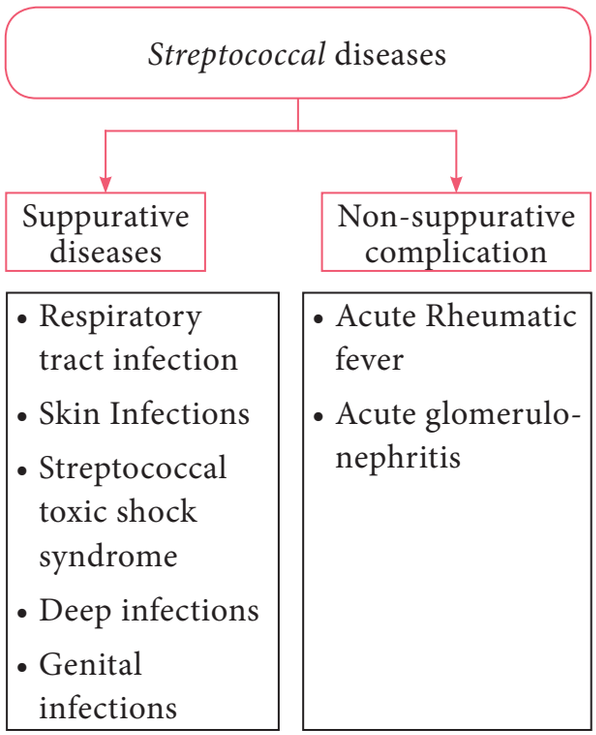
7.4.4 Pathogenesis

Streptococcus pyogenes is intrinsically a much more dangerous pathogen than *Staphylococcus aureus* and has a much greater tendency to spread in the tissues.

Mode of transmission: Streptococcal infections are transmitted by the following ways:



Streptococcal diseases may be broadly classified, and it is shown in flowchart 7.1



Flowchart 7.1: Classification of *Streptococcal* diseases

Suppurative Infections

1. Respiratory tract infection

a. Streptococcal sore throat: Sore throat (acute tonsillitis and pharyngitis) is the

TN_GOVT_XII_Micro_Biology_CH07.indd 117 07-12-2021 15:18:53

most common streptococcal diseases. Tonsillitis is more common in older children and adults. The pathogen may spread from throat to the surrounding tissues leading to suppurative (pus – formation) complication such as cervical adenitis (inflammation of a lymph node in the neck) otitis media (inflammation of middle ear), quinsy (ulcers of tonsils) Ludwig's angina (purulent inflammation around the sub maxillary glands) mastoiditis (inflammation of mastoid process).

b. Scarlet fever: The disease consists of combination of sore throat and a generalized erythematous (redness of skin or mucous membranes) rash.

2. Skin infections

a. Erysipeals: It is an acute spreading lesion. The skin shows massive brawny oedema with erythema it is seen in elderly persons or elders.

b. Impetigo: (Streptococcal pyoderma)

It is a skin infection that occurs most often in young children. It consists of superficial blisters that break down and eroded areas whose surface is covered with pus. It is the main cause leading to acute glomerulonephritis in children.

c. Necrotizing fasciitis: It is an invasive, infection characterized by inflammation and necrosis of the skin, subcutaneous fat and fascia. It is a life-threatening infection.



The strain which cause necrotizing fasciitis to have been named as "Flesh eating bacteria or" killer bacteria.

3. Streptococcal toxic shock syndrome

Streptococcal pyrogenic exotoxin leads to streptococcal toxic shock syndrome (TSS). It is a condition in which the entire organ system collapses, leading to death.

4. Genital infections

Streptococcus pyogenes is an important cause of puerperal sepsis or child bed fever (infection occur when bacteria infect the uterus following child birth)

5. Deep infection

Streptococcus pyogenes may cause pyaemia (blood poisoning characterized by pus forming pathogens in the blood) septicemia (A condition in which bacteria circulate and actively multiply in the bloodstream) abscess in internal organs such as brain, lung, liver and kidney.

Non – Suppurative Complication

Streptococcus pyogenes infections are sometimes followed by two important non – suppurative complications which are, acute rheumatic fever and acute glomerulonephritis. These complications occur 1–4 weeks after the acute infection and it is believed to be the result of hypersensitivity to some streptococcal components.

1. Rheumatic fever

It is often preceded by sore throat and most serious complication of haemolytic streptococcal infection. The mechanism by which *Streptococci* produce rheumatic fever is still not clear. A common cross – reacting antigen exist in some group A streptococci and heart, therefore, antibodies produced in response to the

TN_GOVT_XII_Micro_Biology_CH07.indd 118 07-12-2021 15:18:53

streptococcal infection could cross react with myocardial and heart valve tissue, causing cellular destruction.

2. Acute glomerulonephritis

It is often preceded by the skin infection. It is caused by only a few "nephritogenic types (strains)". It develops because some components of glomerular basement membrane are antigenically similar to the cell membranes of nephritogenic streptococci. The antibodies Formed against *Streptococci* cross react with glomerular basement membrane and damage. Some patients develop chronic glomerulonephritis with ultimate kidney failure.

HOTS

Why are some staphylococcal skin infections similar to streptococcal skin infection?

7.4.5 Laboratory Diagnosis

Specimens: Clinical specimens are collected according to the site of lesion. Throat swab, pus or blood is obtained for culture and serum for serology.

Direct Microscopy: Gram stained smears of clinical specimens is done, where Gram positive cocci in chains were observed. It is indicative of streptococcal infection.

Culture: The clinical specimen is inoculated on blood agar medium and incubated at 37° C for 18–24 hours. After incubation period, blood agar medium was observed for zone of beta – haemolysis around colonies.

Catalase test: *Streptococci* are catalase negative which is an important test to differentiate *Streptococci* from *Staphylococci*.

Serology: Serological tests are done for rheumatic fever and glomerulonephritis. It is established by demonstrating high levels of antibody to streptococci toxins. The standard test is antistreptolysin Otitration. ASOtitres higher than 200 units are indicative of prior Streptococcal infection.

7.4.6 Treatment and Prophylaxis

- Penicillin G is the drug of choice.
- In patients allergic to penicillin, erythromycin or cephalexin is used.
- Antibiotics have no effect on established glomerulonephritis and rheumatic fever.
- Prophylaxis is indicated only in the prevention of rheumatic fever, it prevents streptococcal reinjection and further damage to the heart.
- Penicillin is given for a long period in children who have developed early signs of rheumatic fever.



Myth: Eating chocolate encourages the development of acne.

Fact: It is the oils and fats in many chocolate products, and not chocolate itself, that promote sebum production and subsequent acne. Chocolate in low-fat chocolate milk and in fat-free chocolate candies does not encourage acne. Acne suffers do not need to give up chocolate, they need to reduce their lipid consumption.

TN_GOVT_XII_Micro_Biology_CH07.indd 119 07-12-2021 15:18:53

7.5 *Neisseria Meningitides* (Meningococcus)

The genus *Neisseria* is included in the family *Neisseriaceae* (Figure 7.6). It contains two important pathogens *Neisseria meningitidis* and *Neisseria gonorrhoeae*, both the species are strict human pathogens. *N. meningitides* causes meningococcal meningitis (formerly known as cerebrospinal fever).

The word **Meningitis** is derived from Greek word **'meninx'** means membrane and **'itis'** means inflammation. It is an inflammation of meanings of brain or spinal cord. Bacterial meningitis is a much more severe disease than viral meningitis.

7.5.1 Morphology

They are Gram negative diplococci (0.6µm–0.8µm in size) arranged typically in pairs, with adjacent sides flattened.

They are non – motile, capsulated (Fresh isolates).

Cocci are generally intracellular when isolated from lesions (Figure 7.7).

7.5.2 Cultural Characteristic

They are strict aerobes, but growth is facilitated by 5–10% CO_2 and high humidity. The optimum temperature is 35°C–36°C and optimum pH is 7.4–7.6. They are fastidious pathogens, growth occurs on media enriched with blood or serum. They grow on the following media and show the characteristic colony morphology (Table 7.4).

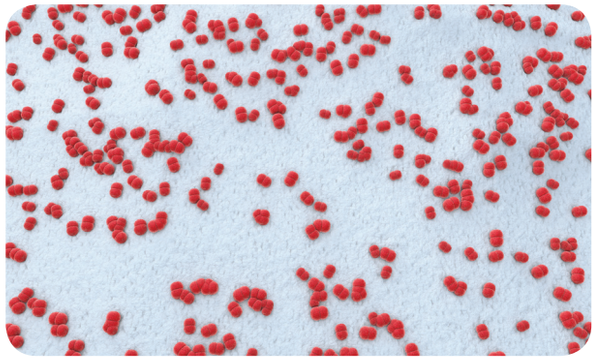


Figure 7.6: Gram staining of *Neisseria Meningitides*

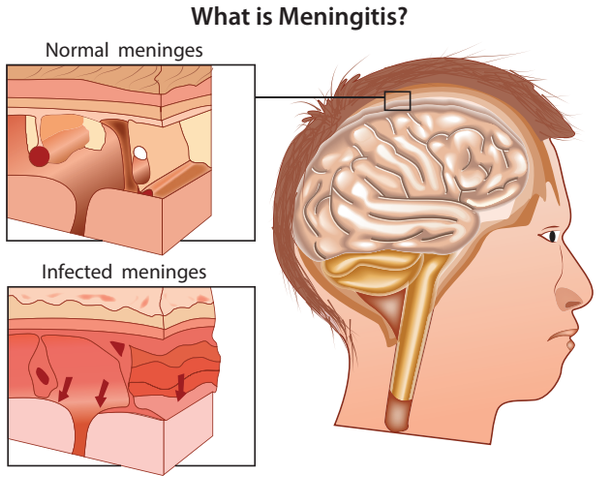


Figure 7.7: Pathogenesis of Meningitis

Table 7.4: Colony morphology of *Neisseria Meningitides* on media

Name of the media	Colony Morphology
Chocolate agar	Colonies are large, colorless to grey opaque colonies.
Mueller Hinton agar	Colonies are small, round, convex grey, translucent with entire edges. The colonies are butyrous in consistency and easily emulsified.

7.5.3 Pathogenesis

N. meningitidis is the causative agent of meningococcal meningitis, also known as pyogenic or septic meningitis. Infection is most common in children

TN_GOVT_XII_Micro_Biology_CH07.indd 120 07-12-2021 15:18:55

and young adults. Meningococci are strict human pathogens. Human nasopharynx is the reservoir of *N.meningitidis.* The pathogenesis is dicussed in the flowchart 7.2.

Source of infection – Airborne droplets

Route of entry – Nasopharynx

Site of infection – Meninges

Incubation period – 3 days

7.5.4 Laboratory Diagnosis

Specimens: CSF, blood, nasopharyngeal scrapings from petechiae lesions are the specimens collected from pyogenic meningitis patients.

Direct Microscopy: CSF is centrifuged, and smear is prepared from the deposit for gram staining. Meningococci are Gram negative diplococci, present mainly inside polymorphs and many pus cells are also seen.

Culture: The centrifuged deposit of CSF is inoculated on chocolate agar. The plate is incubated at 36°C under 5–10% CO_2 for 18–24 hours. After incubation period, meningococcusis identified by gram staining, colony morphology and biochemical reactions. *N. meningitides* is catalase and oxidase positive (Figure 7.8).

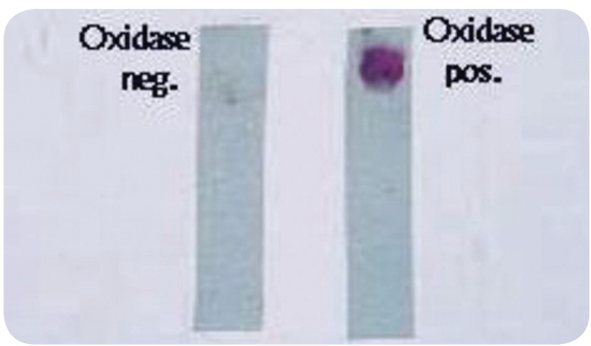


Figure 7.8: Oxidase test

The meningococci gain entry in the nasopharynx and attaches to the epithelial cells with pili. They are engulfed by epithelial cells of mucosa and penetratesintonearby blood vessels, thereby damaging the epithelium and causes pharyngitis.

↓

Cocci spread from the nasopharynx to meninge by travelling along the perineural sheath of the olfactory nerve, through the cribriform plate to the subarachnoid space or through blood stream.

↓

Pathogen entering the blood vessels rapidly permeates the meninges and produce meningitis (most complication in children). It is marked by the following clinical manifestations, which are fever, sore throat, headache, stiff neck, and vomiting convulsions (fits).

↓

The pathogen sheds endotoxin into the generalized circulation, which damages the blood vessels and leads to vascular collapse, hemorrhage, petechiae lesion (a small red or purple spot caused by bleeding into the skin).

↓

A few develop sudden meningococcemia **(water house – friderichsen syndrome)** characterized by shock, disseminated intravascular coagulation and multisystem failure.

↓

It has a violet onset, with fever, chills, shock and coma. Generalized intravascular clotting, cardiac failure, damage to adrenal glands and death occurs within a few hours.

Flowchart 7.2: Pathogenesis of *Neisseria Meningitides*

TN_GOVT_XII_Micro_Biology_CH07.indd 121 07-12-2021 15:18:55

7.5.5 Treatment and Prophylaxis

Penicillin – G is the drug of choice. In penicillin allergic cases, chloramphenicol is recommended.

- Monovalent and polyvalent vaccines (capsular polysaccharide) induce good immunity in older children and adults.
- Conjugate vaccines are used for children below the age of 2 years.

7.6 *Corynebacterium Diphtheriae*

Several species of the genus *Corynebacterium* are normal flora of skin, upper respiratory tract (URT), urogenital and intestinal tract. The most important member of the genus is *C. diphtheriae* the causative agent of diphtheria, a localized inflammation of the throat with greyish white pseudomembrane and a generalized toxemia due to the secretion and dissemination of a highly potent toxin.

The name *Corynebacterium diphtheria* is derived from **Greek word 'Coryne'** – "Club shaped swellings" or"Knotted rod" **'Diphthera'** – Leather.

7.6.1 Morphology

- They are Gram positive slender rods, pleomorphic club shape or coryneform bacterium Non – motile, non – sporing and non – capsulated (Figure 7.9a & b).
- The bacilli are arranged in a characteristic fashion in angular fashion resembling the letters V or L. This has been called Chinese letter or cuneiform arrangement (Figure 7.10).
- They are club shaped due to the presence of metachromatic granules at one or both ends. These granules are composed of polymetaphosphates and represent energy storage depots.

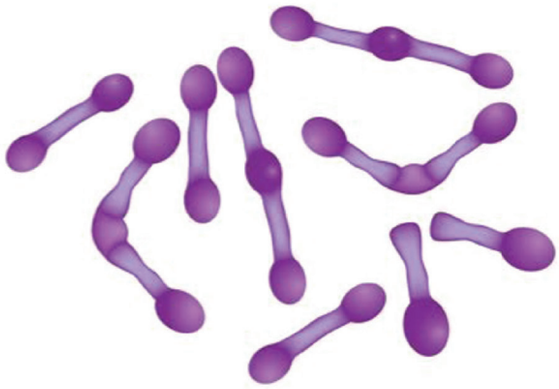


Figure 7.10: Gram staining of *Corynebacterium diphtheriae*

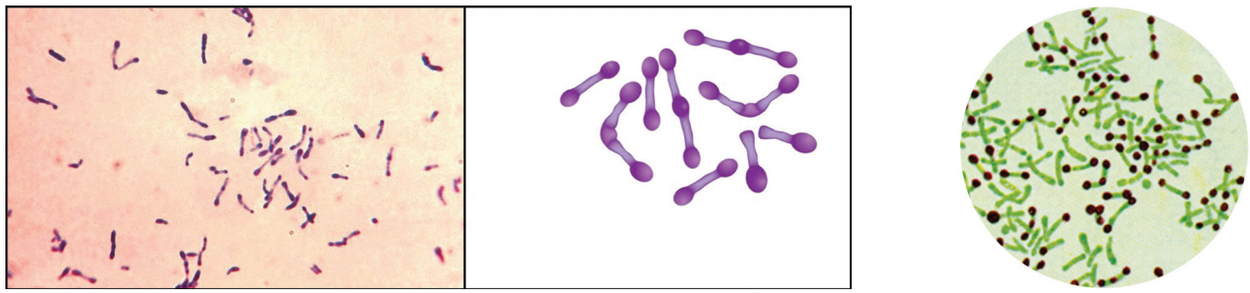


Figure 7.9: (a) Gram staining of *Corynebacterium diphtheriae* **(b)** Albert's staining showing metachromatic granules

TN_GOVT_XII_Micro_Biology_CH07.indd 122 07-12-2021 15:18:58

7.6.2 Cultural Characteristics

- They are aerobic and facultative anaerobe. Optimum temperature is 37°C and pH 7.2.
- They grow on the following media and show the characteristic colony morphology (Table 7.5).

Table 7.5: Colony Morphology of *Corynebacterium diphtheriae* on cultural media

Media	Colony Morphology
Loeffler's Serum slope	They grow on this medium very rapidly. Colonies appear after 6–8 hours of incubation. The colonies are small, circular white or creamy and glistening
Tellurite Blood Agar	Grey or black colonies. Based on colony morphology on tellurite medium, three main biotypes – Gravis, Intermedius and Mitis.

Toxin

- The pathogenicity is due to production of a very powerful exotoxin by virulent strains of diphtheria bacilli.
- The toxigenicity of diphtheria bacillus depends on the presence of a tox^+ gene which can be transferred from one bacterium to another by lysogenic bacteriophages, of which beta phage is the most important.

Properties

The diphtheria toxin is a heat – labile protein and has a molecular weight of about 62,000 Dalton. It consists of two fragments.

a. Fragment A (24,000 Dalton) – It has all enzymatic activity.
b. Fragment B (38,000 Dalton) – It is responsible for binding the toxin to the target cells.

Mode of Action

The toxin acts by inhibiting protein synthesis, specifically fragment A inhibits polypeptide chain elongation in the presence of NAD by inactivating the elongation factor (EF – 2) the toxin has special affinity for myocardium, adrenal gland and nerve endings.

7.6.3 Pathogenicity

Source of infection – Airborne droplets

Route of entry – Upper respiratory tract

Incubation period – 3–4 days

Site of infection – Faucial (nasal, otitis, conjunctival, laryngeal, genital) diphtheria is most commonly seen in children of 2–10years.

Faucial diphtheria is the most common type. The infection is confined to humans only. The toxin has both local (flowchart 7.3) as well as systemic effects.

Systemic effects

The toxin diffuses into the blood stream and causes toxemia. It has got affinity for cardiac muscle, adrenal and nerve endings. It acts on the cells of these tissues.

7.6.4 Clinical Manifestations

1. Laryngeal obstruction, asphyxia (it is a condition of severe deficient supply of oxygen, causing suffocation).
2. Diphtheritic myocarditis (inflammation of heart muscle), polyneuropathy

TN_GOVT_XII_Micro_Biology_CH07.indd 123 07-12-2021 15:18:58

The bacilli enters through URT and it remains confined to the site of entry where they multiply and produces diphtheria toxin

↓

The toxin causes local necrotic changes along with superficial inflammatory reaction. The necrosed epithelium together with fibrinous exudate, leucocytes, erthrocytes and bacteria consitute the greyish psedudomembrane, which is a characteristic feature of diphtheritic infection.

↓

The membrane extends from oropharynx to larynx and into trachae. The mechanical obstruction of the trachae by psedudomembrane can cause suffocation and death.

Flowchart 7.3: Localized effect of diphtheria toxin

(damage of multiple peripheral nerves), paralysis of palatine (the top part of the inside of the mouth) and ciliary muscles.

3. Degenerative changes in adrenal glands, kidney and liver may occur.

7.6.5 Laboratory Diagnosis

Specimen: Two swabs from the lesions are collected. One swab is used for smear preparationand other swab for inoculation on culturemedia.

Direct microscopy: Smears are stained with both Gram stain and Albert stain.

a. Gram Staining – Gram positive slender rods were observed.

b. Albert staining – Club shaped with metachromatic granules were observed.

Culture: The swabis inoculated on Loeffler's serum slope, after overnight incubation at 37°C, the plates were observed for characteristic colonies, which are identified by gram staining.

7.6.6 Prophylaxis

Diphtheria can be controlled by immunization. Three methods of immunization are available (Table 7.6).

7.6.7 Treatment

The specific treatment for diphtheria consists of administration of antitoxin with dose of 20,000–100, 000 units of ADS intramuscularly and antibiotic therapy using penicillin.

7.7 *Clostridium Tetani*

The genus *Clostridium* consists of anaerobic, spore forming Gram positive bacilli. The spores are wider than the bacterial bodies, giving the bacillus a swollen appearance resembling a spindle. The name *Clostridium* is derived from the word **'kluster' (a spindle**). Most species are saprophytes found in soil, water and decomposing plant and animal matter. Some of the pathogens are normal flora of intestinal tract of human and animals.

The genus *Clostridium* includes bacteria that causes 3 major diseases of human – **Tetanus, gas gangrene and food poisoning**. *Clostridium* pathogenicity is mainly due to production of a powerful exotoxin.

Clostridium of medical importance may be classified based on diseases they produce, which is given the Table (7.7).

TN_GOVT_XII_Micro_Biology_CH07.indd 124 07-12-2021 15:18:58

Table 7.6: Immunization for diphtheria

Immunization		
Active	**Passive**	**Combined**
DPT (Triplevaccine) 3 doses of 0.5ml each are given intramuscular route at an interval of 4–6 weeks after birth. Booster dose of DPT are given at 18 months and at the age of 5.	500–1000 units of Antidiphtheritic serum, (ADS) is administered subcutaneously.	It consists of administration first dose of adsorbed toxoid on one arm, while ADS is given on another arm.

Table 7.7: Clostridium sp. causing pathogenic diseases.

Organisms	Diseases
Clostridium tetani	Tetanus
Clostridium perfringens	Gas gangrene
Clostridium botulinum	Food poisoning

7.7.1 Morphology

They are Gram positive spore forming rods. The spores are spherical and terminal in position giving a drumstick appearance. They are motile and non – capsulated.

7.7.2 Culture Characteristics

- They are obligate anaerobes, optimum temperature is 37°C and pH is 7.4.
- It grows on ordinary media, but growth is enhanced by addition of blood and serum. *Clostridia tetani* grows on the following media and show the characteristic colony morphology (Table 7.8).

7.7.3 Toxins

Clostridium tetani produces two distinct toxins namely,

a. Tetanolysis (haemolysin)

b. Tetanospasmin (neurotoxin)

Table 7.8: Colony characteristics of *Clostridium tetani*

Media	Colony Morphology
Blood agar	They produce α – hemolysis which subsequently develop into β – hemolysis (due to tetanolysis) it produces swarming growth.
Cooked meat broth (CMB)	Growth occurs as turbidity with gas formation. The meat is not digested but becomes black on prolonged incubation

Tetanolysis

- Heat labile and oxygen labile toxin.
- It lysis erythrocytes and also acts as neurotoxin.

Tetanospasmin

- It is heat labile and oxygen stable powerful neurotoxin.
- It is protein in nature. consisting of a large polypeptide chain (93,000 Dalton) and a smaller polypeptide chain (52,000 Dalton) joined by a disulphide bond.
- **Mode of Action:** Tetanospasminis a neurotoxin, which blocks the release of

TN_GOVT_XII_Micro_Biology_CH07.indd 125 07-12-2021 15:18:58

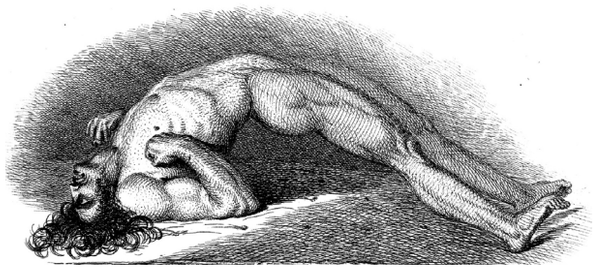


Figure 7.11: Tetanus – opisthotonos

inhibitory neurotransmitters (glycine and gamma – amino butyric acid) across the synaptic junction. The toxin acts presynaptically, the abolition of spinal inhibition causes uncontrolled spread of impulses in CNS (Central Nerves System). This results in muscle rigidity and spasms (due to the simultaneous contraction of agonists and antagonists, in the absence of reciprocal inhibition Figure 7.11).

7.7.4 Pathogenesis

Clostridium tetani is the causative organism of tetanus or lock jaw disease. pathogenesis of *Clostridium tetani* was discussed in detail in flowchart 7.4.

Source of infection – Soil, dust, faeces.

Route of entry – Through wound

Incubation period – 6–12 days

7.7.5 Clinical Feature

It includes, pain and tingling at the site of wound, Lock jaw ortrismus (It is reduced opening of the jaws), Risus sardonicus (mouth kept slightly open), Dysphasia (impairment of the ability to speak or to understand language) and acute asphyxia.

7.7.6 Laboratory Diagnosis

Specimens: Wound swab, exudates or tissue from wound.



Myth: Rust causes tetanus if introduced into a wound – for example, by stepping on a rusty nail.

Fact: Rusty nails are more likely to be contaminated with tetanus endospores because they have been exposed to soil and dust longer than new, rust free nails. Any object that causes a wound, rusty or not, can inoculate the tissue with the bacterial endospores of *C. tetani*. Rust itself neither causes tetanus nor makes it worse.

Microscopy: Gram staining shows Gram positive bacilli with drumstick appearance.

Culture: The clinical specimen is inoculated on blood agar and incubated at 37°C for 24–48 hours under anaerobic conditions. The colonies are confirmed by gram staining, where it shows gram positive bacilli with drumstick appearance.

7.7.7 Treatment

Tetanus patients are treated in special isolated units, to protect them from noise and light which may provoke convulsions. The spasm can be controlled by diazepam



Clostridium difficile is the causative agent of **antibiotic associated colitis.** It is an acute colitis with or without pseudo membrane formation. It is an important complication in patients on oral antibiotic therapy. Many antibiotics have been incriminated but lincomycin and clindamycin are particularly prone to cause pseudomembranous colitis.

TN_GOVT_XII_Micro_Biology_CH07.indd 126 07-12-2021 15:18:58

Table 7.9: Immunization for tetanus

Active immunization	Passive immunization	Combined prophylaxis
a. Tetanus toxoid (TT) b. DPT	Antitetanus serum (ATS) Human Antitetanus immunoglobulins (HIIG)	Tetanus toxoid in one arm and ATS or HTIG in another arm.

Tetanus develops by the contamination of wound with *Clostridum tetani* spores. The spores germinate in reduced oxygen tension (anaerobic environment).

↓

The vegetative cell grows and produces a potent neurotoxin called tetanospasmin. The toxin is absorbed from the site of its production and enters into the blood stream. It ascends to central nervous systems (CNS) through motor nerves.

↓

The toxin blocks synaptic inhibition in the spinal cord. It acts presynaptically.

↓

The toxin affects most of the voluntuary muscles in the body, causing muscle rigidity and spasma due to uncontrolled contractions.

↓

The first symptoms appears in head and neck because of shorter length of cranial nerves. Masseter muscles are first affected causing 'Lock Jaw'.

↓

In severe cases progressive spasm of the back or extensor muscles produces opisthotonos (extreme arching of the back) usually death occurs due to respiratory paralysis.

Flowchart 7.4: Pathogenesis of *Clostridium tetani*

(0.1–0.2 mg/kg) injection. Antibiotic therapy with penicillin or metronidazole should be done for a week or more.

7.7.8 Prophylaxis

It is done by the following methods, which are as follows.

a. Surgical prophylaxis: It aims at removal of foreign body, blood clots and damaged tissue in order to prevent anaerobic conditions favorable for the germination of spores.

b. Immunoprophylaxis: Tetanus is a preventable disease. Immune prophylaxis is of 3 types, which is given in the (Table 7.9).

Infobits

Clostridial Toxins As Therapeutic Agents

Botulinum toxin is the most poisonous substance known, is being used for the treatment of specific neuromuscular disorders characterized by involuntary muscle contraction. Since approval of Botulinum toxin (botox) by the FDA in 1989 for 3 disorders – Strabismus (crossing of the eyes), blepharospasm (spasmodic contraction of eye muscles) and hemifacial spasm (contraction of one side of the face).

In 2000, dermatologists and plastic surgeons began using Botox to eradicate wrinkles caused by repeated muscle contractions as we laugh, smile or frown.

TN_GOVT_XII_Micro_Biology_CH07.indd 127 07-12-2021 15:18:58

7.8 *Shigella Dysenteriae* (Dysentery Bacillus)

The genus *Shigella*are exclusively parasites of human intestine and other primates. *Shigella dysenteriae* is the causative agent of bacillary dysentery or shigellosis in humans. It is a diarrheal illness which is characterized by frequent passage of bloodstained mucopurulent stools. The four important species of the genus *Shigella* are: *Shigella dysenteriae, Shigella flexneri, Shigella sonnei and Shigella boydii.*

7.8.1 Morphology

Shigella are short, Gram negative rods (0.5µm× 1–3 µm in size). They are non – motile, non – sporing and non – capsulated (Figure 7.12).

7.8.2 Cultural Characteristics

- They are aerobes and facultative anaerobes. Optimum temperature is 37°C and optimum pH – 7.4.
- They can be grown on the following media and show the characteristic colony morphology (Table 7.10 & Figure 7.13).

Table 7.10: Colony morphology of Shigella

Media	Colony Morphology
Nutrient Agar	Colonies are circular, convex smooth and translucent
MacConkey Agar	Colourless colonies
SS – Agar	Colourless colonies

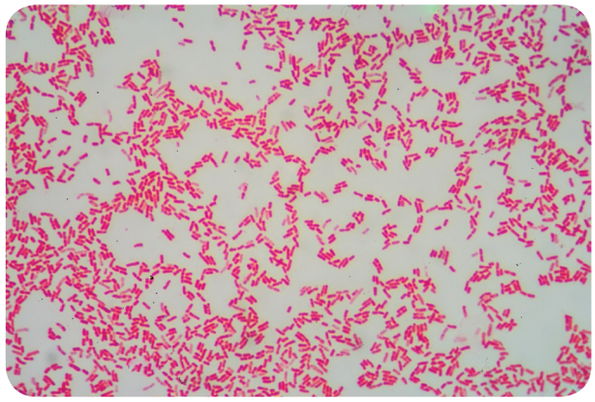


Figure 7.12: Gram staining of *Shigella*

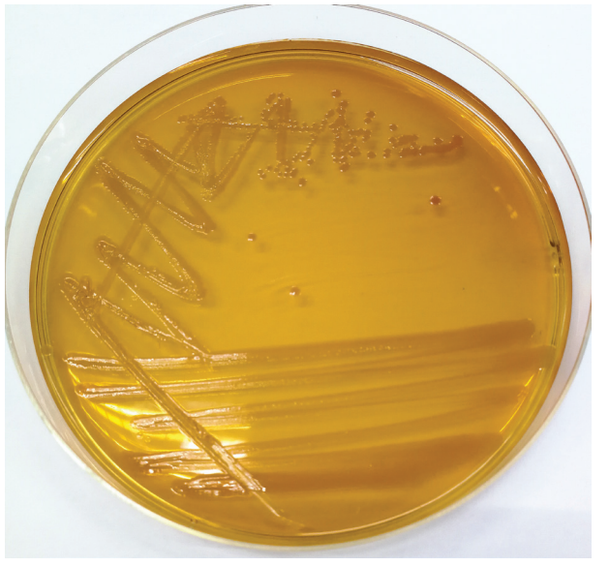


Figure 7.13: Colony morphology of *Shigella* on SS agar

7.8.3 Toxins

Shigella dysenteriae produces toxins, which is of 3 types, namely, endotoxin, exotoxin and verocytotoxin. The mode of action of these toxins is illustrated in the Table 7.11.

7.8.4 Pathogenesis

The pathogenic mechanism of *Shigella dysenteriae*is discussed below in flowchart 7.5.

Source of Infection – Patient or carriers

Route of entry – faecal – oral route

TN_GOVT_XII_Micro_Biology_CH07.indd 128 07-12-2021 15:18:59

Table 7.11: Various toxins of *Shigelladysenteriae*

Toxins	Mode of Action
Endotoxin	It is released after autolysis, it has irritating effect on intestinal wall which causes diarrhea and subsequently intestinal ulcers.
Exotoxin	It is a powerful toxin and acts as Enterotoxin as well as neurotoxin. **As Enterotoxin** – It induces fluid accumulation **As Neurotoxin** – It damages the endothelial cells of small blood vessels of CNS which results in polyneuritis and coma
Vero cytotoxin	It acts on Vero cells

Site of infection – Large intestine

Incubation Period – Less than 48 hours (1–7 days)

Mode of transmission – Food, finger, faeces and flies

7.8.5 Clinical Manifestations

- Frequent passage of loose, scanty faeces containing blood and mucus.
- Abdominal cramps and tenesmus (straining to defecate).
- Fever and vomiting.
- Hemolytic uremic syndrome (It is a condition caused by the abnormal destruction of red blood cells).

Sh. dysenteriae causes bacillary dysentery. The pathogen enters into the host by the ingestion of contaminated food.

↓

The bacilli reaches large intestines and adheres to the epithelial cells of villi. It multiples inside the cell and penetrates into lamina propria.

↓

As the pathogen multiples it produces toxins, it stimulates an inflammatory reaction and causes extensive tissue destruction. It leads to necrosis of surface epithelial cells.

↓

The necrotic epithelia, become soft and friable and are sloughed off leaving behind transverse superficial ulcers.

↓

Abdominal cramps and pain are caused by the disruption of the muscular function of the intestine

↓

The degeneration of intestinal villi and local erosion causes bleeding, heavy mucous secretion resulting in bacillary dysentery

Flowchart 7.5: Pathogenesis of *Shigella dysenteriae*

7.8.6 Laboratory Diagnosis

Specimens: Fresh stool is collected.

Direct Microscopy: Saline and Lugol's iodine preparation of faeces show large number of pus cells, and erythrocytes.

TN_GOVT_XII_Micro_Biology_CH07.indd 129 07-12-2021 15:18:59

Culture: For inoculation, it is best to use mucus flakes (if present in the specimen) on MacConkey agar and SS agar. After overnight incubation at 37°C, the plates are observed for characteristic colonies, which is confirmed by Grams staining and biochemical reactions.

7.8.7 Treatment and Prevention

- Uncomplicated shigellosis is a self – limiting condition that usually recovers spontaneously.
- In acute cases, oral rehydration therapy (ORT) is done.
- In all severe cases, the choice of antibiotic should be based on the sensitivity of prevailing strain.
- Many strains are sensitive to Nalidixic acid and Norfloxacin.
- Improving personal and environmental sanitation.
- The detection and treatment of patients and carriers.

7.9 *Salmonella Typhi* (Eberthella Typhi)

The genus *Salmonella* consists of bacilli that parasites the intestines of vertebrates and human beings. It causes **Enteric fever**, which includes **Typhoid** and **Paratyphoid fever**. The most important species of the genus is *Salmonella typhi* which causes typhoid fever.

7.9.1 Morphology

Salmonellae are Gram – negative rods (1–3µm× 0.5 µm in size). They are motile with peritrichous flagella, non – capsulated and non – sporulated (Figure 7.14).

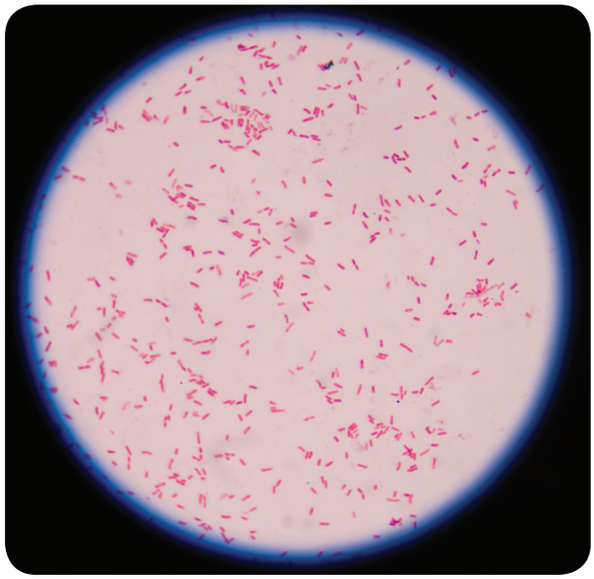


Figure 7.14: Gram staining of *Salmonella typhi*

7.9.2 Cultural Characteristics

They are aerobic and facultative anaerobe, optimum temperature - 37°C and pH is 7–7.5.

They grow on the following media and show the following characteristic colony morphology (Table 7.12).

Table 7.12: Colony morphology of *Salmonella typhi*

Media	Colony Morphology
Nutrient Agar	Colonies are large, circular, smooth, translucent
MacConkey Agar	Colourless colonies (non – lactose fermenters)
SS – Agar	Colourless colonies with black centered.

7.9.3 Pathogenicity

Salmonella typhi causes typhoid fever and its pathogenesis is discussed in flowchart 7.6.

Source of infection – food, feces, fingers, flies

TN_GOVT_XII_Micro_Biology_CH07.indd 130 07-12-2021 15:19:00

Route of entry – faecal oralroute (ingestion)

Incubation period – 7–14 days

The infection is acquired by ingestion of contaminated food and water

↓

The bacilli reaches the small intestine and attach to the epithelial cells of intestinal villi and penetrate to the lamina propria and submucosa

↓

These bacilli are phagocytosed by neutophils and macrophages. They resist intracellular killing and multiply within these cells.

↓

The pathogen enters the mesenteric lymph nodes, multiply there and enters blood stream through the thoracic duct

↓

A transient bacteraemia follows and internal organs like liver, gall bladder, spleen, bone marrow, lungs, lymph nodes and kidneys are infected

↓

The bacilli multiply abundantly in the gall bladder (bile juice) and are dischared continuously into the intestine involving peyer's patches and ileum.

↓

It becomes inflamed, necrosed and slough off, leaving behind typhoid ulcers. These ulcers may lead to two major complication, which are Intestinal perforation and haemorrhage.

Flowchart 7.6: Pathogenesis of *Salmonella typhi*

7.9.4 Clinical Manifestations

- The illness is usually gradual, with headache, malaise (feeling of discomfort), an2orexia (loss of appetite), coated tongue, abdominal discomfort with either constipation or diarrhea.
- Hepatosplenomegaly (enlargement of liver and spleen), step ladder pyrexia (continuous fever) and rose – spots (during 2nd or 3rd week).

7.9.5 Laboratory Diagnosis

Specimens: Blood, stool and urine are the clinical samples collected from typhoid patients. The selection of relevant specimen depends upon duration of illness, which is very important for diagnosis (Table 7.13 & Figure 7.15).

Table 7.13: Specimen collection for typhoid.

Duration of disease	Specimen examination	% Positivity
1st Week	Blood culture	90
2nd Week	Blood culture	75
	Faeces culture	50
	Widal test	Low titer
3rd Week	Widal test	80–100
	Blood culture	60
	Faeces culture	80

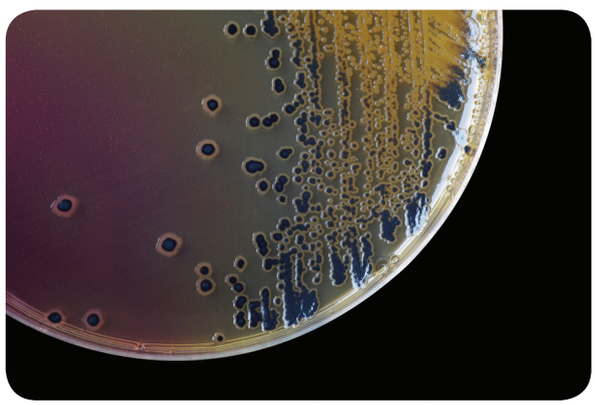


Figure 7.15: Colony morphology of *Salmonella typhi* on SS agar

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The bacteriological diagnosis of enteric fever consists of the following methods, which are:

- Isolation of the bacilli
- Demonstration of antibodies

Isolation of the bacilli

The typhoid bacilli are isolatedfrom the following clinical specimens which are tabulated (Table 7.14).

Demonstration of Antibodies: Slide – agglutination: The isolate is identified by slide agglutination with 'O' and 'H' antisera.

Widal Test: It is an agglutination test for detection of agglutinins 'H' and 'O' in patients with enteric fever. Salmonella antibodies start appearing in the serum at the end of 1st week and rise sharply during the 3rd week of enteric fever.

7.9.6 Prophylaxis

Various types of vaccine and their doses are given in Table 7.15.

Table 7.15: Various types of vaccine and their doses.

Vaccine	Doses
TAB – Vaccine	2 doses of 0.5 ml at an interval of 4–6 weeks
Typhoral	3 doses on alternate days. It gives 65–96% protection for 3–5 years and is safe
typhim – Vi	A single dose of 25µg

Table 7.14: Isolation method of typhoid bacilli from various clinical specimens.

Specimen culture	Isolation methods
Blood culture	5–10 ml of blood is collected and inoculated into blood culture bottle containing taurocholate broth or bile broth. After overnight incubation at 37°C the taurocholate broth is subculture on MacConkey agar. Pale colonies (NLF) appear on MacConkey which is used for motility and biochemical reactions.
Clot Culture (An alternative method to blood culture)	5 ml of blood is collected into a sterile test tube and allowed to clot. The clot is broken up with a sterile glass rod and added to bile broth containing streptokinase, which digests the clot and there by the bacilli are released from the clot. Then it is subcultured on MacConkey agar.
Faeces culture	Faeces sample are inoculated directly on MacConkey's agar, DCA or SS agar. The plates are incubated at 37°C for 24 hours, then characteristic colonies are observed which is confirmed by gram staining.
Urine culture	Urine samples are centrifuged, and the deposit is inoculated into enrichment media and then on selective media.

TN_GOVT_XII_Micro_Biology_CH07.indd 132 07-12-2021 15:19:01

7.9.7 Treatment and Control Measures

- Antibacterial therapy has been very effective in the treatment of patients.
- Ampicillin, amoxicillin and cotrimoxazole are useful in the treatment of typhoid fever.
- At present, ciprofloxacin is the drug of choice.
- Typhoid fever can be effectively controlled by sanitary measures for disposal of sewage, clean water supply and supervision of food processing and handling.

HOTS

Why is proper hand washing considered the most important element in controlling communicable infections?

7.10 *Vibrio Cholerae*

Vibrio is one of the curved rod bacteria, prominent in the Medical Bacteriology. They are present in marine environment and surface waters worldwide. *Vibrio* is a member of the family Vibrionaceae. The most important member of this genus is *Vibrio cholerae*, the causative agent of cholera. The term *Vibrio* is derived from **Vibrare** (Latin word) which means "**to shake or vibrate**" and the word **Cholera** is derived from **Chole** (Greek word) which means, "**to bile**" (Figure 7.16).

7.10.1 Morphology

Vibrio cholerae is gram negative, curved or comma shaped, (1.5um x 0.2–0.4um in size) non – capsulated. The organism is very actively motile with a single polar flagellum and the characteristic movement is called as **darting motility**. In stained smears of mucus flakes from acute cholera patients, the Vibriois seen arranged in parallel rows. This was described by Robert Koch as **"fish in stream"** appearance.

7.10.2 Culture Characteristics

Vibrio cholera is strongly aerobic. It grows best in alkaline media with the optimum temperature 37°C and pH 8.2. It is non-halophilic, therefore, cannot grow in media with a concentration of sodium chloride more than 7% (Figure 7.17). Some of the media in which *Vibrio cholerae* are cultivated are tabulated below in Table 7.16.

Table 7.16: Colony morphology of Vibrio *cholerae* on various media

Media	Colony morphology
Nutrient agar	The colonies are moist, translucent round disks (1–2mm in diameter) with a bluish tinge in transmitted light.
MacConkey agar	The colonies are colorless at first but become reddish on prolonged incubation due to late fermentation of lactose.
Thiosulphate citrate bile sucrose agar (pH 8.6)	It is used as a selective medium for isolation of *Vibrios*. It produces large yellow convex colonies due to sucrose fermentation.

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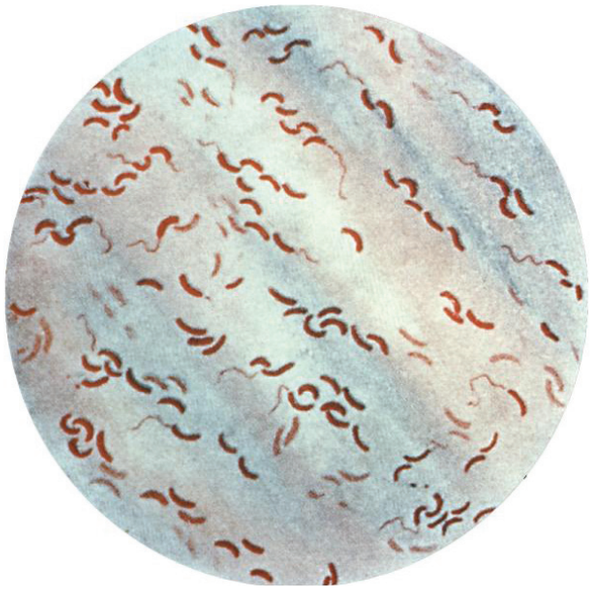

Figure 7.16: Gram staining of *Vibrio cholerae*

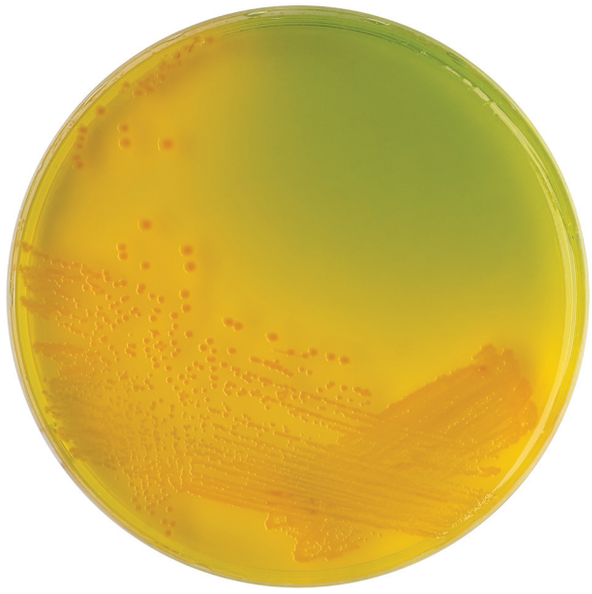

Figure 7.17: Colony morphology of *Vibrio cholerae* on TCBS

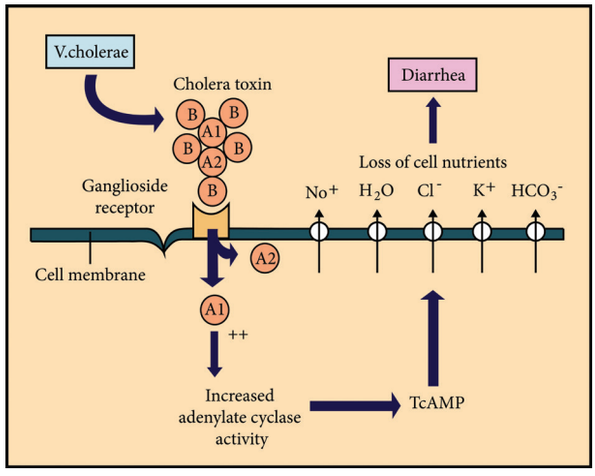

Figure 7.18: Mechanism of action of Cholera toxin

7.10.3 Enterotoxin

Vibrios multiplying on the intestinal epithelium produce an enterotoxin called **Cholera toxin**. It is also known as **Choleragen** (or CT). This toxin molecule is approximately 84,000 Dalton and consists of two major subunits namely A and B There is only one subunit in A (1A) whereas there are five subunits in B (5B) (Figure 7.18).

Mode of Action

- The B (binding) units of enterotoxin get attached to the GM_1 (Ganglioside membrane receptors I) on the surface of jejunal epithelial cells (target cells).
- The A (active) subunits then enters the target cell and dissociates into 2 fragments, A_1 & A_2. The A_2 fragment links biologically active A_1 fragment to the B – subunit.
- The A_1 fragment causes prolonged activation of cellular adenylate cyclase which in turn accumulates cAMP in the target cell. This leads to outpouring of large quantities of water and electrolytes into small intestinal lumen. Thus, resulting in profuse watery diarrhea.



Natural infection of *Vibrio cholerae* occurs only in human beings

TN_GOVT_XII_Micro_Biology_CH07.indd 134 07-12-2021 15:19:03

7.10.4 Pathogenesis

The pathogenic mechanism of *Vibrio cholerae* is discussed below in flowchart 7.7.

Source of Infection – contaminated water or food

Route of entry – fecal – oral route

Site of infection – small intestine

Incubation period – few hours to 5 days (usually 2–3 days)

Vibrio cholerae causes cholera, which is an acute diarrheal disease.

↓

In humans, *Vibrio* enter orally through contaminated water or food. The ingested pathogens pass through the acid barrier of the stomach & multiply in the small intestine.

↓

In the small intestine, the *Vibrio* penetrate the mucous barrier & adhere to the microvilli of the epithelial cells & multiply there.

↓

The *Vibrio*is strictly epipathogen& do not penetrate deep into the guts and there is no bacteremia. The virulence of *Vibrio cholerae* is due to cholera toxin (the mechanism is described earlier in the section 7.10.3 – enterotoxinmode of action)

↓

The toxin also inhibits intestinal absorption of sodium and chloride.

↓

The clinical manifestations & complications are due to massive water and electrolyte depletion.

↓

The voided fluids areodourless and contains flecks of mucus, hence it is known as Rice watery stool

Flowchart 7.7: Pathogenesis of *Vibrio cholerae*



The loss of water during cholera is about 20–30 litres per day.

HOTS

Why is cholera the most severe form of gastroenteritis?

7.10.5 Clinical Feature

Dehydration, anuria (absence of urine excretion), muscle cramps, hypokalemia (low blood potassium) & metabolic acidosis (low serum concentration of bicarbonates).

7.10.6 Laboratory Diagnosis

Specimen: Stool

Direct microscopy: It is not a reliable method for rapid diagnosis, the characteristic darting motility of the vibrio can be observed under dark – field microscope.

Culture: Stool sample is directly inoculated on MacConkey agar and TCBS agar. The plates are examined after overnight incubation at 37°C for typical colonies of *Vibrio cholera*, and the colonies are identified by gram staining and oxidasetest.

7.10.7 Prophylaxis

1. **General Measures**
   - Purification of water supplies
   - Improvement of environment sanitation
   - Infected patients should be isolated, and their excreta must be disinfected

TN_GOVT_XII_Micro_Biology_CH07.indd 135 07-12-2021 15:19:03

2. **Vaccines:** Two types of oral vaccines have been tried recently:
   - Killed oral whole cell vaccines
   - Live oral vaccines

7.10.8 Treatment

1. Oral Rehydrationtherapy: The severe dehydration & salt depletion can be treated by oral rehydration therapy (as recommended by WHO).
2. Antibiotics: It is of secondary importance, oral tetracycline was recommended for reducing the period of Vibrio excretion.



An ideal cholera vaccine is yet to be found.

7.11 *Mycobacterium Tuberculosis* (Tubercle Bacillus)

The genus *Mycobacterium* is distinguished by its thick, complex, lipidrich waxy cell walls. This high lipid content (Mycolic acids) imparts the characteristic of acid fastness or resistance to decolorizationby a strong acid after staining with carbol fuchsin. Many of the Mycobacterial species are saprophytes but several species are highly significant human pathogens. *Mycobacterium tuberculosis* is the causative agent of tuberculosis (TB) It is a killer disease and ranks as one of the most serious infection diseases of the developing countries. TB is primarily a disease of the lungs but may spread to other sites of the body.

The name *Mycobacterium tuberculosis* is derived form,

- Mycobacterium (Greek) – Fungus like bacterium
- Tuberculosis (Latin) – Swelling or Knob

7.11.1 Morphology

They are acid fast bacilli, slightly curved rods, it may occur singly or in small clumps. They are non–motile, non–sporing, and non-capsulated.

7.11.2 Cultural Characteristics

They are obligate aerobe, optimum temperature is 37°C and optimum pH is 6.4–7.0. The pathogen grows on an enriched culture media – Lowenstein Jensen medium. The colonies appear in about 2–3 weeks. The colonies are dry, rough, raised, irregular colonies with a wrinkled surface. Initially creamy white and becoming yellowish later (Figure 7.19).

7.11.3 Pathogenesis

Human tuberculosis is divisible into two form, they are Primary TB and Secondary

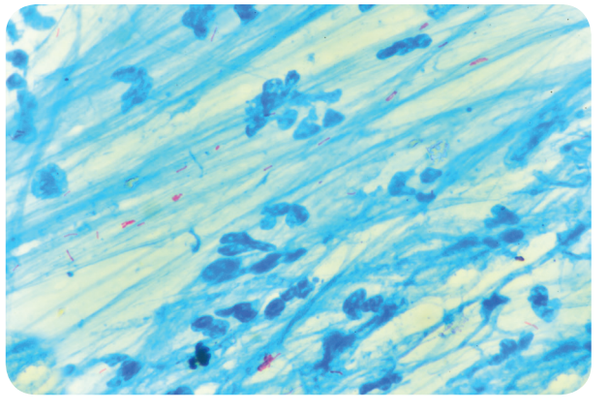

Figure 7.19: Acid fast staining of *Mycobacterium tuberculosis*

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TB. The pathogenesis of Primary Tuberculosis is described in flowchart 7.8.

Source of infection – Airborne droplets

Route of entry – Respiratory tract

Incubation period – 3–6 weeks.

Tubercle bacilli enter the host commonly by inhalation. When bacilli are inhaled, the bacilli enters into the alveolar macrophage, where they can grow and multiply.

↓

Non-resident macrophage are also attracted to the site of infection and these macrophages are engulfs the fubercle bacilli. The bacilli were carried through the lymphatic to the local hilar lymph node.

↓

In the lymph nodes, cell mediated immunity (CMI) is stimulated. The CMI response helps to prevent the further spread of pathogen.

↓

Within 10 days of infection, T-lymphocytes produces lymphokines which activate macrophages and leads to form granuloma, around the foci of infection.

↓

The activated marophages are termed epithelioid cells, which fuse to form multinucleate giant cells.

↓

The granuloma contains necrotic tissue and dead macrophage which is referred as caseation. Granuloma formation is usually sufficient to limit the primary infection.

↓

The lung lesions is frequently found in the lower lobe and called as ghon focus. The ghon focus together with enlarged hilar lymph nodes is called primary complex.

↓

In some persons, the lesions become dormant and produce dense scar tissue which may become calcified. Some bacilli remain in a dormant form as persisters which, when reactivated, cause post primary TB.

↓

In a minority of cases, the ghon focus ruptures into a blood vessel. Then the bacilli spread throughout the body with the formation of numerous granulomas and known as miliary TB.

Flowchart 7.8: The pathogenesis of Primary Tuberculosis

Secondary TB – (Post primary TB) It is caused by reactivation of the primary lesion or by exogenous reinfection. Granulomas of secondary TB most often occur in the apex of the lungs. The necrotic element of the reaction causes tissue destruction and the formation of large area of caseation termed tuberculomas. The presence of caseous necrosis and cavities are two important clinical manifestations of secondary TB. The cavities may rupture into blood vessels, spreading the bacilli throughout the body and break into airways, releasing the pathogen in aerosols and sputum - called as open tuberculosis (Figure 7.20).

7.11.4 Clinical Symptoms

It includes, cough that lasts for more than 2–3 weeks, weight loss, fever, night sweat and loss of appetite.

TN_GOVT_XII_Micro_Biology_CH07.indd 137 07-12-2021 15:19:04



Figure 7.20: Colony morphology of *Mycobacterium tuberculosis*on LJ medium

HOTS

M. tuberculosis the world's most deadly pathogen why?

7.11.5 Laboratory Diagnosis

Specimen: In case of pulmonary tuberculosis the most usual specimen is sputum.

Direct Microscopy: Smear is made from the sputum specimen and stained by Ziehl – Neelson technique. It is examined under oil immersion objective lens. The acid fast bacilli appear as bright red bacilli against a blue background.

Culture: The specimen is inoculated onto LJ – medium and incubated at 37°C for 2 weeks the tubercle bacilli usually grow in 2–8 weeks. The bacterial growth is confirmed by Ziehl – Neelsonstaining.

1. Tuberculin Skin test

Mantoux test: This method has been used routinely. In this test 0.1 ml of PPD (Purified protein derivative) containing 5 TU (Tuberculin unit) is injected intradermally on the flexor aspect of forearm (Figure 7.21) The site is examined after 48–72 hours and induration are measured (diameter in mm).

Positive test: Indurations of diameter d10 mm or more is considered positive.

Negative test: Indurations of 5 mm or less is negative.

2. Gene Xpert MTB

It is an automated diagnosis test it detects DNA sequences specific for *M. tuberculosis* and rifampicin resistance by PCR. Results can be obtained within 2 hours.

7.11.6 Treatment

The antitubercular drugs include two types of agents which are;



Figure 7.21: Mantoux test

TN_GOVT_XII_Micro_Biology_CH07.indd 138 07-12-2021 15:19:05

Bactericidal agents – Rifampicin (R), Isoniazid (H), Pyrazinamide (z), Streptomycin.

Bacteriostatic agents – Ethambutol (E). The regimen for treating TB consists of an intensive phase of 2 months of isoniazid, rifampin, pyrazinamide and ethambutol, followed by a continuation phase of 4 months of isonizid and Rifampin.

7.11.7 Prophylaxis and Control Measures

The BCG (Bacille – Calmette – Guerin) **administered** by intradermal injection of the live attenuated vaccine. The immunity may last for about 10 years.

The prevention of TB can be done by the following general measures such as

1. Adequate nutrition.
2. Practicing good hygiene (washing hands)
3. Health education.
4. Cover the mouth with a tissue when you cough or sneeze.

Infobits

Drug Resistance Tuberculosis

MDR-TB: Multidrug resistance tuberculosis refers to resistance to rifampian and Isoniazid. MDR-TB is a global problem especially in HIV-patients.

XDR-TB: Exensively drug resistance tuberculosis refer M.tuberculosis strains which are resistant to any fluoroquinolone and at lecest one of 3 injectable second line drugs (Kanamycin, Capreomycin and Amikarin), in addition to Isoniazid and rifampicin.



24 March is celebrated as World Tuberculosis day

7.12 *Treponema Pallidum*

Treponema pallidum is included in the family *Spirochaetaceae*. They are slender spirochaetes with fine spirals having pointed ends. Some of them are pathogenic for humans, while others are normal flora in mouth and genitalia. These pathogens are strict parasites and the diseases caused by Treponema are called Treponematoses. *Treponema pallidum* is the causative agent of syphilis. The name *Treponema pallidum* is derived from **Greek** words, which means, **Trepos** – Turn **Nema** –Thread and **Pallidum** – Pale staining.

7.12.1 Morphology

It is thin, delicate spirochete with tapering ends, about 10µm long and 0.1–0.2 µm wide. It has about ten regular spirals, which are sharp and angular, at regular intervals of about 1µm. They are actively motile (endoflagella), exhibiting rotation around the long axis, backward and forward movements and flexion of the whole body. It cannot be seen under light microscope and does not take ordinary bacterial stains. It can be seen under the dark ground (Figure 7.22) or phase contrast microscope. It can be stained by silver impregnation method.

7.12.2 Culture

- Pathogenic Treponema cannot be grown in artificial culture media.

TN_GOVT_XII_Micro_Biology_CH07.indd 139 07-12-2021 15:19:05



Figure 7.22: Dark field microscopy of *Treponema pallidium*

- *Treponema pallidum* strains (Nichol's strain) are maintained in rabbit testes.

7.12.3 Pathogenesis

Source of infection – Human beings (patients)

Mode of transmission – Sexual contact

Site of entry – Through minute abrasions/ cuts on skin or mucosa

Incubation period – 10–90 days

- *Treponema pallidum* causes venereal syphilis, which is acquired by sexual contact. The pathogen enters the human body through cut on the skin or mucosa of genital areas.
- The clinical disease sets in after an incubation period of about a month. There are 3 clinical stage of venereal syphilis, namely – primary, secondary and tertiary syphilis.

Primary syphilis

- A papule appears on the genital area that ulcerates, forming a chancre of primary syphilis called hard chancre.
- The chancre is covered by thick exudates, very rich in spirochetes.
- The regional lymph nodes are swollen, discrete, rubbery and non – tender.
- Even before the chancre appears, the spirochetes spread from the site of entry into the lymph and bloodstream, so the patient may be infectious during the late incubation period.
- The chancre invariably heals within 10–40 days, even without treatment, leaving a thin scar.

Secondary syphilis

- Secondary syphilis sets in 1–3 months after the primary lesion heals. During this interval, the patient is asymptomatic.
- The secondary lesions are due to widespread multiplication of the spirochetes and dissemination through the blood.
- Secondary syphilis is characterized by appearance of papular skin rashes, mucous patches in the oropharynx and condylomata (a raised growth on the skin resembling a wart).
- The lesions are abundant in spirochetes and the patient is most infectious during the secondary stage.
- There may be retinitis (inflammation of the retina of the eye), meningitis, periostitis, and arthritis.
- Secondary lesions usually undergo spontaneous healing, in some cases taking as long as 4 or 5 years.
- After the secondary lesions disappear, there is a period of dormant known as **latent syphilis** the patient does not show any clinical symptoms but with positive serology.

TN_GOVT_XII_Micro_Biology_CH07.indd 140 07-12-2021 15:19:05

Tertiary syphilis

- After several years, manifestations of tertiary syphilis appear. These consist of cardiovascular lesions including aneurysms (enlargement of an artery), gummata (a small rubbery granuloma that has a necrotic centre) and meningovascular manifestations. Tertiary lesions contain few spirochetes.
- In few cases, neurosyphilis such as tabesdorsalis or general paralysis of the insane develops. These are known as late tertiary or quaternary syphilis.

Congenital syphilis

In congenital syphilis, the infection is transmitted from mother to fetus by crossing the placental barrier.

Non – Venereal syphilis

It may occur in doctors or nurses due to contact with patients lesion during examination. The primary chancre occurs usually on the fingers.

7.12.4 Laboratory Diagnosis

The diagnosis of syphilis includes

a. Demonstration of Treponemes

b. Serological tests

Specimen: Exudates are collected from the chancre. Blood (serum) is collected for serology.

Demonstration of Treponemes

a. Dark ground microscopy: The wet film is prepared with exudates and examined under dark ground microscope. Under dark field examination *Treponema pallidum* appears motile spiral organism.

b. Treponemes in tissues: It can be demonstrated by silver impregnation method of staining.

Serological tests

Non – Treponemal tests – In the standard tests for syphilis includes;

a. VDRL – Venereal Diseases Research Laboratory test.

b. RPR – Rapid Plasma Reagain (Figure 7.23).

VDRL or RPR tests are used for serological screening for syphilis and more useful for the assessment of cure following treatment.



TRUST – Toluidine red unheated serum test, modified form of RPR test.

Treponemal Tests: The treponemal tests includes

a. TPHA – Treponema pallidum hemagglutination assay

b. FTA –ABS – Fluorescent treponemal antibody absorption test.

These two tests are used to confirm the diagnosis.



Figure 7.23: Rapid Plasma Reagain test

TN_GOVT_XII_Micro_Biology_CH07.indd 141 07-12-2021 15:19:07

7.12.5 Treatment and Preventive Measure

In early syphilis

a. Benzathine benzyl penicillin, 24 lakhs units intramuscularly in a single dose.

b. Alternatively, doxycycline 100 mg twice a day orally for 15 days.

In late syphilis

Benzathine benzyl penicillin 24 lakhs units, intramuscularly once weekly for 3 weeks.

- Avoiding sexual contact with an infected individual.
- Use of sex barriers (condoms).

7.13 Leptospira Interrogans

Spirochaetes of the genus *Leptospira* are actively motile, delicate and possess numerous closely wound spirals with characteristic hooked ends. Several Leptospires are saprophytes, while many are potential pathogens of rodents, domestic animals and humans. The genus Leptospira consists of two important species, which are *Leptospira interrogans* and *Leptospira biflexa.*

Leptospira interrrogans is the causative agent of leptospirosis, a zoonotic disease. The word Leptospira is derived from Latin word 'Leptos' = fine or thin and 'spira' = Coil and interrogans = Question mark (The shape of this spirochete accounts for its name)

7.13.1 Morphology

- They are spiral bacteria (5–20µm × 0.1µm) with numerous closely set coils. Their ends are hooked and resemble umbrella handles.
- They are actively motile by rotatory movements.
- They cannot be seen under light microscope due to its thinness, best observed by dark fieldmicroscopy (Figure 7.24), phase contrast and electron microscope.
- They stain poorly with aniline dyes, it may be stained with giemsa stain or silver impregnation techniques.

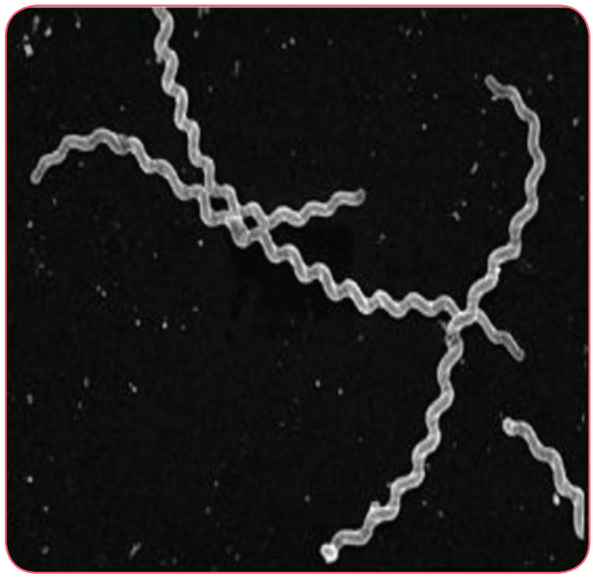


Figure 7.24: Dark field microscopy of *Leptospira interrogans*

7.13.2 Antigenic Structure

Leptospires show considerable antigenic cross reaction.

a. Genus – Specific somatic antigen – It is present in all members of the genus.

b. Surface antigens – This antigen is used to classify Leptospira into serogroups and serotypes.

TN_GOVT_XII_Micro_Biology_CH07.indd 142 07-12-2021 15:19:07

7.13.3 Pathogenicity

Source of infection: Contaminated water

Route of entry: Through cuts or abrasions on skin or mucosa

Incubation period: 6–8 days

- ***Leptospira interrogans*** causes a zoonotic disease named Leptospirosis. It is transmitted to humans by direct or indirect contact with water, contaminated by urine of carrier animals (rat and dog).
- Leptospira enter the body through cuts or abrasions on skin or through mucous membranes of the mouth, nose or conjunctiva.
- After an incubation period of 6–8 days. There is onset of febrile (related to fever) illness with Leptospira in blood (Septicemic phase) which lasts for 3–7 days.
- The organisms disappear from the blood and invades liver, kidney, spleen, meninges producing meningeal irritation such as headache, vomiting.
- The pathogen persists in the internal organs and most abundantly in the kidney. Severe Leptospirosis (Weil's disease) is associated with Fever, conjunctivitis (inflammation of conjunctiva), albuminuria (presence of albumin in the urine), jaundice and hemorrhage. It is a fatal illness with hepatorenal (Kidney failure with severe liver damage).

Clinical manifestations

- In severe cases, vomiting, headache, irregular fever and intense infection of the eyes.
- Jaundice, Albuminuria (The presence of protein Albumin in the urine) and purpuric hemorrhages sometimes occur on skin and mucosa.

7.13.4 Laboratory Diagnosis

The diagnosis of Leptospirosis is made by the following ways

- Direct microscopy of blood or urine
- Isolation of pathogen by culture
- Serological tests.

Direct Microscopy

Blood: Leptospira can be observed in the blood by dark – filed microscope. Blood examination is useful in first week as Leptospira disappear from blood after 8 days.

Urine: Leptospira may be present in urine in the 2nd week of the disease and intermittently thereafterup to 6 weeks. Centrifuged deposit of urine may be observed by Dark filed microscopy.

Culture: **B**lood (1st week) and urine (2nd–6 week) can be cultured in Korthof's medium. Media are incubated at 37°C for 2 days and then left at room temperature for 2 weeks. Culturesare examined every third day for the presence of Leptospira under DFM.

Serological tests

It is very useful method of diagnosis two types of serological tests are used, which are,

a. Screening tests: These tests are genus – specific and done using reactive genus specific antigen (non – pathogenic L. biflexapatoc I strain).

TN_GOVT_XII_Micro_Biology_CH07.indd 143 07-12-2021 15:19:07

Screening test includes – CFT, ELISA, SEL, HAT indirect IF these tests are capable to detect IgM and IgG leptospiral antibodies.

b. Serotype specific tests: These tests identify the infecting serovar by demonstrating specific antibodies.

i. Macroscopic agglutination test

ii. Microscopic agglutination test

7.13.5 Treatment and Preventions

Leptospira are sensitive to penicillin and tetracycline

Preventive measures include rodent control, disinfection of water.

Summary

Pathogenicity refers to the ability of a pathogen to produce disease. Virulence is the ability of the pathogen to cause disease. The various bacterial pathogens, its pathogenesis clinical symptoms, laboratory diagnosis, control, prophylaxis and treatment with appropriate antibiotics are discussed below. S. aureus is a leading cause of hospital acquired infections. Cloxacillin is used against beta lactamase. Producing strains Streptococcus pyogenes is intrinsically a much more dangerous pathogen than Staphylococcus aureus and has a much greater tendency to spread in the tissues. Streptococcal pyrogenic exotoxin leads to streptococcal toxic shock syndrome (TSS). A common cross – reacting antigen exist in some group A streptococci and heart, therefore, antibodies produced in response to the streptococcal infection could cross react with myocardial and heart valve tissue, causing cellular destruction. N. meningitidis is the causative agent of meningococcal meningitis, also known as pyogenic or septic meningitis. Clostridium tetani is the causative organism of tetanus or lock jaw disease. The four important species of the genus Shigella are: Shigella dysenteriae, Shigella flexneri, Shigella sonnei and Shigella boydii. Several Leptospires are saprophytes, while many are potential pathogens of rodents, domestic animals and humans.

TN_GOVT_XII_Micro_Biology_CH07.indd 144 07-12-2021 15:19:07

Evaluation

Multiple choice questions

1. Which of the following toxin is responsible for staphylococcal scalded skin syndrome?
   a. Epidermolytic toxin
   b. Enterotoxin
   c. Coagulase
   d. Haemolysin



2. The most important bacterial cause of sore throat is
   a. *Staphylococcus aureaus*
   b. *Streptococcus pyogenes*
   c. *Haemophilus influenzae*
   d. *Escherichia coli*
3. The causative agent of waterhouse – friderichsen syndrome is
   a. *Neisseria meningitidis*
   b. *Streptococcus pyogenes*
   c. *Treponema pallidum*
   d. *Staphylococcus aureus*
4. A gram-positive bacilli possessing metachromatic granules, showing Chinese letters arrangement are characteristic of
   a. *Corynebacterium diphtheriae*
   b. *Mycobacterium tuberculosis*
   c. *Bacillus anthracis*
   d. *Clastridium perfringens*
5. Drumstick appearance of spores is a characteristic feature of
   a. *Clostridium difficle*
   b. *Clostridium perfringens*
   c. *Clostridium botulinum*
   d. *Clostridium tetani*
6. Shigella dysenteriae causes
   a. Bacillary dysentery
   b. amoebic dysentery
   c. Traveller's diarrhoea
   d. Colitis
7. The most important specimen for isolation of Salmonella typhi in the first week of enteric fever is
   a. Blood b. Urine
   c. CSF d. Faeces
8. Which of the following methods can be used to demonstrate T. pallidum?
   a. Silver impregnation method
   b. Dark ground microscopy
   c. Immuno fluorescence staining
   d. All of the above
9. Weil's disease is caused by which of the following bacteria
   a. Salmonella typhi
   b. Escherichia coli
   c. Leptospira interrogans
   d. Vibrio cholerae
10. BCG Vaccine is
    a. Live attenuated vaccine
    b. Toxoid
    c. Killed vaccine
    d. None of the above

Answer the following

1. Enlist the toxins and enzymes produced by Staphylococcus aureus.
2. Write a short note on non-suppurative complications of streptococcus pyogenes infections.
3. Write about Meningococcal vaccines.
4. Write the prophylaxis for diphtheria

TN_GOVT_XII_Micro_Biology_CH07.indd 145 07-12-2021 15:19:07

5. Enlist the difference between bacillary dysentery and amoebic dysentery/
6. What is enteric fever?
7. Write the prophylaxis for Salmonella typhi.
8. Write the mode of action of cholera toxin/
9. Tubercubin skin test.
10. Define chancre.
11. Write the morphology of Clostridium tetani
12. List out the antituberculosis drugs.
13. Write the colony morphology of salmonella typhi shigella dynsenteriae on ss-agar.
14. Discuss the pathogenesis of typhoid fever.
15. Describe the pathogenesis of pulmonary tuber culosis.
16. Write briefly about Lab/diagnosis of tuberculosis.
17. Explain the pathogenesis of V. Cholerae
18. Write the mode of action of tetanospasmin
19. Describe the pathogenesis of lock-jaw disease.
20. What is staphylococcal toxic shock syndrome (STSS).
21. Describe the mode of entry of Meningococci from nusopharymx to brain.
22. Why antibiotics are avoided in dynsentery except in severe cases.
23. Define zoonotic bacterial disease and give two examples.
24. Describe the pathogenesis of primary syphilis.
25. Enlist the recent serologtical methods used to diagnose tuberculosis.
26. Write the colony morphology of Lowenstein – Jensen.

TN_GOVT_XII_Micro_Biology_CH07.indd 146 07-12-2021 15:19:07

Chapter 8

Medical Parasitology

Learning Objectives

After studying this chapter the students will be able to,

- *Know the various types of parasites and hosts.*
- *Discuss the classification of medically important parasites.*
- *Discuss the pathogenesis and clinical aspects of parasitic infections.*
- *Describe the general epidemiological aspects and transmission patterns of diseases caused by protozoa and helminths.*
- *Identify the methods and procedures of laboratory diagnosis of pathogenic protozoans and helminths in clinical specimens.*
- *Know the treatment options for parasitic infections.*
- *Implement the preventive and control measures of protozoans and helminthic infections.*

Chapter Outline

8.1 Parasite and Host

8.2 *Entamoeba histolytica*

8.3 *Giardia lamblia*

8.4 *Leishmania donovani*

8.5 *Plasmodium falciparum* and *P. vivax*

8.6 *Ascaris lumbricoides*

Medical Parasitology is the branch of Medical Science that deals with the study of parasites living in or on the body of human, their geographical distribution, the diseases caused by them, clinical features and the response generated by human against them. It is also concerned with various methods of their diagnosis, treatment, prevention and their control. Parasitology is a dynamic field, as these parasites constantly change their morphology, hosts, and host relationships. For this reason, Parasitology is an active field of study, which has raised expectations for the development of new drugs, vaccines and other control measures through biotechnology. However, these expectations are reduced by the inherent complexity of parasite and their relationship with host, the firm establishment of parasite and vectors in their environments and the vast socio economic problems in the geographical areas where parasites are most prevalent.

Before learning in detail about a few medically important parasites of human, let us know what is a parasite?

TN_GOVT_XII_Micro_Biology_CH08.indd 147 07-12-2021 15:22:12

8.1 Parasite and Host

Parasites are living organisms, which depend on living host for their nourishment and survival. They multiply or undergo development in the host. Host is defined as an organism, which harbors the parasite, provides nourishment and gives shelter to parasite. Host is relatively larger than the parasite.

8.1.1 Association between Host and Parasite

The relationship between host and the parasite can be of the following types:

- Symbiosis
- Commensalism, and
- Parasitism.

Flowchart 8.1 describes the types of host – parasite relationship

8.1.2 Types and Classification of Parasite

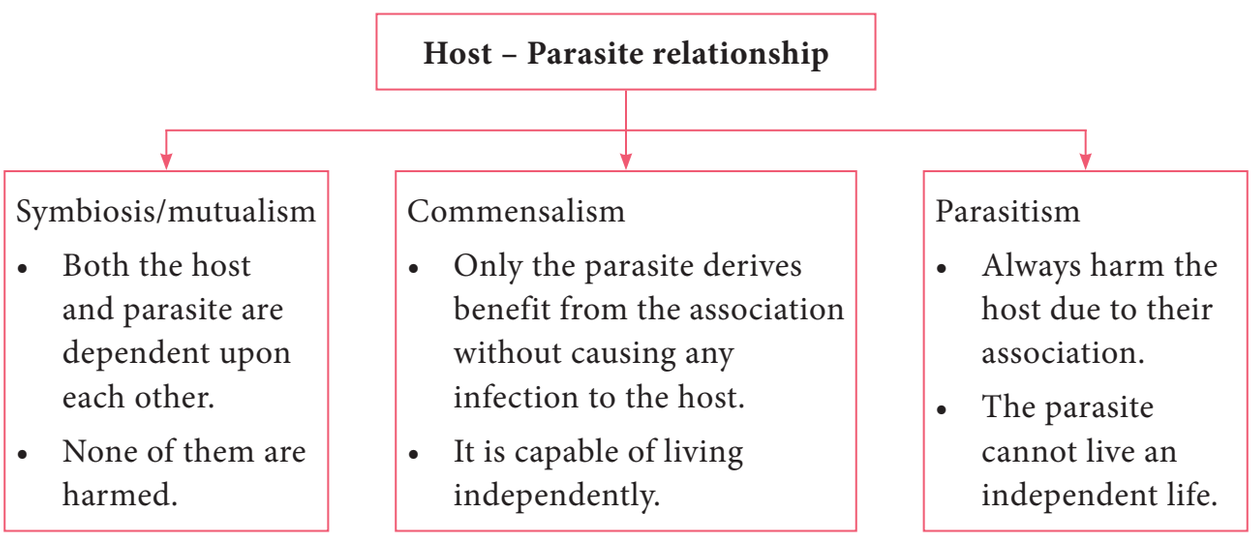
According to the nature of the host- parasite interaction and the environmental factors, the parasite may be one of the following,

Ectoparasite: These parasites live on the outer surface or in the superficial tissues of the host (Example: Lice). The infection by these parasites is called **infestation**.

Endoparasite: The parasite which lives within the host is called Endoparasite. Invasion by the parasite is called **Infection**. Most of the protozoan and helminthic parasites causing human diseases are endoparasites.

Endoparasites can be further classified as:

- **Obligate parasite:** This parasite is completely dependent on its host and cannot survive without it. Example: *Hookworms*.
- **Facultative parasite:** This parasite may either live as free living form or as a parasite when the opportunity arises. Example: *Naegleria fowleri*.
- **Opportunistic parasite:** This parasite is capable of producing disease in an immune deficient host (like AIDS and cancer patients). Example: *Toxoplasma gondii*.
- **Zoonotic Parasite:** This parasite primarily infects animals and is



Flowchart 8.1: The types of host – parasite relationship

TN_GOVT_XII_Micro_Biology_CH08.indd 148 07-12-2021 15:22:12

transmittable to humans. Example: Fasciola species.

- **Accidental parasite:** This parasite infect an unusual host are known as accidental parasites. Example: *Echinococcus granulosus* infects man accidentally.
- **Wandering or Aberrant parasites:** Parasites which infect a host migrate to the site where it cannot live or develop further are called aberrant parasites. Example: Dog roundworm infecting humans.

8.1.3 Types of Host

Definite host: The host which harbour the adult parasites or in which parasites undergo sexual method of reproduction is referred to as a definite host. The definite host may be a human or any other living organism. Example: Mosquito acts as a definite host for *Plasmodium* spp. in Malaria.

Intermediate host: The host in which the larval stages of the parasite live or in which asexual reproduction of parasite takes place is called the intermediate host. Example: Man acts as an intermediate host for *Plasmodium* spp. in Malaria.

Reservoir host: The host which harbour the parasite and acts has an important source of infection to other susceptible hosts is known as reservoir host. It is also called temporary host. Example: Dog is the reservoir host for disease kala azar.

Natural host: The host which is naturally infected with a certain species of parasite, is called natural host. Example: Pig is the natural host of *Balantidium coli*.

Paratenic host or transport host: some parasites enter a host in which they do not undergo any development but remains alive till they gain entry into the definitive host or intermediate host. Such a host is termed as paratenic host or transport host or carrier host.



Common name of medically important parasite

Intestinal flagellates – *Giardia intestinalis*

Oral Flagellates – *Trichomonas tenax*

Genital flagellates – *Trichomonas vaginalis*

Blood and Tissue flagellates – *Leishmania and Trypanosoma*

Ciliated protozoa – *Balantidium coli*

Dog roundworm – *Toxocara canis*

Cat roundworm – *Toxocara cati*

Roundworm – *Ascaris lumbricoides*

Hookworm – *Ancylostoma duodenale*

Liver fluke – *Fasciola hepatica*

Blood fluke – *Schistosoma haematobium*

Lung fluke – *Paragonimus westermani*

Pork tapeworm – *Taenia solium*

Beef tapeworm – *Taenia saginata*

Eyeworm – *Thelazia spp*

Threadworm or Human pinworm – *Enterobius vermicularis*

Human whipworm – *Trichuris trichiura*

8.1.4 Classification of Medical Parasitology

The most acceptable taxonomic classification of human parasites includes Endoparasites and Ectoparasite. Endoparasites are subclassified into protozoan parasite (unicellular organisms) and helminthic parasite (multicellular

TN_GOVT_XII_Micro_Biology_CH08.indd 149 07-12-2021 15:22:12

organism). Parasites of medical Importance come under the Kingdom called Protista and Animalia. Protista includes the microscopic single- celled eukaryotes known as protozoa. In contrast, helminths are microscopic, multicellular worms possessing well differentiated tissues and complex organs belonging to the kingdom Animalia. Classification of medically important parasites is given in Flowchart 8.2.

8.1.5 Life Cycle of Parasites

Direct life cycle

The life cycle of parasite that requires only single host to complete its development, is called direct life cycle. Example: *Entamoeba histolytica* requires only human host to complete its life cycle.

Indirect life cycle

The life cycle of parasite that requires two or more species of hosts to complete its development, the life cycle is called as indirect life cycle. Example: Malarial parasite (Plasmodium spp.) requires both human host and mosquito to complete its life cycle.

8.1.6 Transmission of Parasites

It depends upon Source or reservoir of infection, and mode of transmission.

1. Sources of infection

A. Human: Human is the source or reservoir for a majority of parasitic infection. The condition in which the infection is transmitted from one infected human to another human is called **anthroponoses**.

B. Animals: Animals act as the source of infection in many parasitic diseases. The condition where infection is transmitted from animals to humans is called **zoonoses**.

2. Mode of transmission

A. Oral transmission: This is through ingestion of contaminated food, water, vegetables, soiled fingers or fomites contaminated by faeces that contain the infective stage of parasite. This mode of transmission is referred to as faecal-oral route. Example: Cysts of *Entamoeba histolytica*.

B. Skin transmission: This is another important route. The infective larvae of hookworm enter the skin of persons walking bare footed on contaminated soil.

C. Vector transmission: It could be a biological or a mechanical means. Many parasitic diseases are transmitted by insect bite. Example: sandfly is vector for *Leishmania*.

D. Direct transmission by person to person contact. Frequently, *Entamoeba*, *Giardia* and *Trichomonas* are transmitted by sexual contact among homosexuals.

E. Vertical transmission: It is the transmission from mother to fetus. Example: Toxoplasmosis.

So far, we have learnt about the general introduction and classification of parasites. Now, let us learn a few important human parasites in detail.

Introduction to Protozoa

General characteristics of protozoa:

1. They are microscopic unicellular eukaryotes.
2. The single cell has a relatively complex internal structure and it performs

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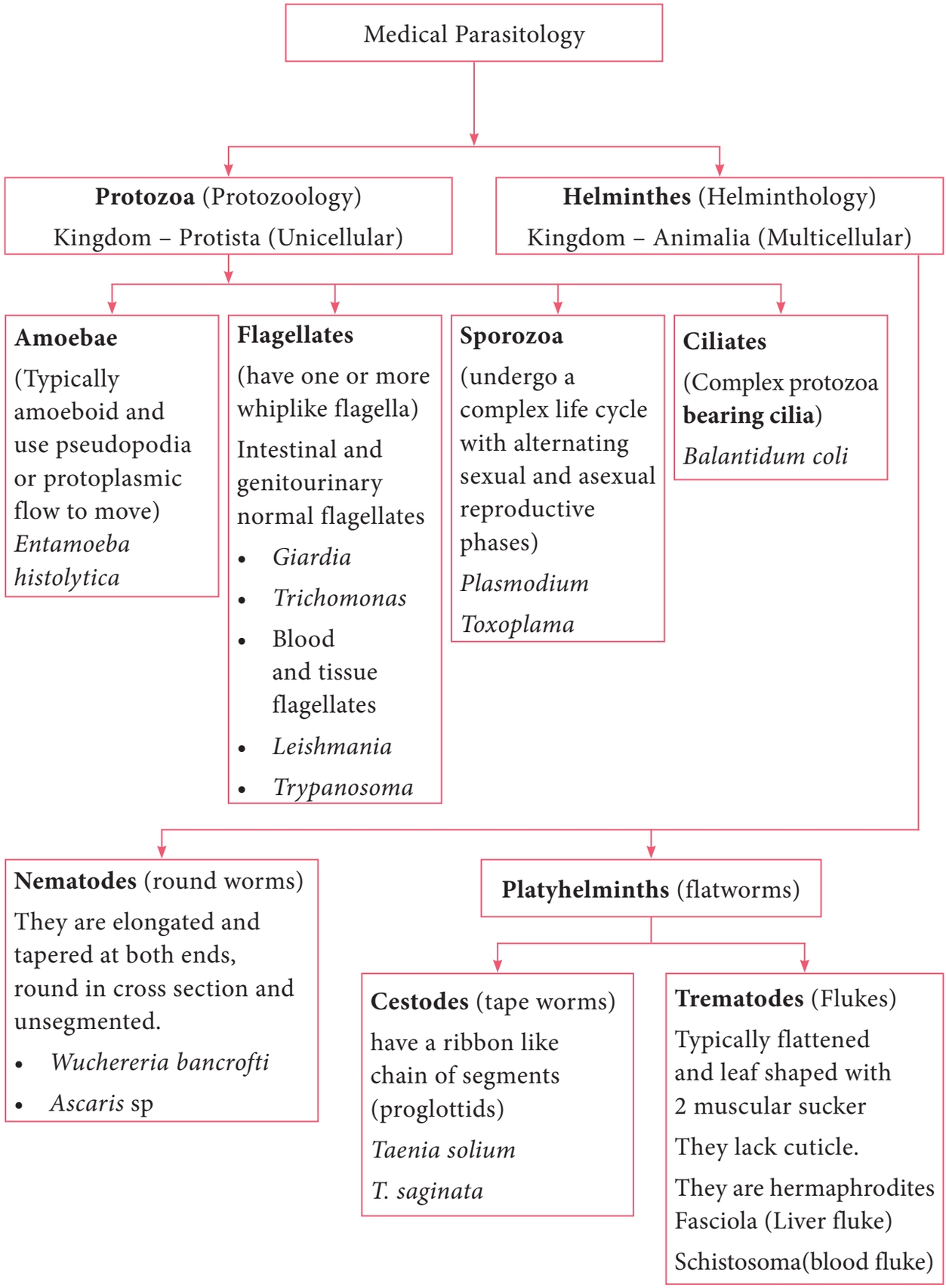

Flowchart 8.2: Classification of medically important parasites

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Infobits

Parasites having direct life cycle

Protozoa	Helminths
• *Giardia lamblia*	• *Ascaris lumbricoides*
• *Trichomonas vaginalis*	• *Trichuris trichiura*
• *Balantidium coli*	• *Ancyclostoma duodenale*

Parasites having indirect life cycle

S.No	Protozoa	Definite host	Intermediate host
1.	*Plasmodium spp.*	Female *Anopheles* mosquito	Man
2.	*Toxoplasma gondii*	Cat	Man
3.	Cestodes *Taenia solium*	Man	Pig
4.	Trematodes *Fasciola hepatica*	Man	Snail
5.	Nematodes *Wuchereria bancrofti*	Man	Mosquito

various complex metabolic activities such as digestion, reproduction, respiration and excretion.

3. Each cell consists of nucleus and cytoplasm.
4. A protozoa parasite during its life cycle may exist in two stages such as trophozoite and cyst.

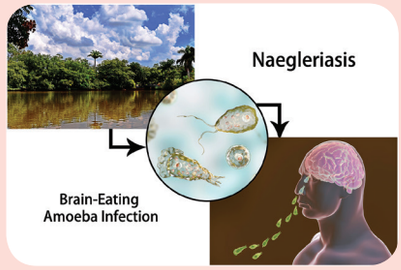
Amoebae

Amoebae are structurally simple protozoans which have no fixed shape. The cytoplasm of amoeba is bounded by a membrane and can be differentiated into an outer ectoplasm and inner endoplasm. Pseudopodia (false foot) are formed by the amoebae by throwing out ectoplasm followed by endoplasm. These are employed for locomotion and engulfment of food by phagocytosis.

Reproduction occurs by fission and budding. Amoebae are classified as either free living or intestinal amoebae.

Infobits

Naegleria fowleri (Brain eating amoeba) is a thermophilic, free living amoebae occasionally act as human pathogens producing meningoencephalitis known as primary amoebic meningoencephalitis (PAM). Infections most often occur when water containing *Naegleria fowleri* is inhaled through the nose, where it then enters the nasal and olfactory nerve tissue traveling to the brain. *N. fowleri* occurs in three forms -as a cyst, trophozoite (amoeboid) and a biflagellate (it has two flagella). The flagella form can exist in the cerebrospinal fluid.



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8.2 Intestinal Amoeba – *Entamoeba histolytica*

8.2.1 Geographical Distribution

It is Worldwide in distribution they are more common in the tropics than elsewhere. It is found wherever sanitation is poor.

8.2.2 Habitat

Trophozoites of *E.histolytica* live in the mucous and submucous layers of the large intestine of human.

8.2.3 Morphology

E.histolytica occurs in 3 forms as Trophozoite, Precyst and Cyst.

Trophozoite: It is the growing or feeding stage of the parasite. It is the only form present in tissues. It has no fixed shape. They vary in size from 18 to 40µ, average being 20 to 30µ. The cytoplasm is usually described as outer ectoplasm and inner endoplasm (Figure 8.1). The endoplasm contains nucleus, food vacuoles, erythrocytes, occasionally leucocytes and tissue debris. The nucleus is characterised by evenly arranged chromatin on the nuclear membrane and the presence of a small, compact, centrally located karoyosome (It is a DNA containing body, situated peripherally or centrally within the nucleus). Trophozoites exhibits active crawling or gliding motility by forming finger-like projections called Pseudopodia.

The trophozoite reproduce by binary fission in every 8 hours. Trophozoites survives upto 5 hours at 37°C and are killed by heat, drying and chemical sterilization. Even if live trophozoites from freshly passed stools are ingested, they are rapidly destroyed in stomach and cannot initiate infection. Therefore, the infections is not usually transmitted by trophozoites.

Precyst

Trophozoites undergo encystment in the intestinal lumen. Encystment does not occur in the tissue or in feces outside the body. Precyst is smaller in size about 10 -20 µm in size. It is round or oval in shape. The endoplasm is free of red blood cells and other ingested food particles (Figure 8.1). The nuclear structure retains the characteristics of the trophozoite.

Cyst

Precyst secretes a highly refractive cyst wall around it and becomes a cyst. A mature cyst is a quadrinucleate spherical body. The cyst begins as a uninucleate body but soon divides by binary fission and develops into binucleate and quadrinucleate bodies (Figure 8.1). The cytoplasm of the cyst is clear and hyaline (translucent) and the nuclear structure retain the characteristic of the trophozoites.

The mature quadrinucleate cyst, passed in the stool, does not undergo any further development and remain alive for several months in the soil or in environment where they were deposited. The mature quadrinucleate cysts are the infective forms of the parasite.

8.2.4 Life – Cycle of Entamoeba histolytica

E. histolytica passes its life cycle only in one host, the human.

Infective form: Mature quadrinucleate cyst.

Mode of transmission: Ingestion of food and water contaminated with cyst.

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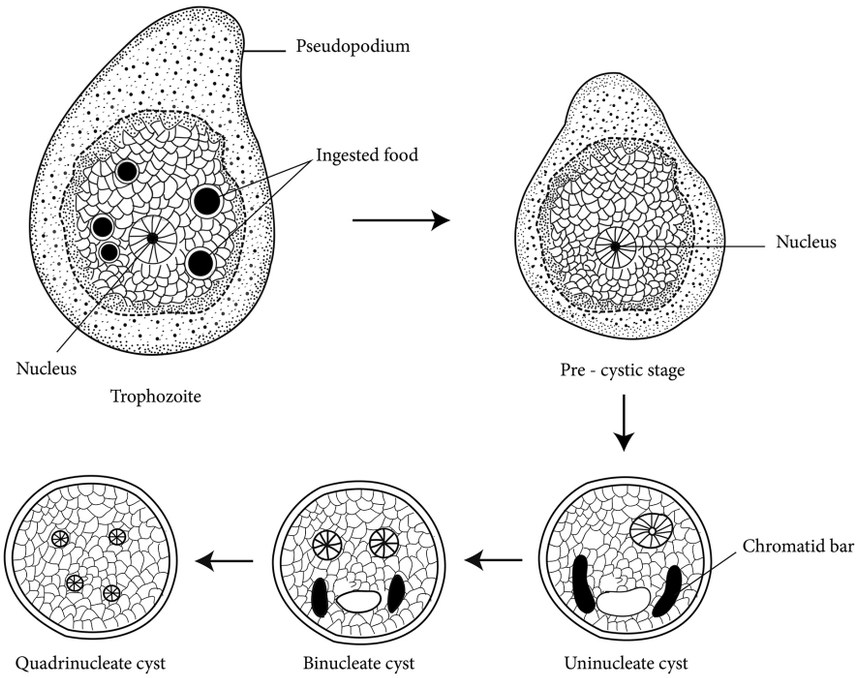

Figure 8.1: Trophozoite, precyst and cyst of *Entamoeba histolytica*

- The cysts that are swallowed along with food and water enter into the alimentary canal. The cyst wall is resistant to action of gastric juice. The cysts pass through the stomach undamaged and enters the small intestine (Figure 8.2).
- When the cyst reaches caecum or lower part of the ileum, due to alkaline medium, the cyst wall is damaged by trypsin leading to excystation.
- The cytoplasm gets detached from the cyst wall and an amoeboid movement appear causing a tear in the cyst wall, through which quadrinucleate amoeba is liberated. This stage is called the metacyst.
- The nuclei in the metacyst immediately undergo division to form 8 nuclei, each of which gets surrounded by its own cytoplasm to become 8 small amoebulae or metacystic trophozoites.
- These metacystic trophozoites are carried to the caecum and colon. They invade the tissues and lodge in the submucous tissue of the large intestine which is their normal habitat.
- Trophozoite grow and multiply by binary fission. The trophozoite phase of the parasite is responsible for producing the characteristic lesion of amoebiasis.



The amoeba infecting man may be classified according to their pathogenicity and habitat.

A. Pathogenic

Intestinal Amoeba: *Entamoeba histolytica*

B. Non pathogenic

1. Mouth Amoeba: *Entamoeba gingivitis*
2. Intestinal Amoeba: *Entamoeba coli*
   Entamoeba nana

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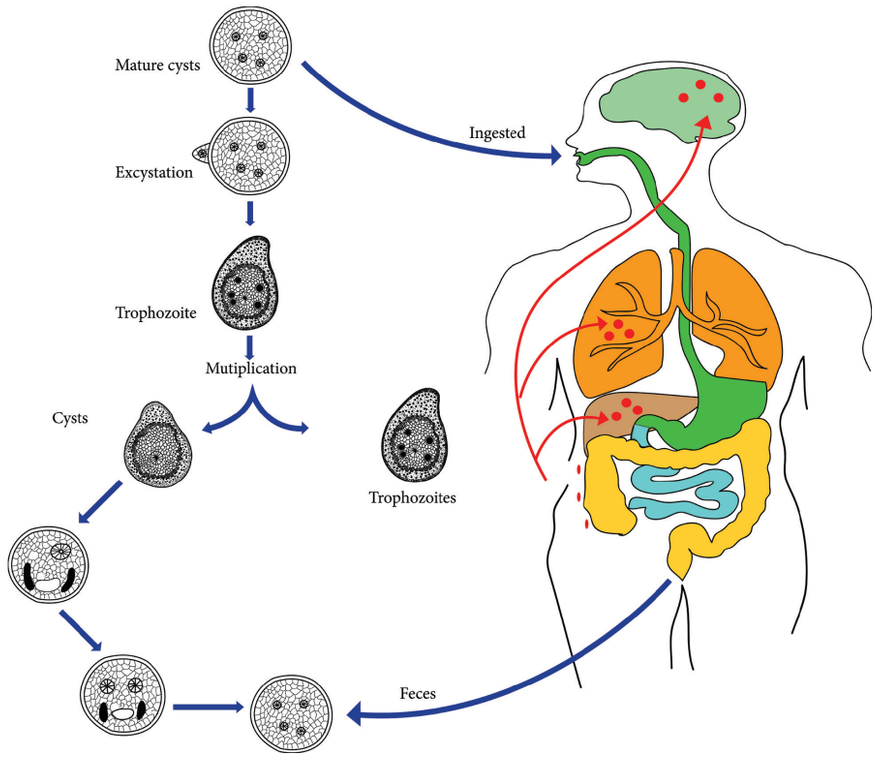


Figure 8.2: Life cycle of *Entamoeba histolytica*

- Some of the trophozoites in colon develop into precystic forms and cysts, which are passed in feces to repeat the cycle.

8.2.5 Pathogenesis

E. histolytica causes intestinal and extra intestinal amoebiasis (Flowchart 8.3).

E. histolytica can live in the intestine without causing symptoms. But, they can also cause severe disease. These amoebas may invade the wall of the intestine leading to amoebic dysentery, an illness that causes intestinal ulcers, bleeding, increased mucus production and diarrhoea. The ulcers are strictly confined to the large intestine being most numerous in the caecum and next in the sigmoid-rectal regions. The lesions may be generalized or localised. A typical amoebic ulcer varies from pin's head to one inch or more in diameter in size. The shape of ulcer may be round or oval. On vertical section, the ulcer appears like flask, with mouth and neck being narrow and base being large and rounded (Figure 8.3 shows the flask – shaped ulcer). The base of ulcer is generally formed by the muscular coat and filled up by the necrotic material. The ulcers generally do not extend deeper than submucosal layer.

8.2.6 Clinical Features

Incubation period is highly variable, but is generally 4 to 5 days.

A wide spectrum, from asymptomatic infection (luminal amoebiasis), to invasive intestinal amoebiasis (dysentery, colitis, appendicitis, toxic mega colon, amoebomas), to invasive extraintestinal amoebiasis occurs. Flowchart 8.4 classifies the clinical outcomes of infection with *Entamoeba histolytica*. Only about 10% to 20% of people who are infected with *E. histolytica* become sick from the infection.

TN_GOVT_XII_Micro_Biology_CH08.indd 155 07-12-2021 15:22:15

Infection of *E. histolytica*

Intestinal amoebiasis

Infection is limited entirely to the large intestine, the initial site of infection of the parasite.

Extra intestinal amoebiasis

The trophozoites migrate and produce lesions in

Liver – Hepatic Amoebiasis

Lungs – Pulmonary Amoebiasis

Brain – Cerebral Amoebiasis

Spleen – Splenic Abscess

Flowchart 8.3: Infection Caused by E.*histolytica*

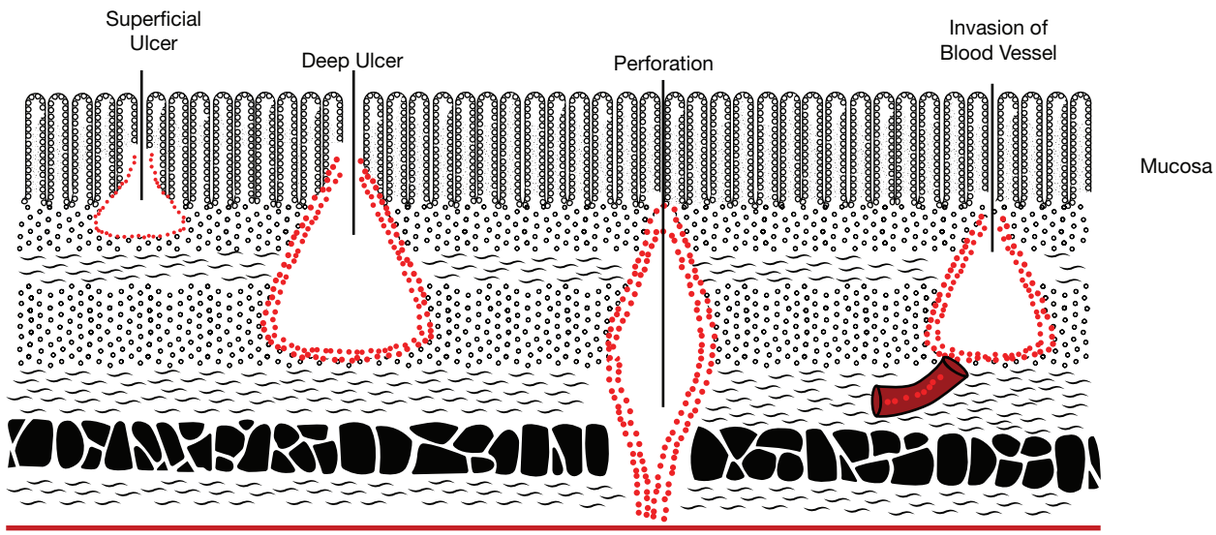
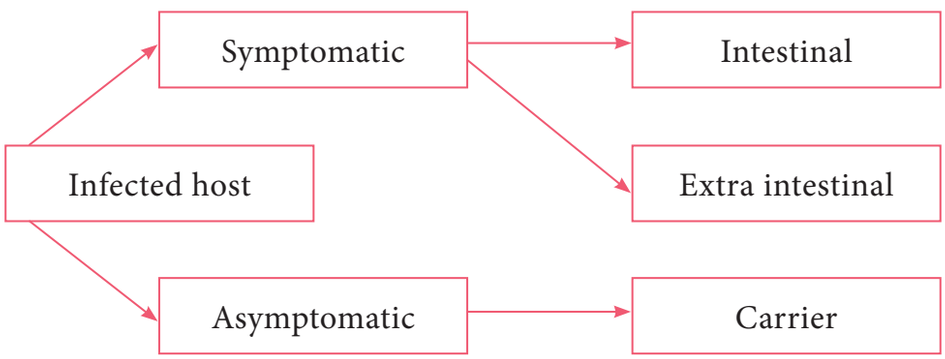


Figure 8.3: Ulcers in intestinal amoebiasis



Flowchart 8.4: The clinical outcomes of infection with *Entamoeba histolytica*

TN_GOVT_XII_Micro_Biology_CH08.indd 156 07-12-2021 15:22:15

The typical manifestation of intestinal amoebiasis is amoebic dysentery. The symptoms are often quite mild and can include loose feaces, stomach pain and stomach cramping. In acute amoebic dysentery, the symptoms include abdominal pain, bloody stool, fever, tenderness, rectal tenesmus and hepatomegaly (enlargement of liver). People affected may develop anemia due to loss of blood. On clinical and laboratory ground, amoebic dysentery should be differentiated from bacillary dysentery. A Table 8.1 shows the difference between the stools of amoebic and bacillary dysentery.

Extra intestinal amoebiasis

1. **Hepatic amoebiasis:** This is the most common form of extra intestinal invasive amoebiasis. Liver abscess may be multiple or more often solitary, usually located in the upper right lobe of the liver (Figure 8.4). Amoebic liver abscess (ALA) contains an odour less and thick chocolate brown pus called anchovy sauce pus. ALA is associated with an abrupt onset of high fever, right upper abdominal pain and tenderness. Anorexia (loss of appetite for food), nausea (the sensation to vomit), vomiting, fatigue (extreme tiredness) and weight loss are also frequent.

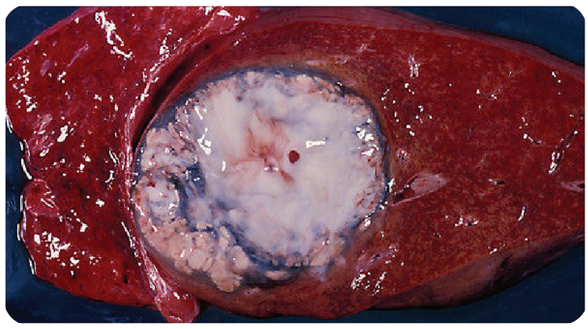


Figure 8.4: Amoebic liver abscess

2. **Pulmonary Amoebiasis:** It is very rare, but this may occur by direct hematogenous spread from the colon. The patient presents with severe chest

Table 8.1: Difference between the stools of amoebic and bacillary dysentery

	Amoebic dysentery	Bacillary Dysentery
Macroscopic observation		
Number	6–8	Over 10 motions a day
Amount	Relatively large	Small
Odour	Offensive	Odorless
Colour	Dark red	Bright red
Nature	Blood and mucus mixed with faeces	Blood and mucus no faeces
Microscopic observation		
RBC	In clumps, reddish – yellow in colour	RBC in rouleaux, bright red in colour
Pus cells	Scanty	Numerous
Parasite	Trophozoites of *E. histolytica*	Nil
Charcot – Leyden crystals	Present	Nil

TN_GOVT_XII_Micro_Biology_CH08.indd 157 07-12-2021 15:22:15

pain and have dyspnoea (shortness of breath). The sputum of patient is chocolate brown. Amoebic trophozoites may be demonstrated in the sputum.

3. **Cerebral amoebiasis:** The condition is unusual. In cerebral amoebiasis, the abscess is single, small and is located in the cerebral hemisphere. The patient may die of rupture or involvement of cerebellam within 12–72 hours. Biopsy of the brain shows the amoebic trophozoites.
4. **Cutaneous amoebiasis:** It can be caused by perforation of an amoebic abscess or surgical wound infected with amoebae. It is less frequent condition.
5. **Genitourinary Amoebiasis:** This condition includes amoebiasis of the kidney and genital organs. Amoebiasis of the genital organs is a rare condition. Lesions of amoebiasis is shown in Figure 8.5.

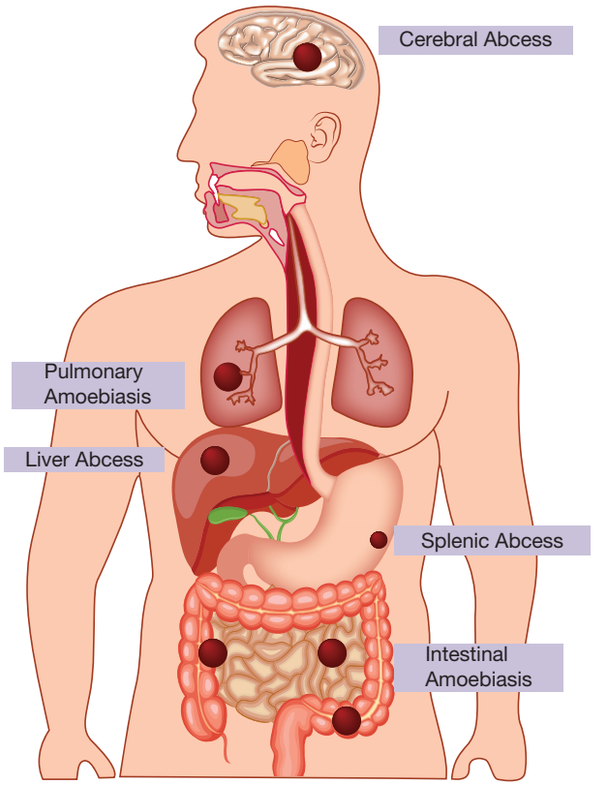


Figure 8.5: Extra-intestinal amoebiasis

8.2.7 Laboratory Diagnosis

Specimens: Stool is the specimen of choice. Other specimens collected includes blood, rectal exudates and rectal ulcer tissue collected from the base by endoscopies.

Methods in examination of stool

A. Direct wet mount examination of stool: Demonstration of mature quadrinucleate cysts or trophozites in stool is diagnostic of intestinal amoebiasis. The wet mount of stool is prepared in the saline, iodine or lacto phenol cotton blue.

B. Examination of stool after concentration: Demonstration of amoebic cysts by Formalin – ether is the method of choice.

C. Examination of stained stool smears: Staining by iron haematoxylin, Periodic Acid – Schiff (PAS) stains demonstrate the presence of both trophozoites and cyst.

Amoebic liver abscess (ALA): Demonstration of amoebic trophozoites in the aspirated liver pus establishes the diagnosis of ALA.

Serology: Detection of amoebic antigens in the serum by Enzyme Linked Immunosorbent Assay (ELISA).

Molecular diagnosis: PCR (Polymerase chain reaction) is employed to detect amoebic genome in the aspirated liver pus for the diagnosis of ALA.

Imaging methods: X – Ray magnetic resonance imaging (MRI) scan and computerized Axial Tomography (CAT) Scan are the imaging methods used.

TN_GOVT_XII_Micro_Biology_CH08.indd 158 07-12-2021 15:22:16

Treatment: Eradication of amoebae by the use of amoebicidal drugs and replacement of fluid and electrolyte is the treatment for amoebiasis. Listed below the drugs used in the treatment for amoebiasis.

- Paramomycin and iodoquinol acts in the intestinal lumen but not in tissues.
- Emetine, chloroquine are effective in systemic infection. They act only on trophozoites.

Metronidazole is the drug of choice which acts as both luminal and tissue amoebicides. It is low in toxicity and is effective against intestinal as well as extra -intestinal amoebic infections.

8.2.8 Prevention and Control

- Proper sanitation is the key to avoid amoebiasis. Washing hands with soap and water after using the bathrooms and before handling food.
- Drinking safe and boiled water.
- Avoid eating unwashed fruits and vegetables.
- Prevention of water supplies from faecal contamination.
- Early rapid detection of diseased people and subsequent treatment with amoebicidal drugs. No vaccine is available yet against amoebiasis in humans.

8.3 Intestinal Flagellates – *Giardia Lamblia*

(Also known as *Giardia duodenalis, Giardia intestinalis*)

8.3.1 Geographical Distribution

It is the most common protozoan pathogen and is worldwide in distribution. The disease is very high in areas with low sanitation, especially tropics and subtropics.

8.3.2 Habitat

Giardia lamblia lives in the duodenum and upper jejunum of human. It is the only protozoan parasite found in the lumen of the human small intestine.

8.3.3 Morphology

It exists in two forms

- Trophozoite and
- Cyst



Antoine van Leeuwenhoek observed and illustrated *Giardia lamblia* in his own loose stool. This was the first protozoan parasite of human that is recorded and the first to be seen under a microscope.

Trophozoite

The trophozoite is in the shape of a tennis or badminton racket. It is rounded anteriorly and pointed posteriorly. The size of the trophozoite is 14 µ long by 7µ broad. Dorsally, it is convex and ventrally, it has a concave sucking disc which helps in its attachment to the intestinal mucosa. It is bilaterally symmetrical. All the organs of the body are paired. Trophozoite of Giardia possess,

- 1 pair of nuclei
- 4 pairs of flagella
- Parabasal body (Blepharoplast), from which the flagella arise (4 pairs)

TN_GOVT_XII_Micro_Biology_CH08.indd 159 07-12-2021 15:22:16

- 1 pair of axostyles, running along the midline
- Two sausage – shaped parabasal or median bodies lying transversely posterior to the sucking disc
- The trophozoite is motile, with a slow oscillation about its long axis, often resembling falling leaf (Figure 8.6a).

Cyst

It is the infective form of the parasite. The cyst is small and oval, measuring 12 µm × 8 µm and is surrounded by a hyaline cyst wall.

Its internal structure includes 2 pairs of nuclei grouped at one end. A young cyst contains 1 pair of nuclei. The axostyle lies diagnonally, forming a dividing line within cyst wall (Figure 8.6b).

8.3.4 Life Cycle: Giardia Life Cycle in Host (Human)

Infective form: Mature cyst

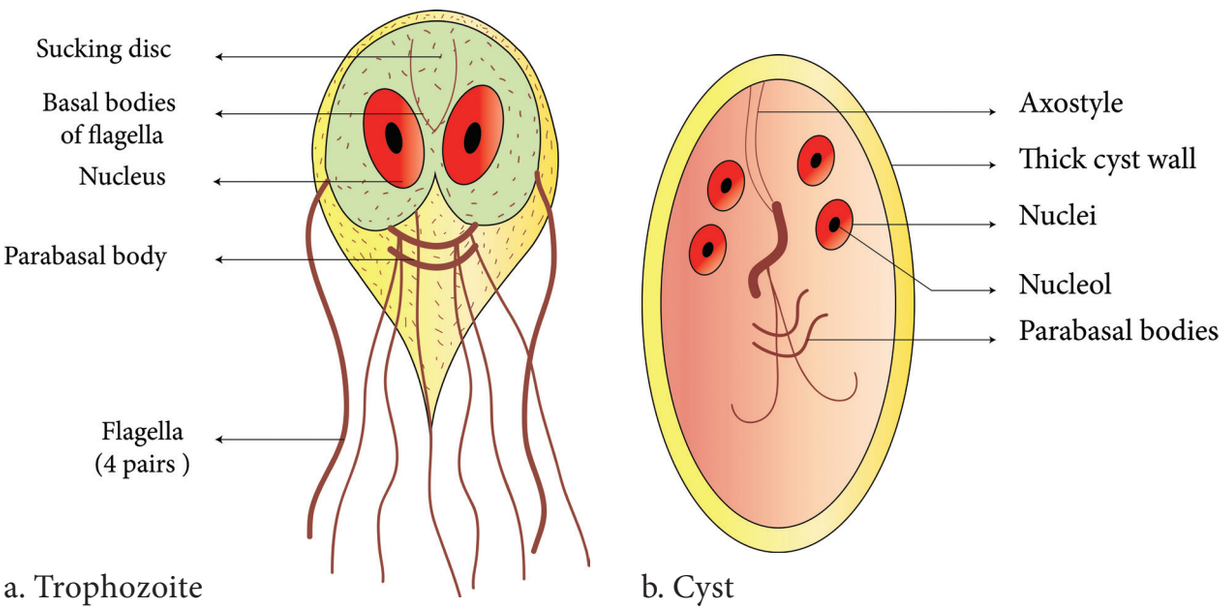
Mode of transmission: Human acquires infection by ingestion of cyst in contaminated water and food. Direct person – to person transmission occurs in children. Transmission occurs through oral-anal and oral-genital route in sexually active homosexual males. Within half an hour of ingestion, the cyst hatches out into two trophozoites, which multiply by binary fission and colonize in the duodenum. The trophozoites live in the duodenum and upper part of jejunum, feeding by pinocytosis. When conditions in duodenum are unfavourable, encystment occurs, usually in large intestine. Cysts are passed in stool and remain viable in soil and water for several weeks (Figure 8.7).

8.3.5 Pathogenicity

Giardia lamblia does not invade the tissue, but remains attached to intestinal epithelium by means of the sucking disc. It causes a disturbance of intestinal function leading to malabsorption of fat.

8.3.6 Clinical Manifestations

Incubation period is variable, but is usually about 2 weeks.



a. Trophozoite b. Cyst

Figure 8.6: Trophozoite and cyst of *Giardia*

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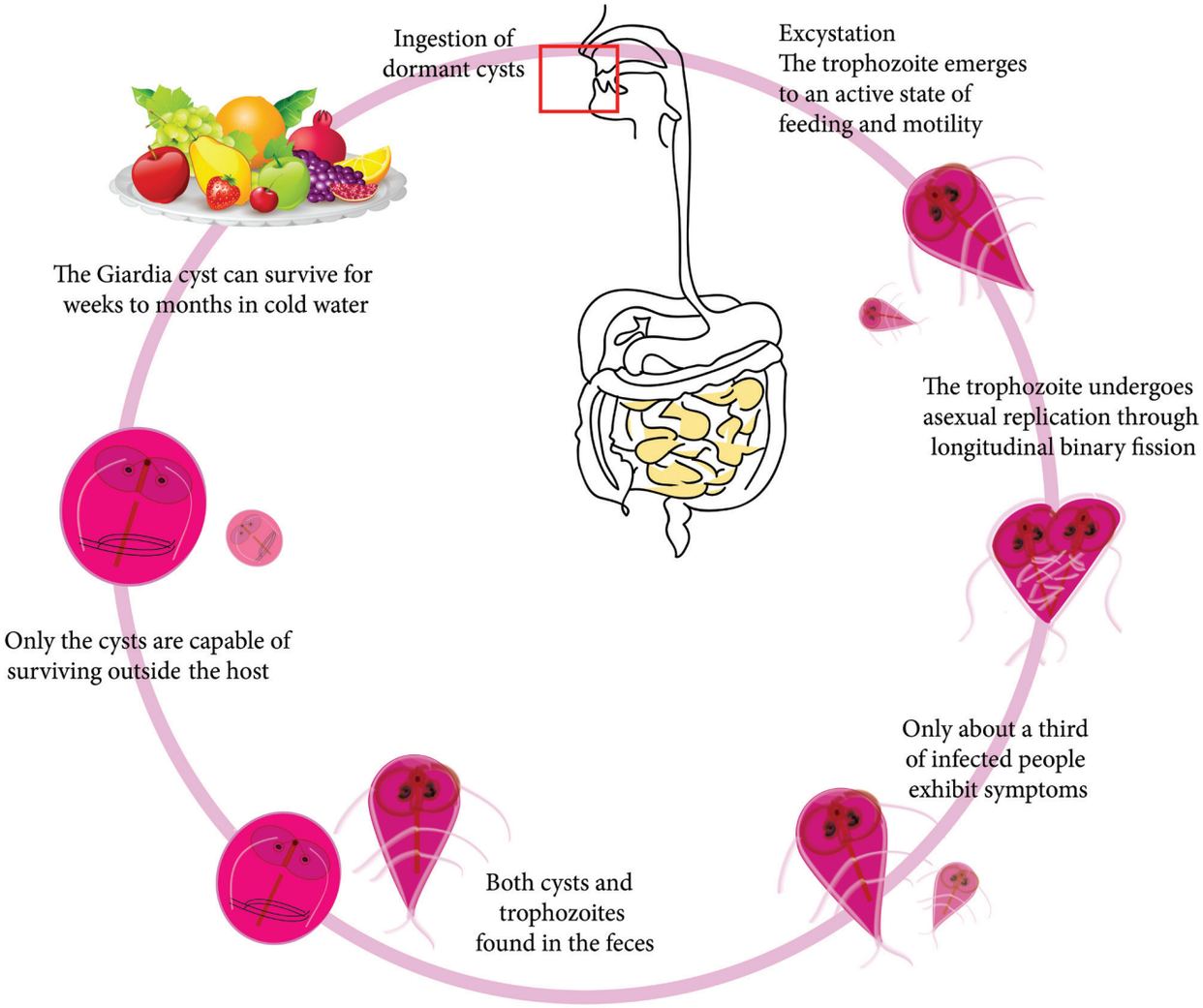

Figure 8.7: Life cycle of *Giardia lamblia*

The disease is asymptomatic, but in some cases it may lead to abdominal cramps, flatulence, looseness of bowels, foul smelling stool and mild steatorrhoea (passage of yellowish and greasy stools in which there is excess of fat). The stool contains excess mucus and fat but no blood and pus. Children may develop chronic diarrhoea, malaise (discomfort), nausea, anorexia (loss of appetite for food), malabsorption of fat, vitamin A and protein. Occasionally, Giardia may colonize the gall bladder causing biliary colic and jaundice.

8.3.7 Laboratory Diagnosis

Specimens: Stool and blood

Examination of stool sample: Giardiasis can be diagnosed by identification of cysts of *Giardia lamblia* in the formed stools and the trophozoites and cyst of the parasite in diarrhoeal stools.

Macroscopic examination of stool: Fecal specimens containing *Giardia lamblia* may have an offensive odor. It is pale coloured with fatty substance floating in water.

Microscopic examination of stool: Cysts and trophozoites can be found in

TN_GOVT_XII_Micro_Biology_CH08.indd 161 07-12-2021 15:22:20

diarrheal stools by saline and iodine wet preparations (Figure 8.8).

Serodiagnosis: Immuno chromatographic strip tests and indirect immunofluorescence (IIF) tests are readily available. For antigen and antigen detection ELISA, Commercially available ELISA kits detects Giardia – Specific antigen.

Molecular methods: DNA probes and polymerase chain reaction (PCR) have been used to demonstrate parasitic genome in the stool specimen.

8.3.8 Treatment

Metronidazole and Tinidazole are the drugs of choice.

8.3.9 Prevention and Control

Giardiasis can be prevented and controlled by,

- Proper disposal of human faeces, maintenance of food and personal hygiene and health education.
- After using the bathroom and before eating, the hands should be washed thoroughly with soap and warm water. Boiling of water is the best and effective method in killing the viable cysts.
- To reduce the risk of venereal transmission, patients should avoid risky sexual behavior.
- No vaccine or effective chemo prophylactic drug is available for prevention of Giardiasis.

8.4 Tissue Flagellates – *Leishmania donovani*

The genus is named after the scientist Leishman, who first described the parasite in London in May 1903.

8.4.1 Geographical Distribution

Leishmania species is found in the Mediterranean, the Middle East, Africa and Asia including India.

8.4.2 Habitat

Leishmania donovani is an obligate intracellular parasite of human and other mammalian hosts. They are always found as intracellular amastigotes in the reticuloendothelial cells of the spleen, bone marrow, liver, intestinal mucosa and mesenteric lymph nodes of hosts.

8.4.3 Morphology

The parasite exists in two forms:

Amastigote: It is the form found in human and other mammalian hosts. They are found inside monocytes, polymorphonuclear leucocytes or endothelial cells. They are small, round to oval bodies measuring 2–3µm in length (Figure 8.8). They are also known as LD (Leishman donovan) bodies.

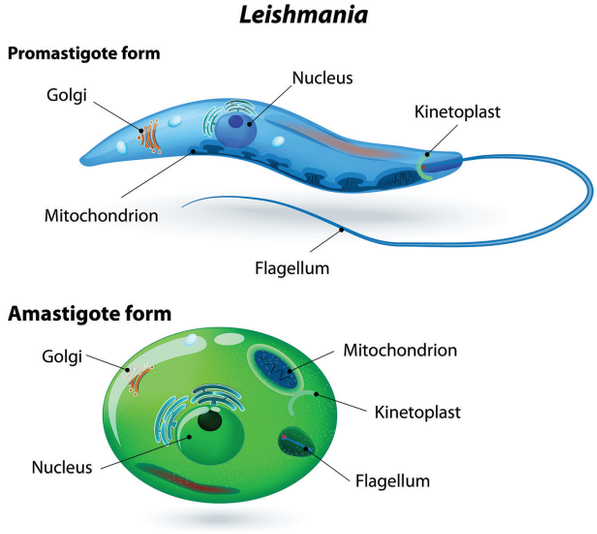

Figure 8.8: Promastigote and Amastigote form of Leishmania

TN_GOVT_XII_Micro_Biology_CH08.indd 162 07-12-2021 15:22:20

Promastigote: These forms are found in the mid-gut of sand fly and in the culture media. The fully developed promastigotes are long, slender and spindle – shaped. They measure 15µm to 25µm in length and 1.5µm to 3.5µm in breadth. A single nucleus is situated at the centre. The kinetoplast lies near the anterior end. The flagellum is single, delicate and measures 15µm–28µm (Figure 8.8).

Infobits

There are 3 main forms of Leishmaniases – Visceral (also known as Kala-agar and the most serious form of the disease), cutaneous (the most common) and mucocutaneous. The disease affects some of the poorest people on earth, and is associated with malnutrition, population displacement, poor housing, a weak immune system and lack of financial resources. Leishmaniasis is linked to environmental changes such as deforestation, building of dams, irrigation schemes, and urbanization.

PKDL occurs in all areas endemic for L.donovani but is commonest in East Africa and on the Indian subcontinent, where up to 50% and 10% of patients with kala-azar, respectively develop the condition. The frequency is reported to be declining in India.

Cutaneous Leishmaniasis

The clinical spectrum of cutaneous leishmaniasis (oriental sore) is broad and may mimic that of other skin conditions, such as staphyloccal or streptococcal infection, mycobacterial ulcer, leprosy, fungal infection, cancer, sarcoidosis and tropical ulcer.

8.4.4 Life – Cycle of Leishmania donovani

Leishmania donovani completes its life cycle in two different hosts. The complete life cycle is given in Figure 8.9.

Host	Forms
Human and other mammals (Example: Dogs)	Amastigote
Sandfly of Genus *Phlebotomus*	Promastigote

Development in Human

The parasite is transmitted to human and other vertebrate hosts by the bite of blood sucking female sandfly. During the blood meal, the sandfly deposists promastigotes on surface of the skin. These promastigotes are immediately phagocytosed by fixed macrophages of the host, in which they are transformed into amastigotes. The amastigotes multiply by binary fission within the macrophages. As many as 50 to 200 amastigotes may be present inside the enlarged cell. These are called LD bodies. The rupture of cell releases amastigotes in large numbers which inturn are free to infect other cells. Free amastigotes are subsequently carried by circulation. These forms invade monocytes of the blood and macrophages of the spleen, liver, bone marrow, lymph nodes and other tissues of the reticuloendothelial cells.

Development in sandfly

Female sandfly during a blood meal ingest free, as well as intracellular amastigotes in the blood. In the mid gut of the sandfly, the amastigotes are transformed within 72 hours to flagellated promastigotes.

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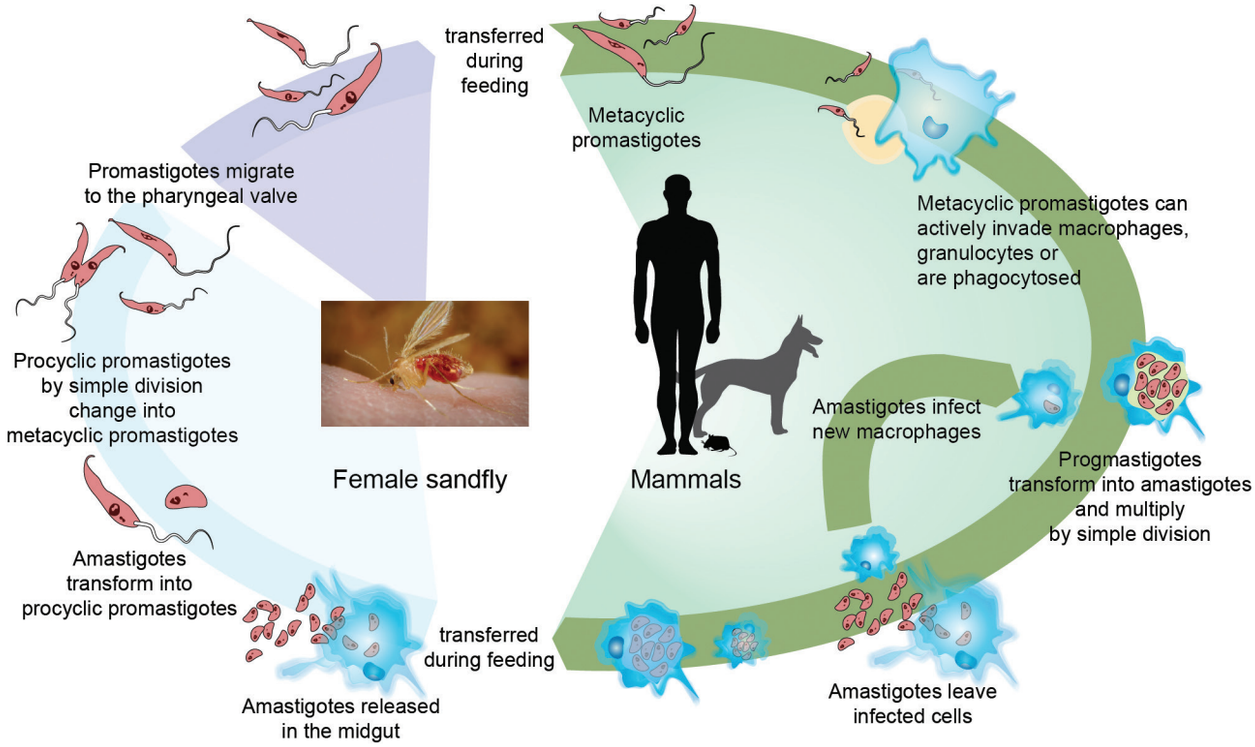

Figure 8.9: Life cycle of *Leishmania donovani*

These promastigotes multiply by binary fission. After a period of 6 to 9 days, these forms migrate from the midgut to the pharynx and buccal cavity of sandfly. Bite of the infected sandfly transmits infection to susceptible persons and the life – cycle is repeated.

8.4.5 Pathogenesis

Leishmania donovani causes visceral Leishmaniasis. The disease is also known as Dum – Dum fever, Asian fever, Assam fever, or infantile splenomegaly. Leishmaniasis is a disease of the reticuloendothelial system. Proliferation and destruction of reticuloendothelial cells of the internal organs are responsible for the pathological changes in visceral leishmaniasis.

Spleen, liver and lymphnodes are enlarged in this condition. Bone marrow is dark red in colour and shows extensive proliferation of reticuloendothelial cells. Kidney shows cloudy swelling and is invaded by macrophages parasitized by amastigotes.

8.4.6 Clinical Features

Incubation period: It is usually 3–6 months but can be months or years.

Visceral Leishmaniasis is a serious and fatal systemic disease. In India, the disease is called Kala – azar meaning "black disease".

The disease is characterized by the presence of fever, hepatosplenomegaly (Figure 8.10) (the simultaneous enlargement of both liver and the spleen), hypergammaglobulinemia (a condition in which increased levels of a certain immunoglobulin in blood serum), Leucopenia, Thrombocytopenia (deficiency of platelets in the blood), Cachexia (a condition that causes extreme weight loss)

TN_GOVT_XII_Micro_Biology_CH08.indd 164 07-12-2021 15:22:20

with marked anemia, emaciation and loss of weight. Epistaxis (bleeding from nose) and bleeding from gums are common. In Indian patients, the skin on the hands, feet, abdomen, around the mouth and fore – head becomes grayish and dark coloured. This hypo – pigmentation of the skin is unique in Indian patients giving the disease name Kala – azar.



Figure 8.10: Splenomegaly

Post kala – azar dermal leishmaniasis (PKDL): It is a non – ulcerative lesion of the skin, which is seen after completion of treatment of the kala – azar. This condition is characterized by multiple, hypopigmented, erythematous macules involving the face and trunk (Figure 8.11). In Indian forms, PKDL appears after a latent period of 2 years and may even persist as long as 20years, creating a persistent human reservoir of infection.

Laboratory diagnosis

Specimens: Aspiration from spleen, bone marrow, lymph node, liver biopsy and peripheral blood.



Figure 8.11: Post kala – azar dermal leishmaniasis -PKDL

Methods of examination: This includes, microscopy and culture

1. Direct microscopy

The amastigotes of *Leishmania donovani* (known as LD bodies) can be demonstrated in the smears of spleen, bone marrow, liver, lymph node and peripheral blood stained in Leishman, Giemsa or wright stains. Splenic aspiration is the most sensitive method to detect LD bodies. Examination of peripheral blood smear and buffy coat smear is more commonly used to find LD bodies in the circulating monocytes.

2. Culture

Promastigotes are found in the culture media. Tissue samples and aspirates are inoculated in the NNN (Novy–MacNeal–Nicolle) medium for demonstration of promastigotes.

Laboratory diagnosis of kala – azar is briefly discussed in Flowchart 8.5.

Treatment: Pentavalent antimonials are the drugs of choice. Pentamidine, Amphotericin B and Miltefosine (oral drug) are recommended.

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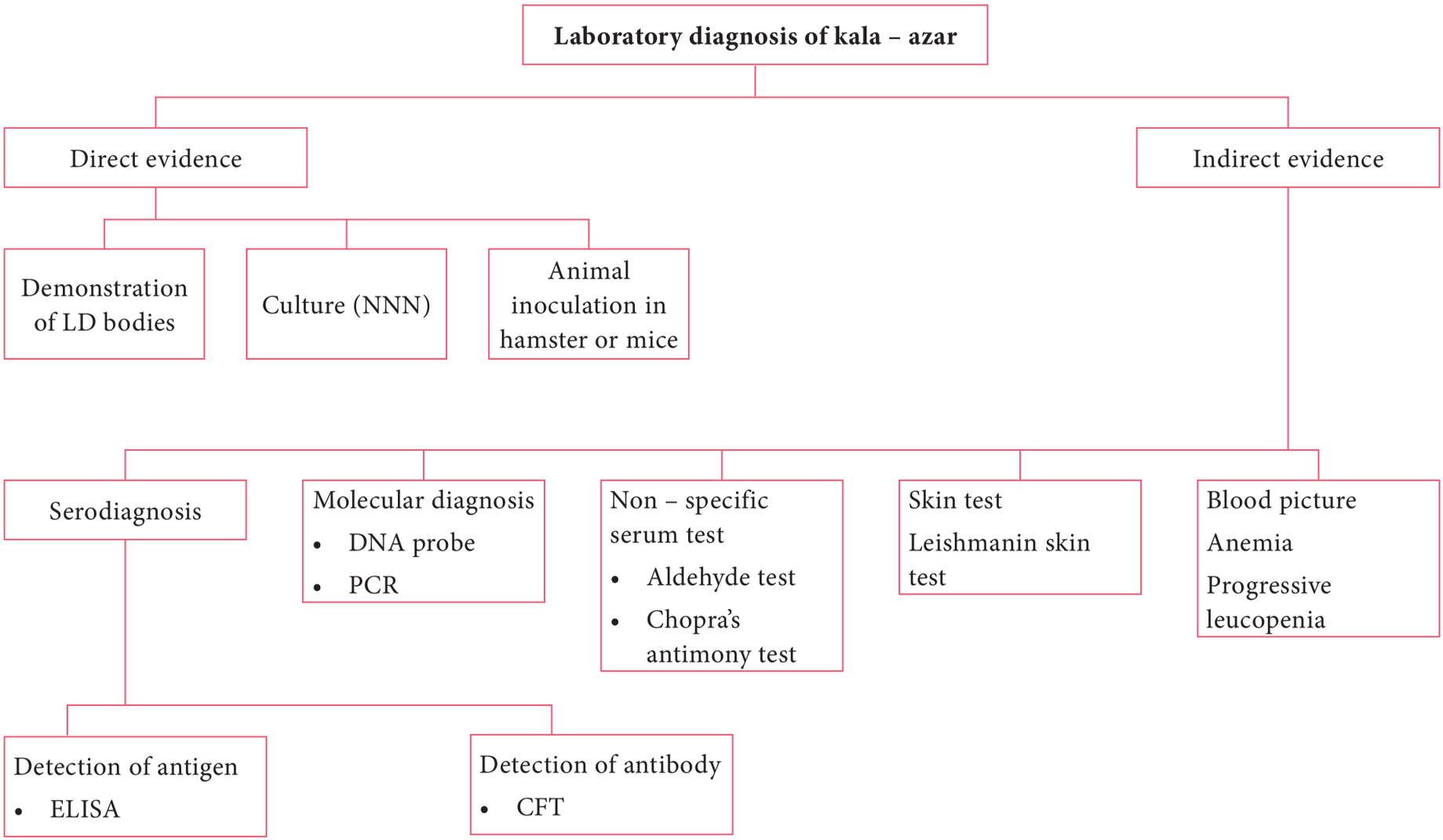

Flowchart 8.5: Laboratory diagnosis of kala-azar

07-12-2021 15:22:21

8.4.7 Prevention and Control

Integrated insecticidal spraying (DDT and Malathion) to reduce sandfly population.

Reduction of reservoir by killing all the infected dogs.

Personal prophylaxis by using anti – sandfly measures like using thick clothes, bed nets, window mesh or insect repellants and keeping the environment clean.

No vaccine is available against kala – azar.

8.5 Sporozoa – *Plasmodium*

Protozoan parasites characterised by the production of spore – like oocysts containing sporozoites were known as sporozoa. The parasites belonging to this group of protozoa do not possess any special organs of locomotion, such as flagella or cilia. The medically important parasite of this class that is given in the text is malarial parasite.

Malaria

It is the disease condition with seasonal intermittent fevers, chills and shivering. The name malaria (Mal: bad, aria: air) was given in the 18th century in Italy. The specific agent of malaria was discovered in RBC's of a patient in 1880 by Alphonse Laveran. In 1897, Ronald Ross identified the developing stages of malarial parasites in mosquitoes in Secunderabad, India. This led to various measures for the control and possible eradication of malaria by mosquito control. Both Ross (1902) and Laveran (1907) won the Nobel Prize for their discoveries in malaria.



The single most important protozoan disease is malaria, which causes 1.5 million deaths each year.

Different species of malaria parasites can develop in the same mosquito and such an infected mosquito can transmit the infection to man giving rise to cases of "mixed infection" the commonest being *P. falciparum* with *P. vivax*.

Infobits

Three basic types of malaria

1. Benign tertian (P. vivax and P. orale) with a fever every 2nd day (Example: Monday – fever, Tuesday – no fever, Wednesday – fever).
2. Benign quartan (P. malariae) with a fever every 3rd day. (Example: Monday; fever, Tuesday – no fever, Wednesday – no fever, Thursday – fever.
3. Malignant tertian (P. falciparum), in which the cold stage is less pronounced and the fever stage is more prolonged and severe. This type of malaria is more dangerous because of the complications caused by capillary blockage (i.e, convulsion, coma, acute pulmonary insufficiency and cardic failure). Large numbers of erythrocytes are parasitized and destroyed, which may result in dark-coloured urine. (black water fever); intravascular hemolysis, hemoglobinuria, and kidney failure).

   Two species of plasmodium, P. vivax and P. ovale, can remain in the liver, if not treated properly. The organism leave the liver and re-infect erythrocytes, causing the symptoms.

TN_GOVT_XII_Micro_Biology_CH08.indd 167 07-12-2021 15:22:21

Causative agents of human malaria:
The organisms: Four species of Plasmodium cause malaria in humans.

- *Plasmodium vivax:* (Benign Tertian malaria)
- *Plasmodium falciparum:* (Malignant tertian malaria)
- *Plasmodium malaria:* (Benign Quartan malaria)
- *Plasmodium ovale:* (Benign tertian malaria)

The two most common species are P. vivax and P. falciparum, WHO reports (2018) that falciparum being the most pathogenic of all.

8.5.1 Geographical Distribution

Malarial parasites are found in all countries. In India, malaria continues to be a major public health threat.

8.5.2 Habitat

The malarial parasites infecting man, after passing through a developmental phase in the parenchyma cells of the liver, reside inside the red blood corpuscles and are carried by the circulating blood to all the organs.

8.5.3 Vectors

Human malaria is transmitted by over 60 species of female Anopheles mosquito.

Human malarial parasite – *Plasmodium falciparum*

Of all the human malaria parasites, *P. falciparum* is the most highly pathogenic and responsible for malignant tertian malaria. This is a form of disease which runs an acute course in non-immune patients and is frequently fatal if untreated.

8.5.4 Life Cycle

The malaria parasite passes its life cycle in two different hosts and comprises of two phase as follows,

Definitive host: Female Anopheles mosquito (a sexual phase of parasite occurs).

Intermediatehost: Human (an asexual phase of parasite occurs).

Thus, life cycle of malaria parasite show alternation of generations- asexual and sexual generation in two different hosts (Figure 8.12).

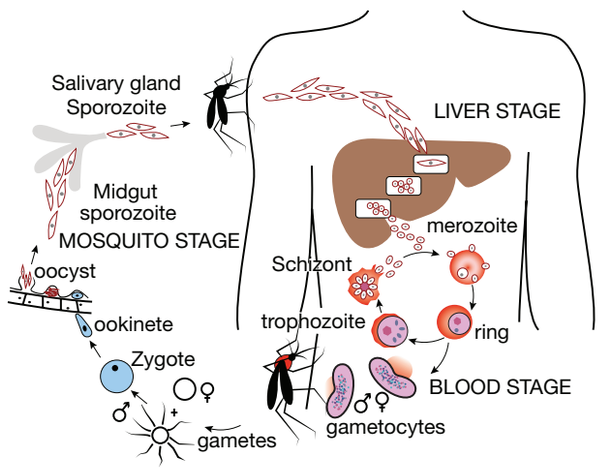

Figure 8.12: Lifecycle of Plasmodium spp

8.5.5 Human Cycle (Asexual Phase – Schizogony)

Human infection occurs when the sporozoites (the infective forms of the parasite are present in the salivary gland of the mosquito) are injected into blood capillaries when the mosquito feeds on blood after piercing the skin. The malarial parasite multiplies by division and the process designated as Schizogony (schizo: to split, gone: generation).

TN_GOVT_XII_Micro_Biology_CH08.indd 168 07-12-2021 15:22:21

Sporozoites are minute thread-like curved organisms with tapering ends. Measuring 9–12μ in length with a central elongated nucleus while, the cytoplasm reveals no pigment as seen with a light microscope. In human, schizogony occurs in two locations. One in the red blood cells (erythrocytic schizogony) and other in the liver cells (pre – or exoerythrocytic schizogony).

A. Pre–erythrocytic or Exoerythrocytic schigony

- Sporozoites do not directly enter the RBC's to initiate erythrocytic schizogony, but undergo developmental phase in other human tissues.
- This cycle lasts for about 8 days in *Plasmodium vivax*, 6 days in *P. falciparum* and 9 days in *P. ovale*.
- This pre–erythrocytic schizogony occurs within parenchymal cells of the liver.
- The Sporozoites, which are elongated spindle – shaped bodies, become rounded inside the liver cells.
- They enlarge in size and undergo repeated nuclear division to form several daughter nuclei, each of which is surrounded by cytoplasm.
- This stage of the parasite is called the pre–erythrocytic or exoerthrocytic schizont or merozoites.
- The heptocyte is distended by the enlarging schizont and the liver cell nucleus is pushed to the periphery.
- Mature liver stage schizonts are spherical multinucleate and contain 2000–50,000 uninucleate merozoites.
- These normally rupture in 6–15 days and release thousands of merozoites into the blood stream.
- They do not return from red blood cells to liver cells.

Plasmodium vivax and *P. ovale* – parasites in liver tissue are called hypnozoites.

B. Erythrocyticstage

- The merozoites released by pre–erythrocytic schizonts invade the red blood cells (Parasitaemia).
- Merozoites are pear – shaped bodies, about 1.5 μm in length.
- In the erythrocyte, the merozoite loses its internal organelles and appears as rounded body having a vacuole in the center with the cytoplasm pushed to the periphery and the nucleus at one pole. These forms are called ring forms or young trophozoites.
- The parasite feeds on the hemoglobin of the erythrocyte. They incompletely metabolize hemoglobin therefore, hematin – globin pigment or haemozoin pigment is left behind.
- The malaria pigment released when the parasitized cells rupture is taken up by recticuloendothelial cells.
- The ring form develops and becomes irregular in shape and shows amoeboid motility. This is called the amoeboid form.
- When the amoeboid form reaches a certain stage of development, its nucleus starts dividing by mitosis followed by a division of cytoplasm to become mature schizonts or merozoites.

TN_GOVT_XII_Micro_Biology_CH08.indd 169 07-12-2021 15:22:21

- A mature schizont contains 8–32 merozoites and haemozoin. The mature schizont bursts releasing the merozoites into the circulation.
- The merozoites invade fresh erythrocytes within which they go through the same process of development. This cycle is called erythrocytic schizogony.
- The rupture of the mature schizont releases large quantities of pyrogens. This is responsible for the febrile paroxysms characterising malaria.
- In *P. falciparum*, erythrocytic schizogony always takes place inside the capillaries and vascular regions of internal organs. Therefore, in these infections, schizonts and merozoites are usually not seen in the peripheral blood.

C. Gametogony

- Some of the merozoites, after a few erythrocytic cycles do not develop into trophozoites and schizonts but they undergo sexual differentiation to develop into the gametocytes.
- Development of gametocytes takes place within the internal organs and only the mature forms appear in circulation.
- The mature gametocytes in *P. falciparum* are crescent shaped.
- Female gametocytes are generally more numerous and larger.
- Male gametocytes and female gametocytes are called micro gametocytes and macro gametocytes respectively.
- Gametocyte appears in 10–12 days in *P. falciparum*.
- The gametocytes do not cause any clinical illness in the host, but are essential for transmission of the infection.
- A person who harbors the gametocytes is referred to as a carrier or reservoir.

Infobits

Sir Ronald Ross Institute of Parasitology is a malaria research institute located in Begumpet, Secunderabad, Hyderabad, India. Established in 1955, the institute is a division of Osmania University. The institute is named after Sir Ronald Ross, winner of Nobel Prize for Physiology or Medicine, 1902. Though he was a surgeon by qualification, Ross was attracted towards research in tropical diseases, especially malaria. During his posting, he worked on his research from a laboratory in the old Begumpet military hospital building. It was in this building on 20 August 1897 that he made the discovery of the malarial parasite inside the body of a mosquito.His study confirmed that mosquitoes were the carriers of malaria parasite.

8.5.6 Mosquito Cycle (Sexual Cycle – Sporogony)

- A Female Anopheles mosquito during its blood – meal from an infected person, sucks up both the sexual and asexual forms of parasite. But, only the mature sexual forms develop and the rest die.
- The gametocytes are set free in the midgut (stomach) of mosquito and undergo further development.

TN_GOVT_XII_Micro_Biology_CH08.indd 170 07-12-2021 15:22:21

- The nuclear material and cytoplasm of the male gametocyte divides to produce long, actively motile, whip – like forms of 8 microgametes. This process is called exflagellation of male gametocytes.
- The Exflagellation is completed within 15–30 minutes for *P. falciparum*.
- The female gametocyte does not divide but maturation involves by condensation of nucleus to become the female gamete.
- Female gamete is fertilized by one of the microgametes to produce the zygote. The zygote is formed in 20–120 minutes after the blood meal. The zygote is initially is a non – motile round body, but within 18–24 hours, it gradually elongates into a vermicular motile form. This is called the ookinete.
- Ookinete penetrates the epithelial lining of stomach wall. Their anterior end comes in close contact to the cell membrane by secretion of some proteolytic substances which causes lysis of cell membrane. Later, the ookinete come to lie just beneath the basement membrane.
- It becomes rounded into a sphere with an elastic membrane. This stage is called the oocyst. The oocyst increase in size and undergo numerous nuclear multiplication which develops a large number of sickle shaped bodies known as sporozoites.
- Number of oocysts in the stomach wall varies from a few to over a hundred.
- Around the 10th day of infection the oocyst ruptures, releasing sporozoites in the body cavity of the mosquitos.
- The sporozoites are distributed through the circulating fluid into various organs and tissues of the mosquito except the ovaries.
- The sporozoites have a special affinity towards the salivary glands. The mosquito at this stage is capable of transmitting infection to man.

8.5.7 Pathogenesis

In malaria, typical pathological changes are seen primarily in the spleen, liver, bone marrow, lungs, kidney and brain.

Liver: The liver is enlarged. The organ becomes more firm and pigmented. Pigments are found in parenchymal cells.

Spleen: The spleen is markedly enlarged. If the infection lasts over a long period, the spleen is usually grayish, dark brown or even black and is commonly known as 'ague cake'.

Bone marrow, Lungs, Kidneys and Brain are enlarged and pigmented. They are filled with parasitized erythrocytes. Anemia is caused by destruction of large number of red cells by complement mediated and autoimmune hemolysis. It is also due to the increased clearance of both parasites and parasitized RBCs by the spleen.

8.5.8 Clinical Manifestations

The incubation period is generally 9–14 days but, it can be as short as 7 days. The most malignant form of malaria is caused by P. *falciparum* hence, variable clinical syndromes are associated with falciparum malaria. That include,

1. Prodromal (initial indication of the onset of disease) period: Non – specific symptoms such as malaise (condition of

TN_GOVT_XII_Micro_Biology_CH08.indd 171 07-12-2021 15:22:21

general weakness or discomfort), myalgia (severe muscle pain) headache and fatigue (feeling of tiredness) are usually seen during the prodromal period.

2. Malarial paroxysm (sudden onset of disease): It is the classical manifestation of acute malaria. It is characterised by fever, chill and rigor (sudden feelings of cold with shivering).The fever is caused by rupture of red blood cells that contain malarial parasites. The fever occurs every 48 hours in falciparum malaria.
3. Anemia (A condition in which the blood does not have enough healthy Red Blood cells) and
4. Hepatosplenomegaly (simultaneously enlargement of both the liver and the spleen)

The symptoms are non – specific with headache, pains in back and limbs, anorexia, nausea and a feeling of chill rather than a distinct cold phase. Hyponatremia (A condition that occurs when the level of Sodium in the blood is too low) occur in both uncomplicated and severe malaria.

Infobits

Transfusion Malaria

Malaria can be transmitted by transfusion of blood from infected donors. First reported in 1911, transfusion malaria is one of the most common transfusion-transmitted infections today. Blood transfusion can accidentally transmit malaria, if the donor is infected with malaria. The parasites may remain viable in blood bank for 1–2 weeks. As this condition is induced by direct infection of red cells by the merozoites. Pre-erythrocytic schizogony and hypnozoites are absent.

8.5.9 Complications of Severe Falciparum Malaria

1. Black water fever

The syndrome is the manifestation of repeated infections of falciparum malaria, which were inadequately treated with quinine. The condition is associated with haemoglobinaemia (excess of hemoglobin in the blood plasma) and haemoglobinuria (excretion of free haemoglobin in the urine). The syndrome is known as black water fever due to the dark red to brown – black appearance of the urine in this condition (Figure 8.13). It is dark due to presence of free haemoglobin as methaemoglobin or oxyhaemoglobin in it. Kidney failure is the immediate cause of death.

2. Cerebral malaria

Cerebral malaria is the most common presentation of severe malaria in adult. Cerebral malaria may be sudden in onset. Clinically, the condition manifests with fever for 4–5 days, slowly lapsing into coma, with or without convulsions. It is marked by a severe headache, high fever even above 180°F, and changes in mental status. Death may occur within few hours. Algid malaria and septicemic malaria are also other serious complication of falciparum malaria.

3. Pernicious malaria

The term pernicious malaria is referred to as a series of phenomena that occur during the course of an in treated *P. falciparum* infection within 1 to 3 days.

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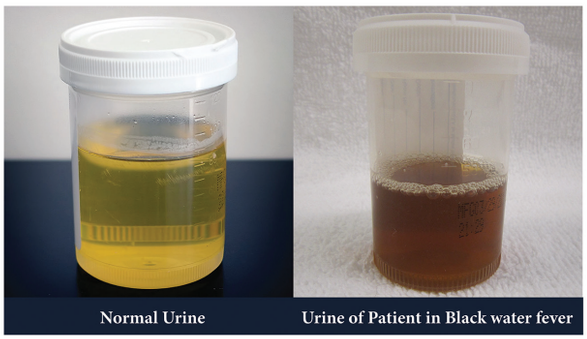



Figure 8.13: Urine in Black water Malaria

4. Anaemia: An individual suffering from an attack of malaria, after a few paroxysms becomes temporarily anaemic. The reduction in red blood cells is greater in *P. falciparum* infection than in infection with *P. vivax* and *P. malariae*. This is because *P. falciparum* invades young and mature erythrocytes and the infection rate of red blood cells is also greater.

HOTS

Which stage is infective in Blood transfusion malaria?

8.5.10 Recrudescence

In *P. falciparum* and *P. malariae* infections after the primary attack, sometimes there is a period of latency, during which there is no clinical illness. But some parasites persist in some erythrocytes and gradually increase in numbers. Fresh malarial attacks then develop. It appears after a period of latency usually within weeks after the primary attacks. Persistence of the erythrocytic cycle of the parasites are called recrudescences. In *P. falciparum* infections, recrudescences are seen for 1–2 years, while in *P. malariae* infection, they may last for long periods, even upto 50 years.

Infobits

The global technical strategy for malaria 2016-2030 was adopted by the World health Assembly in May 2015. It provides a comprehensive framework to guide countries in their efforts to accelerate progress towards malaria elimination. The strategy sets the target of reducing global malaria incidence and mortality rates by at least 90% by 2030.

8.5.11 Plasmodium vivax

P. vivax shows a similar life cycle in humans and mosquitoes like that of *P. falciparum*. Except in *P. vivax*, a latent tissue stage, the hypnozoites present in the liver parenchyma.

Relapse in vivax malaria is caused by these hypnozoites. Hypnozoites are the dormant stages of the parasites. These are single – nucleated parasites measuring 4µm–6µm in diameter. These become active and develop into tissue schizonts after a short period of dormancy. This relapse may occur at intervals up to 3 years or more after the first attack. *P. vivax* merozoites invade only young erythrocytes and the reticulocytes.

8.5.12 Clinical Manifestations

P. vivax is the most wide spread species causing malaria in man. However, unlike

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Table 8.2: Comparison of course of infection – *P. falciparum* and *P. vivax* in man

Stage	P. falciparum	P. vivax
Pre – erythrocytic schizogony	Stage lasts for 6 days. Each Schizont produces about 40,000 merozoites approximately	Lasts for 8 days. Each Schizont produces about 12,000 approximately
Erythrocytic schizogony	Each cycle lasts for 36–48 hours. First temperature peak occurs by 12thday of infection. Primary attack last for 10–14 days.	Each cycle lasts for 48 hours. First fever peak occur by 16th day of infection. Primary attack lasts for 3–4 weeks.
Gemotogony	Gametocytes in peripheral blood may be seen on 21st day of infection.	Gametocytes in peripheral blood may be seen on 16th day of infection.
Exo – erythrocytic schizogony	Absent. Relapses do not occur	Present. Can continue for up to 3 years. Relapses often occur.

falciparum malaria, vivax malaria, is less severe and death from the condition relatively is less common. Table 8.2 describes the comparison of course of infection in Falciparum malaria with Vivax malaria

8.5.13 Laboratory Diagnosis

Diagnosis of malaria includes:

a. Parasitic diagnosis

b. Serodiagnosis, and

c. Molecular diagnosis

Parasitic diagnosis – Demonstration of parasite by microscopy

Specimen: Blood

Conventional light microscopy of stained blood smear is the gold standard for confirmation of malaria.

Two types of smears are prepared from the peripheral blood. They are thin and thick smears (Figure 8.14). Ring forms and gametocytes are most commonly seen in the peripheral blood smear.

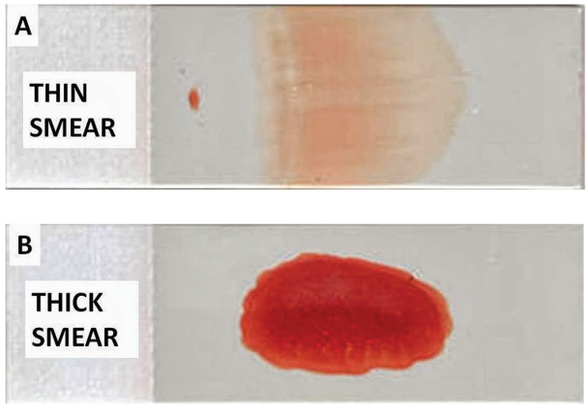

Figure 8.14: Blood smear

Thin smear

They are prepared from capillary blood of fingertip and spread over a good quality slide by a second slide (spreader slide) held at an angle of 30°–45° from the horizontal such that a tail is formed.

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Thin smears thus prepared are air dried, fixed in alcohol and stained by one of the Romanowsky stains such as Leishman, Giemsa or JSB (Jaswant singh and Bhattacharjee) stain.

Thin smears are used for:

a. Detecting parasites, and

b. For determining the species of the infecting parasite.

Thick smear

They are prepared usually with 3 drops of blood spread over a small area of about 10mm. The thick film is dried. This smears consist of a thick layer of dehemoglobinized (lysed) red blood cells. It is not fixed in methanol.

Thick film is stained similar to thin film. Thick smears have the advantage of concentrating the parasites and therefore increase the sensitivity of diagnosis. Thick smears are used for:

a. Defecting parasites,

b. Quantitating parasitaemia, and

c. Demonstrating malarial pigments.

Fluoroscence microscopy

The method is mainly used for mass screening in field laboratory. Fluorescent dyes like acridine orange is used to stain the blood smears. It stains DNA as fluorescent green and cytoplasmic RNA as red.

QBC (Quantitative Buffy coat smear)

This is a sensitive method for detection of malaria parasites. In this method, blood is collected in a capillary tube coated with fluorescent dye and is subjected to centrifugation. After centrifugation, the Buffy coat in the centrifuged capillary tubes is examined under a fluorescent microscope. Acridine orange – stained malaria parasites appear brilliant green.

Serodiagnosis

It is not helpful in clinical diagnosis. It is used mainly for epidemiological survey and to identify the infected donors in transfusion malaria. The test used are indirect haemagglutination (IHA), Indirect fluorescent antibody (IFA) and Enzyme – linked immunosorbent assay (ELISA) for the detection of serum antibodies.

Rapid Antigen detection tests kits are available commercially like the dipstick, card and cassette bearing monoclonal antibody. These tests are based on the detection of antigens using immune chromatographic methods. These tests can detect plasmodium in 15 minutes.

Molecular diagnosis

DNA probe and PCR are highly sensitive methods for the diagnosis of malaria. It is more sensitive than that of thick blood smear. It is highly species specific.

Other tests includes the measurement of hemoglobin, total WBC and platelet count in severe falciparum malaria, urine can be tested for free hemoglobin, if black water fever is suspected. Blood urea and serum creatinine has to be monitored for renal failure.

8.5.14 Treatment

The most commonly used drugs are Chloroquine, Quinine, Pyrimethamine and Doxycycline.

8.5.15 Prevention and Control

The preventive measures to control malaria mainly depend on treatment of

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infected individuals and reducing the transmission of malaria.

The control measures include the use of insecticides such as DDT (Di chlorodiphenyl tri chloromethane) or Malathion for controlling the populations of adult mosquitoes.

Proper use of mosquito nets, wearing protective clothings and use of mosquito repellants can prevent the mosquito bite.

Introduction to Helminths

General characteristics of Helminthic parasite:

1. Helminths are multicellular worms. They are bilaterally symmetrical animals having 3 germ layers and belong to the kingdom Metazoa.
2. They are invertebrates characterised by elongated, flat or round bodies.
3. Helminths develop through egg, larval and adult stages. Flowchart 8.1 describes the classification of helminthes.

8.6 Nematode: *Ascaris Lumbricoides*

8.6.1 Geographical Distribution

It is the most common of human helminthes and is distributed worldwide.

8.6.2 Habitat

The adult worms lives in the small intestine particularly in jejunum and in ileum.

8.6.3 Morphology

Adult worm

Ascaris lumbricoides resembles and sometimes confused with the earthworm. Its specific name *lumbricoides* means earthworm in Latin. Male and Female worm of Ascaris lumbricoides are shown in Figure 8.15.

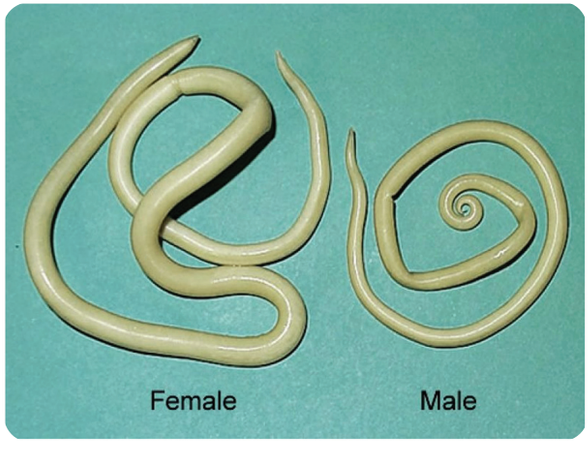

Figure 8.15: Adult worms of *Ascaris Lumbricoides*

- They are large cylindrical worms with tapering ends. The anterior end being thinner than the posterior end. It is the largest intestinal nematode parasitizing man.
- The life – span of the adult worm is less than a year.

Male worm

- The adult male worm is smaller than female worms.

DO YOU KNOW?

The roundworm, *Ascaris lumbricoides* is the largest nematode parasite in the human intestine. An editorial in the lancet in 1989 observed, that if all the round worms in all people worldwide were placed end-to-end. They would encircle the world 50 times. Soil-transmitted intestinal nematodes are called Geohelminths.

TN_GOVT_XII_Micro_Biology_CH08.indd 176 07-12-2021 15:22:22

- The tail – end (Posterior end) of the male worm is curved ventrally to form a hook and 2 curved copulatory spicules.

Female worm

- The adult female worm is larger (20–40 cm) and thicker (3–6 mm) than male worm.
- The posterior end is conical and straight. The anus is in the sub terminal part and opens like a transverse slit on the ventral surface.
- The vulva is situated mid – ventrally, near the junction of the anterior and middle thirds of the body. This part of the worm is narrow and is called the vulvar waist.
- A single worm lays up to 200,000 eggs per day.

Egg: Two types of eggs are passed in feces by the worms.

Fertilized Egg

- The fertilized eggs are produced by fertilized females.
- The eggs are round or oval in shape and measures 45 µm in length and 35µm to 50µm in breadth.
- They are bile – stained and appear as golden brown (brownish) in colour.
- The egg is surrounded by a thick smooth shell with an outer albuminous coat (corticated eggs). Sometimes this outer coat is lost in few eggs. Those eggs are called as decorticated eggs (Figure 8.16).
- Each egg contains a large unsegmented ovum with a clear crescentic area at each pole. The eggs float in saturated solution of common salt.

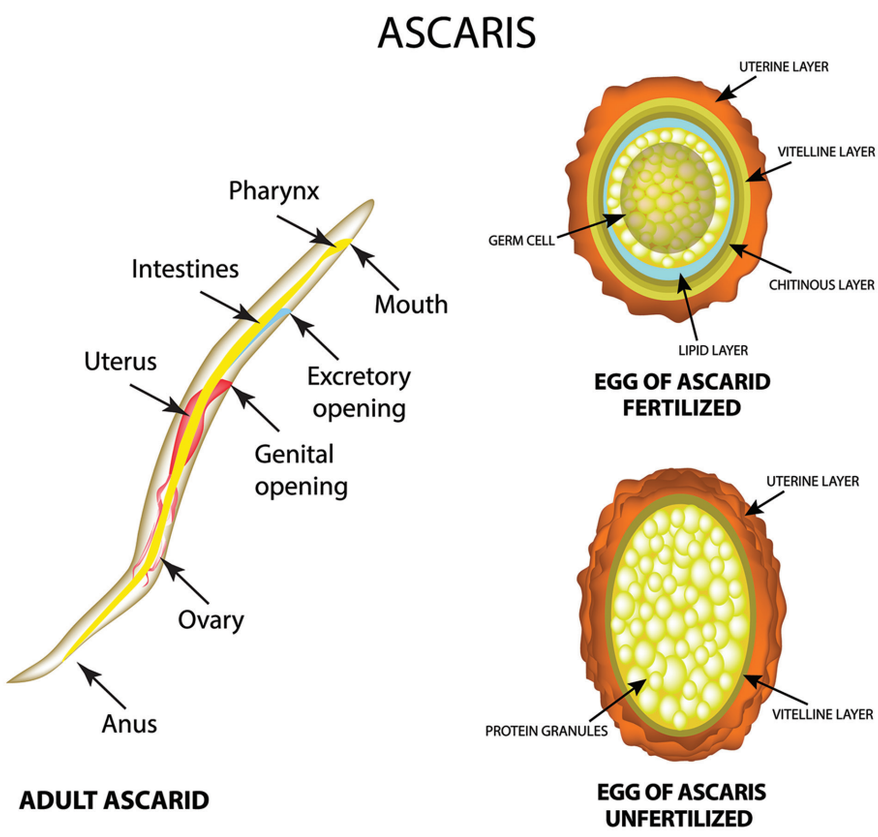

Figure 8.16: Fertilized and Unfertilized egg of *Ascaris lumbricoides*

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Unfertilized egg

- The female even not fertilized by male is capable of liberating eggs. These unfertilized eggs are narrower, longer and elliptical in shape.
- These are heaviest of all the helminthic eggs – It measures about 80µm × 105µm in size.
- The eggs have a thinner shell with an irregular coating of albumin (Figure 8.16).
- These eggs do not float in saturated solution of common salt.

HOTS

What makes worm's egg float or sink?

8.6.4 Life – Cycle

The life – cycle of *A. lumbricoides* is completed in a single host, human (Figure 8.17).

Infective form: Ermbryonated eggs. The fertilized egg passed in feces is not immediately infective. It has to undergo a period of development in soil. The development usually takes from 10–40 days. The embryo moults twice during the time and becomes the infective rhabditiform larva.

Mode of transmission: Man acquires the infection by ingestion of food, water or raw vegetables contaminated with embryonated eggs of the round worm.

The ingested eggs reach the duodenum to liberate the larvae by hatching. These

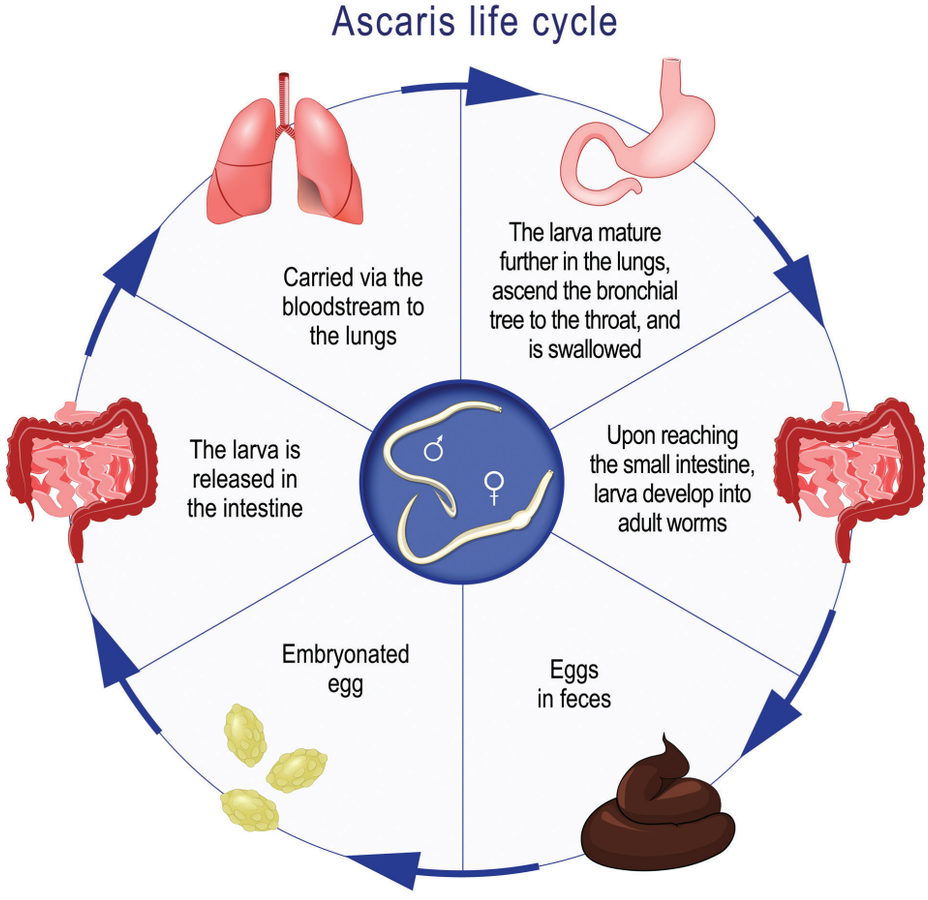


Figure 8.17: Lifecycle of *Ascaris lumbricoides*

TN_GOVT_XII_Micro_Biology_CH08.indd 178 07-12-2021 15:22:22

larvae then penetrate the intestinal wall and are carried by the portal circulation to the liver. They live in liver for 3 to 4 days. Then they are carried to the right side of the heart, then to lung. In the lung, they grow and moult twice.

After development in the lungs, in about 10–15 days, the larvae pierce the lung capillaries and reach the alveoli. Then they are carried up the respiratory passage to the throat and swallowed back to the small intestine.

In the small intestine, the larvae moult finally and develop into adults. They become sexually mature in about 6–12 weeks. The fertilized female start laying eggs which are passed in the faces to repeat the cycle.

8.6.5 Pathogenesis

Infection of *A. lumbricoides* in human is known as ascariasis. The adult worm may produce its pathogenic effects in the following ways.

a. The spoliative or nutritional effects is usually seen when the worm burden is heavy. Presence of enormous numbers (sometime exceeds 500) often interferes with proper digestion and absorption of food. Ascariasis may contribute to protein – energy malnutrition and vitamin A deficiency.

b. The toxic effects is due to the metabolites of adult worm. Ascaris allergens produce various allergic manifestations such as fever, urticaria and conjunctivitis.

c. The mechanical effects are the most important manifestations of ascariasis. In heavy infections, adult worms can cause obstruction and inflammation of intestinal tract, particularly of the terminal ileum.

d. Ectopic ascariasis (Wander lust) is due to the adult male worms. They are restless wanderers. The wandering happens when the host temperature rises above 39°C. The worm may wander up or down along the gut. It may enter the biliary or pancreatic duct causing acute biliary obstruction or pancreatitis. It may enter the liver and lead to liver abscesses. The worm may go up the esophagus and come out through mouth or nose. It may crawl into the trachea and the lung causing respirator obstruction or lung abscesses. Migrating downwards, the worm may cause obstructive appendicitis. The worm may also reach kidneys. "Larva migrans" is a term used when the larval sworms migrate to various parts of the body.

8.6.6 Clinical Manifestations

Incubation Period is 60–70 days. Clinical manifestations due to adult worm vary from asymptomatic to severe and even fatal infection. Clinical manifestation in ascariasis can be caused either by the migrating larvae or by the adult worms.

Symptoms due to the migrating larvae: leads to ascaris pneumonia and larvae may enter the general circulation, disturbances have been reported in the brain, spinal cord, heart and kidneys.

Symptoms due to the adult worms: Diffuse or epigastric abdominal pain, abdominal cramping, abdominal swelling (especially in children), fever, nausea,

TN_GOVT_XII_Micro_Biology_CH08.indd 179 07-12-2021 15:22:22

vomiting and passing roundworms and their eggs in the stool.

8.6.7 Laboratory Diagnosis

Specimen collected: Stool, sputum and blood.

Detection of parasite

Adult worm: It can be detected in stool or sputum of patient by naked eye. Pancreatic or biliary worms can be detected by ultra-sound and endoscope.

Larvae: Larvae can be detected in sputum and often in gastric washings. Chest X – ray may show pulmonary infiltrates.

Eggs: Detection is through demonstration of eggs in feces. Detection of both fertilized and unfertilized eggs are made after staining. Eggs may be demonstrative in the bile obtained by duodenal aspirates.

Blood Examination

Complete blood count may show eosinophilia in early stage of infection.

Serological tests

Ascaris antibody can be detected by IHA, IFA and ELISA

8.6.8 Treatment

Commonly used drugs are Albendazole and Mebendazole.

8.6.9 Prevention and Control

a. Proper health education should be given for improved sanitation and personal hygiene.



The National Deworming Day (Febuary 10th) is an initiative of India to make every child in the country worm free. This is one of the largest public health programs reaching large number of children during a short period.

More than 836 million children are at risk of parasitic worm infections worldwide. According to World Health Organization 241 million children between the ages of 1 and 14years are at risk of parasitic intestinal worms in India, also known as Soil-Transmitted Helminths (STH).

b. Avoid eating of uncooked green vegetable, food preparation and fruits that may contain faecal eggs.

c. Treating infected persons especially children. Deworming of school children have been found effective in control of ascariasis.

Summary

Medical Parasitology deals with the study of parasites infecting humans. The diseases caused by them and the clinical manifestations produced in infected humans. It is also concerned with various methods of their diagnosis, treatment and their prevention and control. There are different types of parasites and hosts. Parasites lives on its host for its nourishment and survival. The relationship between host and the parasite can be symbiotic,

TN_GOVT_XII_Micro_Biology_CH08.indd 180 07-12-2021 15:22:22

commensalic or parasitic. Parasites of medical importance comes under the kingdom called Protista and Animalia. Protista includes the microscopic single – celled eukaryotes known as protozoa. In contrast, helminths are macroscopic, multicellular worms possessing well differentiated tissues and complex organs belonging to the kingdom Animalia. Protozoa includes *Entamoeba* and *Giardia* which cause intestinal infections (dysentery and diarrhoea) Leishmania donovani, the unicellular tissue flagellatis causes Leishmaniasis. Plasmodium spp., the protozoan parasite which causes malaria are transmitted by female Anopheles mosquito carring sporozoites forms of the parasite. The four species infective to humans are *P. falciparum*, *P. malariae*, *P. vivax* and *P. ovale*. Multicellular organisms and intestinal worms. The helminths such as Ascaris lumbricoides causes Ascariasis, an infection of the small intestine. Ascariasis is the most common roundworm infection.

TN_GOVT_XII_Micro_Biology_CH08.indd 181 07-12-2021 15:22:22

Evaluation

Multiple choice questions

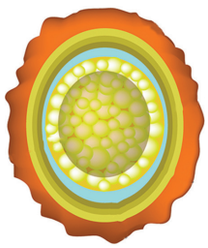
1. A host in which a parasite undergoes asexual reproduction is _____.
   a. Definitive host
   b. Intermediate host
   c. Reservoir host
   d. perfect host
2. Which of the following statement is true concerning Entamoeba histolytica.
   a. It has no cystic stage
   b. It is non – pathogenic
   c. It is not transmitted through faecal – oral route
   d. Trophozoites live in large intestine of human
3. Animals that are routinely infected with a protozoan or parasite are termed as _____.
   a. Definitive b. Intermediate
   c. Reservoir d. Parasite
4. The schizonts enter which body part?
   a. Blood stream b. Spleen
   c. Mouth d. Liver
5. Leishmania organism are transmitted to human by _____
   a. Sandflies b. tsetse files
   c. Mosquitoes d. Reduviid bug
6. The _____ parasite is capable of producing disease in an immune deficient host.
   a. Entamoeba histolytica
   b. Toxoplasma gondii
   c. Ascaris
   d. Taenia
7. Which of the following parasitic infection can lead to malabsorption of fat.
   a. Amoebiasis
   b. Ascariasis
   b. Hookworm infection
   d. Giardiasis
8. Flask shaped ulcers in human intestine are related to _____.
   a. Giardiasis b. Amoebiasis
   c. Leishmanian d. Chaga's disease
9. This disease was first observed by Leeuwenhoek often he discovered parasitic organisms in his stool under the microscope.
   a. Chaga's diease b. Gardiasis
   c. Malaria d. Ascariasis
10. The common name for A. *lumbricoides* is round worm.
    a. Round worm b. Pin worm
    c. Tape worm d. Whip worm

Answer the following

1. Which laboratory findings are diagnostic for leishmaniasis?
2. How is amoebiasis diagnosed.
3. Following ingestion, what is the life cycle of E.histolytica?
4. What is the clinical spectrum of amoebiasis?
5. What is the role of microscopy in the diagnosis of amoebiasis.
6. What is ALA?
7. How Female Ascaris worm is differentiable from male worm?

TN_GOVT_XII_Micro_Biology_CH08.indd 182 07-12-2021 15:22:22

8. Why do some parasites need definitive and intermediate hosts rather than just one host to complete its life-cycle.
9. What is the difference between reservoir and paratenic hosts?
10. Why is the mosquito a definitive host in malaria?
11. A fecal sample was subjected to Saturated salt flotation from a 14 year old boy according to doctor's advice and check-up. The results of this test are shown under the microscope. Identify the parasite egg and comment on it.



12. With neat diagram describe the trophozoite of *Giardia*.
13. Explain the erythrocytic stage of Plasmodium falciparum.
14. What complication arises due to inadequately treated patient with quinine suffering from malignant malaria?
15. Describe the life-cycle of large roundworm which grows to a length of up to 40cm that infects humans.
16. For which parasite mosquito acts as definitive and intermediate host?
17. Fill in the blanks in the column given below.

Human Nematode Infection			
Parasite	Acquired by	Site in humans	Transmitted through
Ascaris lumbricoides	Ingestion of eggs	_____	Person to person

TN_GOVT_XII_Micro_Biology_CH08.indd 183 07-12-2021 15:22:22

Chapter 9

Medical Mycology

Learning Objectives

After studying this chapter the students will be able to,

- *Identify the pathogenic fungi most commonly causing disease by using advanced techniques. Fungal infection is common in developing countries so we should be aware of prevention and treatment of fungal infections.*
- *Study the taxonomy, structure and classification of medically important fungi.*
- *Study mycosis, its pathogenesis, clinical feature, treatment and prophylaxis.*
- *Study about the collection, processing of the sample and molecular diagnosis of fungal infections.*

Chapter Outline

9.1 Classification of fungi based on the Host parasitic Relationship

9.2 Superficial Cutaneous Mycosis

9.3 Subcutaneous Mycoses

9.4 Systemic Mycosis

9.5 Opportunistic Mycosis

The branch of biology that deals with the study of fungi is known as **"Mycology"**. The term is derived from Greek work **'Mykes'** means **mushroom** and **'Logos'** means **study**. Medical Mycology is the study of fungal infection, epidemiology, ecology, pathogenesis, diagnosis and treatment in human beings**. Raymond Jacques Sabouraud (1864–1936)** is the father of Medical Mycology.

9.1 Classification of Fungi based on the Host Parasitic Relationship

Based on the host parasitic relationship the fungi are grouped into three types.

a. Commensalism: The fungus neither gets benefit nor harmed by the host parasitic relationship.

b. Mutualism: The fungus benefited from the host parasitic relationship.

c. Parasitism: The host is harmed by the fungus in host parasitic relationship.

9.1.1 Mycoses

Diseases caused by the medically important fungi are called Mycoses. Based on their wide spectrum of adaptability, fungi causing human mycoses can be categorized into:

TN_GOVT_XII_Micro_Biology_CH09.indd 184 07-12-2021 15:25:48

a. **Pathogenic fungi:** The ability of the fungi to adapt to skin flora and cause infection.

b. **Opportunistic fungi:** When the immune status of the host is reduced, fungi will induce or cause infection.

c. **Toxigenic fungi:** Toxins produced by fungi are responsible for the illness or death of patients after ingestion of the contaminated food.

d. **Allergenic fungi:** Allergens are secreted by the fungi which cause allergic reaction in the human beings.

Mycoses are classified according to the specific site of involvement.

a. **Superficial Mycoses:** The infection is limited to the outer most layers of the skin and its appendages. Example: Malassezia and Piedra infection

b. **Cutaneous Mycoses:** The infection extends deeper into the epidermis and it also invades hair and nails. Example: Dermatophytoses.

c. **Sub cutaneous Mycoses:** The infection extends to dermis, subcutaneous tissue and muscles by any traumatic injury. Example: Mycetoma

d. **Systemic Mycoses**: The infection originates from lungs and later spreads systemically to other organs. Systemic mycoses along with the opportunistic fungal infection are known as deep mycoses. Example: Cryptococcosis

e. **Opportunistic Mycoses:** The infection occurs when the immune status of the individuals is altered. It is common among immune compromised and immune suppressed patients. Example: Candidiasis

Aeromycology

The Aeromycology is the study of air borne fungi, its types and the seasonal variations of allergenic fungal spores in the environment.

There are certain fungal pathogens which cause infections associated with workers in mycological laboratories. To avoid this safety procedures and equipments safety levels or bio safety levels (BSL) are used. BSL - 1 is used for low - risk microorganisms and BSL - 4 is used for highly risk pathogens.

Infobits

Medical Mycology in India

In India, the fungal infections are known since the ancient civilization mentioned in **Aryan documents such as Atharva Veda** Mycetoma is described as Padavamikam meaning ant hillfoot this was observed by John Gill in Madurai district of Tamil Nadu in 1842 which was designated as '**Madura foot**'.

9.1.2 Characteristics of Fungi

Fungi are heterotrophic organisms that exist as saprophytes, commensal or parasites. They are found on decaying vegetative matter and also in soil. Morphological features, cell structure, reproduction, nutritional requirement and thermal dimorphism in the pathogenic fungi are described as follows:

i. Morphological Features

Fungi are eukaryotic with well defined cell wall and intra cellular membrane bound organelles. The cell wall is composed of polysaccharides and chitin. Fungi vary in

TN_GOVT_XII_Micro_Biology_CH09.indd 185 07-12-2021 15:25:48

size and shape. They are broadly divided into two main groups.

a. **Yeasts:** The yeasts are unicellular organisms which reproduce by asexual process known as **budding** or by **fission**. The cell develops a protuberance that enlarges and separates from the parental cell. The yeasts produce chains of elongated cells known as **Pseudohyphae**. Some yeasts reproduce by sexual process Example: *Cryptococcus neoformans*. Germ tube is special morphology found in *Candida albicans*. Some are commensal without any medical significance.

b. **Molds:** The molds grow by apical extension, forming an interwoven mass called as **Mycelium,** branching filaments known as **hyphae**. Hyphae that grow on the surface are called **vegetative hyphae**. They are responsible for the absorption of nutrients. The hyphae that project above the surface are called **aerial hyphae** and they produce specialized reproductive structures called as **conidia**.

HOTS

Can you cultivate the molds at home?

Depending on cell morphology fungi are divided into four types, they are **Yeasts:** These are unicellular organisms that divide by budding (Figure 9.1a & b). Example: *Cryptococcus neoformans* (Pathogenic), *Saccharomyces cerevisiae* (Non pathogenic). **Yeast – like fungi:** These fungi reproduce by budding but fails to separate and hence elongation takes place forming pseudohyphae. Example: *Candida* species (Pathogenic). **Molds:** These fungi produce spores which germinate to form vegetative hyphae (Figure 9.2). Example: Dermatophytes, *Aspergillus, Penicillium, Mucor*. **Dimorphic fungi:** These Fungi exist in both yeast at 37°C and filamentous form at 25°C. This Phenomenon is known as **Fungal dimorphism** (Figure 9.3). Example: *Histoplasma capsulatum*, *Blastomyces dermatitidis*.

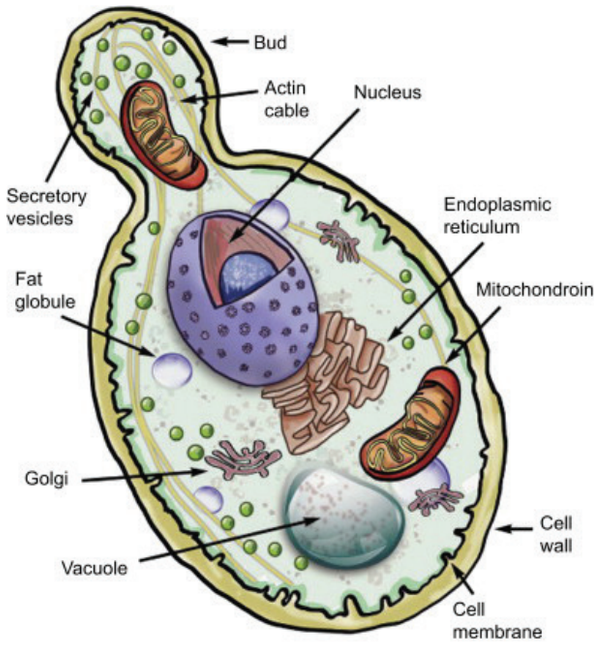
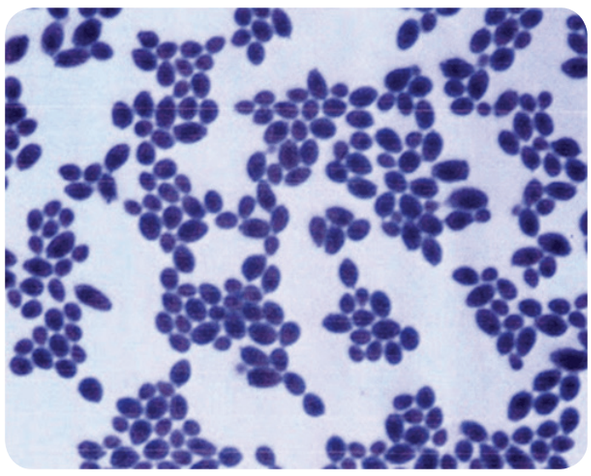


Figure 9.1: (a) Morphology of yeast



(b) Microscopic view of yeast

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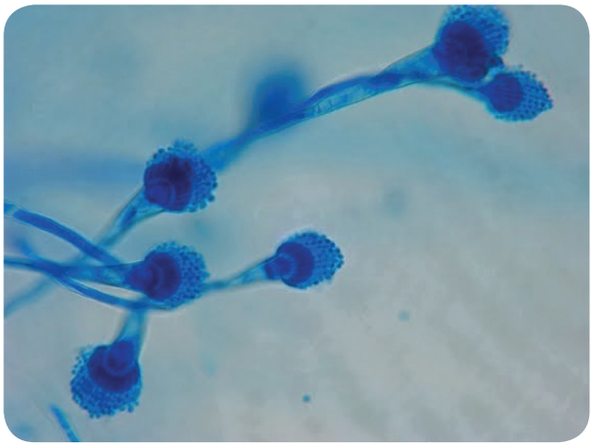

Figure 9.2: Microscopic view of yeast moulds

Phaeoid fungi: Most of true pathogenic fungi are dimorphic fungi which are composed of darkly coloured hyphal form known as **dematiaceous fungi**. Some are yeast like and also known as black yeasts.

Vegetative Structures: Several structures are formed by the vegetative mycelia that have no reproductive value but are important for the differentiation of fungi eg. Chlamydospores and Arthrospores. Chlamydospores are thick walled, resistant to adverse conditions and are larger than other cells. Arthrospores are rectangular spores which are thick walled that are disposed on maturity.

ii. Cell structure

a. Capsule: Fungi produce an extra cellular polysaccharide in the form of capsule. Example: *Cryptococcus*.

b. Cell wall: Fungi possess a multilayered rigid cell wall exterior to the plasma lemma. The cell wall is made up of chitin, a water insoluble, homopolymer of N-acetyl glucosamine. Chitin synthase is responsible for the bio synthesis of chitin.

c. Plasmalemma: Cytoplasmic membrane or plasmalemma encloses complex cytosol. It is composed of glycoprotein, lipids and ergosterol.

d. Cytosol: Cytosol comprises of mitochondria, microtubules, ribosomes, golgi apparatus, double membrane endoplasmic reticulum and Nucleus. The nuclei of the fungi are enclosed by a membrane and contain most of cellular DNA.

iii. Reproduction of fungi

Spores play a major role in reproduction. There may be asexual or sexual cell divisions.

a. Asexual Reproduction: The asexual reproduction involves, budding or fission or mitosis. Fungi produce more than one type of asexual spores. They are microspores (microconidia) and macrospores (macroconidia). Spores that are present inside sporangium are

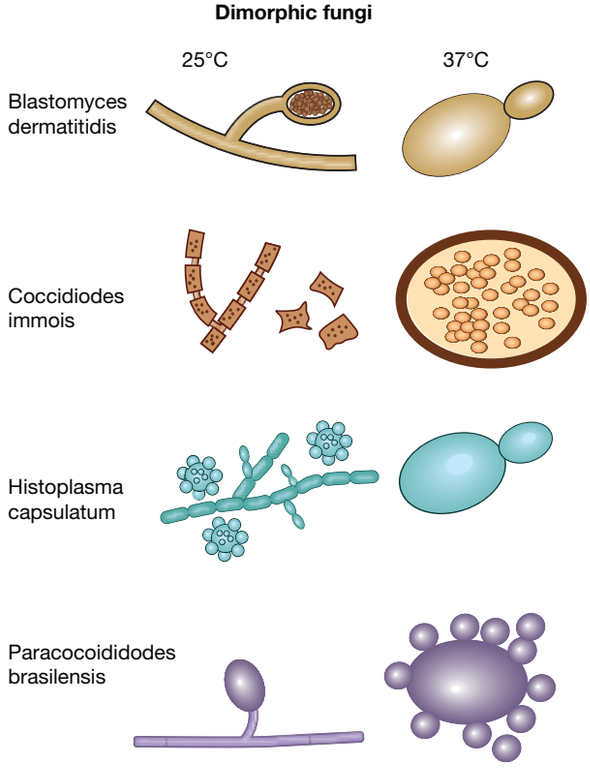

Figure 9.3: Dimorphic Fungi

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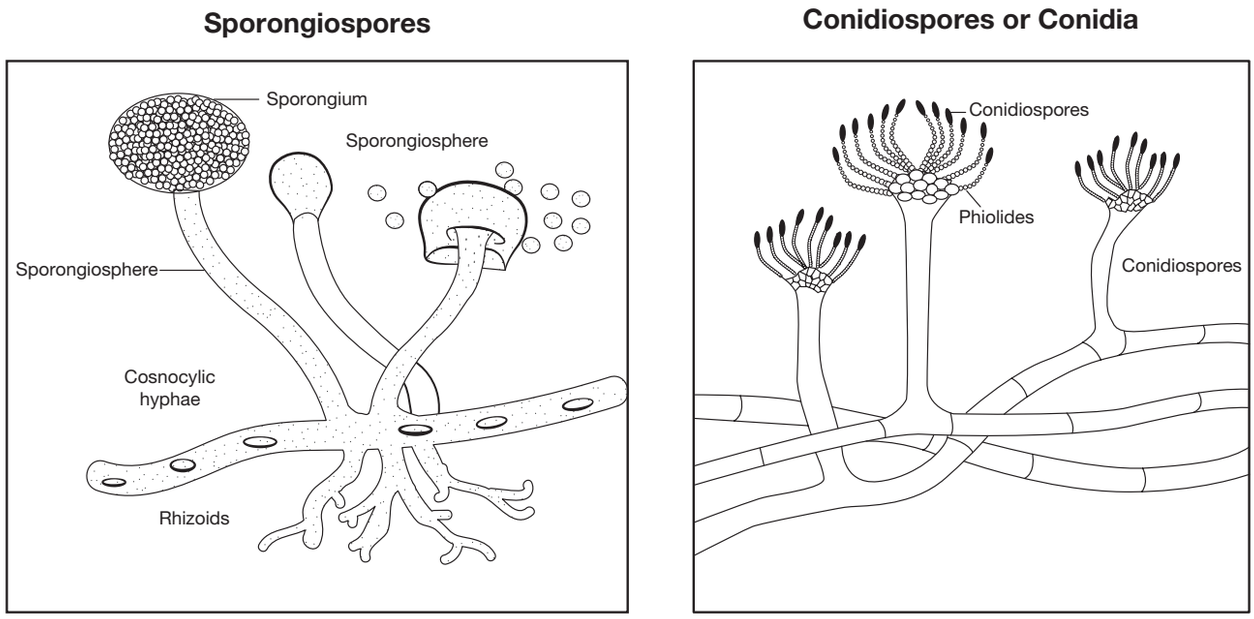

Figure 9.4: Asexual Spores of fungi

known as **sporangiospores** and those that are borne exogenously are called **conidiospores** (Figure 9.4)**.** Based on the arrangement of conidia they are classified as Acropetal, Basipetal and Sympodial.

b. Sexual Reproduction: The process of sexual reproduction typically consists of plasmogamy (cytoplasmic fusion), Karyogamy (union of two nuclei) and meiosis (haploid formation). Anamorphs and Telomorphs are the 2 phases of sexual reproduction

c. Mycelia Sterile: Mycelia sterile are fast growing molds that do not produce spores or conidia. They are medically significant fungi and are difficult to identify

iv. Growth and nutrition

Fungi are ubiquitous in nature and grow readily in the presence of nitrogen and carbohydrates. Medically significant fungi are Mesophilic. The optimum temperature *invitro* for majority of the pathogenic fungi is between 25°C and 37°C. The fungi prefer acidic pH; do not require light for their growth. All fungi are heterotrophs requiring organic nutrients. They absorb their nutrient and do not ingest food. Medically significant fungi are facultative parasites, capable of causing disease or living on dead organic matter.

9.2 Superficial Cutaneous Mycoses

The superficial cutaneous fungal infections involve the outer most layers of skin and its appendages like hair and nails. The causative agents colonize on epidermis or supra - follicular portions of hair and do not penetrate into deeper layers.

The genus ***Malassezia*** is responsible for the superficial infection of the skin. *Malassezia furfur* is lipophilic yeast. It is a commensal of normal skin in the sebaceous glands of warm - blooded vertebrates. It may be pathogenic under certain conditions usually causing skin

TN_GOVT_XII_Micro_Biology_CH09.indd 188 07-12-2021 15:25:52

conditions like Pityriasis versicolor, Seborrheic dermatitis, Atopic dermatitis, Malassezia folliculitis and systemic infection. Symptoms include macular, erythematous, hyper pigmented or hypo pigmented lesions with fine scaling.

Tinea nigra is responsible for the superficial cutaneous infection of the skin. *Hortaea werneckii* is the phaeoid (dematiaceous) fungi causes infection on the palms and soles. It is also commonly termed as *Tinea nigra palmaris* and *Tinea nigra plantaris*. Symptoms includes brown to black deeply pigmented non - scaly, macular lesions affecting skin of the palms and occasionally soles.

Piedra causes superficial infection of hair shaft. The word **Piedra** is derived from Spanish word **Stone**. There are two types of Piedra based on causative fungi and characteristics of nodules. They are **Black piedra** caused by *Piedraia hortae* and **White piedra** caused by *Trichosporon* species. The symptoms include development of firm, irregular nodules of fungal elements cemented to the hair. The piedra can be distinguished on the basis of shape, size and pigmentation of fungal cells of nodules which are found around hair cortex.

9.3 Cutaneous Mycoses

Dermatophytoses are the most common cutaneous fungal infection seen in man and animals affecting skin, hair and nails. The fungi can invade the keratinized tissues of skin and its appendages and they are collectively known as **Dermatophytes** or **Tinea** or **ring worm** infection. The dermatophytes are hyaline septate molds. They are divided into three main anamorphic genera depending on their morphological characteristics.

i. ***Trichophyton*** [Cause infection in skin, hair and nails]

ii. ***Microsporum*** [Cause infection in skin and hair]

iii. ***Epidermophyton*** [cause infection in skin and nail]

The fungal species affecting humans are known as **anthropophilic**. Those inhabitating domestic and wild animals as well as birds are called **zoophilic**. Fungi species from soil are known as **geophilic** dermatophytes.

HOTS

What are the sources of dermatophytes?

9.3.1 Pathogenesis and Pathology

The dermatophytes grow within dead keratinized tissue and produce keratinolytic proteases, which provide means of entry into living cells. Fungal metabolic products cause erythema, vesicles and pustule on the site of infection. Some dermatophytes species like soil saprobes digest the keratinaceous debris in soil and are capable of parasitizing keratinous tissues of animals.

9.3.2 Clinical Features

The clinical manifestations of Dermatophytoses are also called **Tinea or Ringworm** depending on the anatomical site involved. Following are the common clinical conditions produced by dermatophytes:

TN_GOVT_XII_Micro_Biology_CH09.indd 189 07-12-2021 15:25:52

1. **Tinea Capitis:** This is an infection of the **shaft of scalp hairs**. It can be inflammatory (eg. Kerion, Favus) or non - inflammatory (Black dot, Seborrheic dermatitis). The infected hairs appear dull and grey (Figure 9.5a). Breakage of hair at follicular orifice which creates patches of alopecia with black dots of broken hair. It is caused by *Trichophyton* species.
2. **Tinea Corporis:** This is an infection on the **glabrous (non - hairy) skin** of body. Erythematous scaly lesions with sharply marginated raised border appear on the infected areas (Figure 9.5b). It is caused by *Trichophyton rubrum*.
3. **Tinea Imbricata:** It forms concentric rings of scaling on the **glabrous skin**, leading to lichenification. It is caused by *Trichophyton concentricum*
4. **Tinea Gladiatorum:** This infection is common among wrestlers and athletes. Lesions are seen on **arms, trunk or head and neck**. It is caused by *Trichophyton tonsurans*.
5. **Tinea Incognito:** It is steroid modified Tinea caused as a result of misuse of corticosteroids in combination with topical antimycotic drugs.
6. **Tinea Faciei:** This is an infection of **skin of face** except beard. Erythematous annular plaques are formed. It is one of the forms of Tinea incognito.
7. **Tinea Barbae:** This is the infection of the **beard and moustache** areas of the face. This is also called **barber's itch.** It is caused by *Trichophyton mentagrophytes*, *Trichophyton rubrum* and *Microsporum canis*. Erythematous patches on the face with scaling appear and these develop folliculitis.
8. **Tinea Pedis:** This is an infection of the **foot, toes and interdigital web spaces**. This is seen among the individuals wearing shoes for long hours and known as **Athlete's foot** (Figure 9.5c). Erythema and scaling associated with itching and burning sensation appear with thin fluid discharging from small vesicles. It is caused by *Trichophyton mentagrophytes*, *Trichophyton rubrum* and *Epidermophyton floccosum*.
9. **Tinea Cruris:** This is an **infection of the groin** in men who use long term tight fitting garments. Erythematous



(a) Tinea Capitis



(b) Tinea Corporis



(c) Tinea Pedis

Figure 9.5: Clinical conditions of Dermatophytes

TN_GOVT_XII_Micro_Biology_CH09.indd 190 07-12-2021 15:25:53

sharp margin lesions known as Jock itch. It is caused by *Trichophyton rubrum* and *Epidermophyton floccosum*.

10. **Tinea Manuum:** This is an infection of the **skin of palmar** aspect of hands. It causes hyperkeratosis of the palms and fingers. It is caused by *Trichophyton mentagrophytes*, *Trichophyton rubrum* and *Epidermophyton floccosum*.
11. **Tinea Unguium:** This is an infection of the **nail plates**. The infection spreads on the entire nail plate infecting the nail bed. It results in opaque, chalky or yellowish thick ended nail. It is caused by *Trichophyton mentagrophytes*, *Trichophyton rubrum* and *Epidermophyton floccosum*.

Figure 9.6 shows the microscopic view of major determatophytes

Infobits

How do dermatophytes cause disease in humans?

Dermatophytosis is a common contagious **disease caused** by fungi **known as dermatophytes**. **Dermatophytes** belong to a group of **organisms** that are able to break down the keratin in tissues such as the epidermis, hair, nails, feathers, horns and hooves.

9.3.3 Laboratory Diagnosis

i. Samples

Skin scrapings, hair and nail samples were collected

a. Direct Examination

Samples are subjected to KOH (10%) wet mount, the affected site were disinfected with alcohol before collecting the clinical specimen.

b. Fungal culture

The samples are inoculated on Sabouraud dextrose agar (SDA) with antibiotics and cycloheximide and are incubated at 25°C–35°C. The colony morphology can be identified.

The three genera of dermatophytes are *Trichophyton*, *Microsporum* and *Epidermophyton* (Table 9.1). They are identified based on morphology of the macro conidia, micro conidia, their shape, position on the spore bearing hyphae such as spiral hypha, racquet hypha, nodular pectinate body.

ii. Special Techniques

1. Wood's Lamp Examination

Clinical samples are exposed to Wood's lamp. Wood's glass consists of Barium silicate containing 9% Nickel oxide. It transmits long wave ultra violet light with a peak of 365nm that shows a characteristic fluorescence produced by the samples. The patterns of fluorescence are bright green, golden yellow and coral red. *Microsporum* species and *Trichophyton* species are differentiated using this technique.

2. Hair brush sampling Technique

It involves brushing the scalp with a sterile plastic hair brush, which is then inoculated into an appropriate culture medium by plates, is incubated at 25°C–35°C. The colony morphology can be identified.

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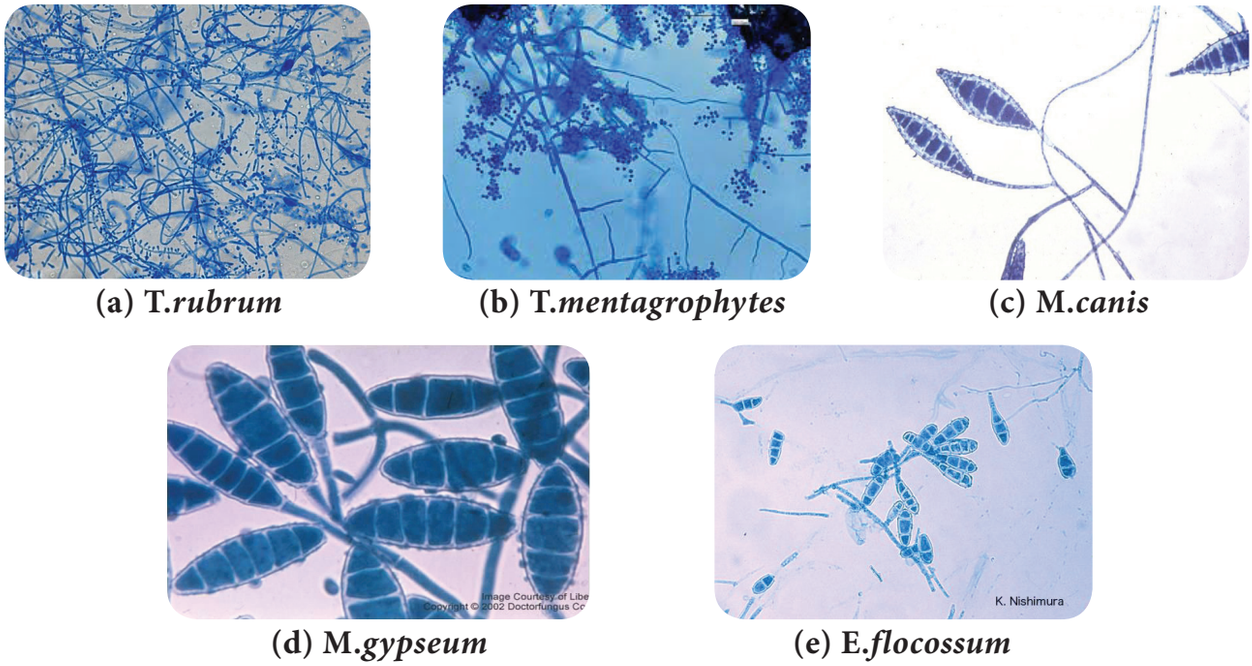
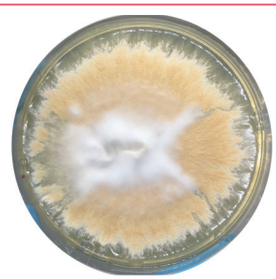


(a) T.*rubrum* (b) T.*mentagrophytes* (c) M.*canis*

(d) M.*gypseum* (e) E.*flocossum*

Figure 9.6: LPCB wet mount of major Dermatophytes

Table 9.1: Microscopic and macroscopic characteristics of Dermatophytes.

S.No	Dermatophytes	Macro conidia	Micro conidia	Macroscopic Morphology – SDA
1.	*Trichophyton*	Rare, thin-walled, smooth	Abundant	
2.	*Microsporum*	Numerous, thick-walled, rough	Rare	
3.	*Epidermophyton*	Numerous, smooth-walled	Absent	

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Dermatophyte infections, also known as tinea, are the most common fungal **infections** of the skin, hair, and nails. The term "**dermatophyte**" refers to fungal species that infect keratinized tissue, and includes members of the *Trichophyton, Microsporum*, and *Epidermophyton* genera

3. Hair perforation Test

It is used to differentiate *T. mentagrophytes and T. rubrum*. Wedge-shaped perforations in the hair shaft are observed in hair infected with *T. mentagrophytes*.

4. Urease Test

It is used to differentiate between *T. mentagrophytes* and *T. rubrum*. *T. mentagrophytes* hydrolyzes urea and becomes deep red, showing positive result.

iii. Treatment

Whitfield's ointment is used for all Tinea infections. Oral griseofulvin is the drug of choice for nails and scalp infections. Itraconazole and terbinafine may be given as pulse therapy.

9.4 Subcutaneous Mycoses

The fungal infections are characterized by development of lesions at the site of infection by the traumatic inoculation in the subcutaneous tissues. Examples are Mycetoma, Sporotrichosis, Chromoblastomycosis and Rhinosporidiosis.

9.4.1 Mycetoma

Mycetoma is a slowly progressive, chronic granulomatous infection of skin and subcutaneous tissues with involvement of under lying fasciae and bones usually affecting the extremities. Mycetoma is commonly called **Madura foot or Maduramycosis** (Figure 9.7). They are classified into two categories, namely **eumycetoma** cased by **fungi** and **actinomycetoma** caused by higher bacteria of class **actinomycets**.

9.4.2 Pathogenesis and Pathology

The causative agent of Mycetoma is commonly present in saprobic soil source and is transmitted by accidental trauma by thorns or by injury into the subcutaneous tissue. It is common among farmers with minor trauma and abrasions of the skin. Use of wicks for removal of earwax is responsible for Mycetoma of the ear.

HOTS

Is mycetoma occupational disease?



Figure 9.7: Madura foot

TN_GOVT_XII_Micro_Biology_CH09.indd 193 07-12-2021 15:25:58

9.4.3 Classification of Mycetoma

Mycetoma is classified on the basis of the causative agent. **Aerobic actinomycetes** causes **actinomycetoma** whereas **hyaline and phaeoid fungi** cause **eumycetoma.**

9.4.4 Clinical Features

The clinical entity depends upon the age of the lesions and to size, shape and color of the grains. The painless localized swollen lesions with purulent fluid lead to the secondary bacterial infections. Important features of Mycetoma are as follows:

i. Tumor like swelling
ii. Multiple draining sinuses
iii. Presence of grains or granules in sinuses.

9.4.5 Laboratory Diagnosis

i. Samples

The clinical samples collected in Mycetoma is usually grains, pus exudates or biopsy.

a. Direct Examination

Grams staining, modified Ziehl – Neelson staining, LPCB and KOH wet mount are used to visualize the organisms.

The grains should be washed, crushed and cultured on different media. Crushed grains are examined (Figure 9.8a).

KOH mount

Eumycotic grains show thick 2–6 µm hyphae with large globose swollen cells with or without chlamydospores. Actinomycotic grains show thin filaments of 0.5–1 µm with coccoid or bacillary forms.

Gram stain

Actinomycetoma grains show Gram-positive branching filamentous bacteria with branches (Figure 9.8b).

Ziehl - Neelson stain

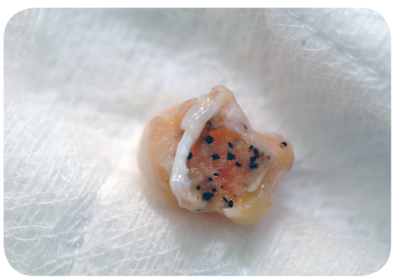
Nocardia species show red pink acid fast filamentous bacteria.

b. Culture

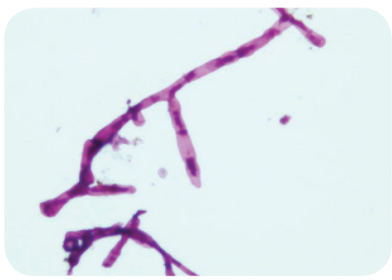
Crushed grains are washed several times with normal saline without antibiotics and inoculated on to **Sabouraud dextrose agar, blood agar, Lowenstein -Jensen** media and brain-heart infusion agar. The plates are incubated at 25°C, 37°C and 44°C for various organisms (Figure 9.8c).

ii. Treatment

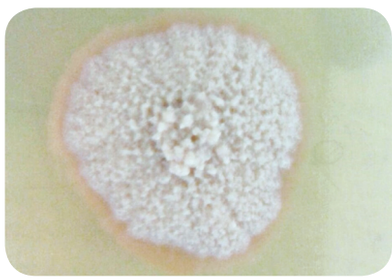
1. Ketoconazole 200 mg and Itraconazole 100mg are given for 8–24 months to treat eumycetoma.
2. Sulfonamides, tetracylines, streptomycin, amoxicillin are administered to treat actinomycetoma.



(a) Biopsy-Black grains



(b) Microscopic Morphology



(c) Macroscopic Morphology

Figure 9.8: Laboratory Diagnosis of Mycetoma

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9.5 Systemic Mycoses

Systemic mycoses are caused by dimorphic fungi; these infections are acquired by inhalation of spores. These primarily involve the respiratory system and are self-limiting and asymptomatic. If symptomatic, it spreads to other parts of body through circulation. These infections are caused by **true fungal pathogens**. Systemic and opportunistic infections together cause **Deep mycoses**.

The organisms have a mycelial form when grown on fungal culture and have yeast form in the tissue. The examples of systemic mycoses are Histoplasmosis, Blastomycosis.

9.5.1 Histoplasmosis

Histoplasmosis is caused by dimorphic fungus ***Histoplasma capsulatum***. The fungi live inside the cells of the reticuloendothelial system, where they grow within macrophages and giant cells. This infection is also known as **Darling's disease**.

9.5.2 Pathogenesis and Pathology

The infection with H. *capsulatum* develops when conidia or mycelial fragments are inhaled and converted into yeasts in alveolar macrophages in the lungs. The oval yeast cells parasitize macrophages, which are activated by T lymphocytes resulting in localized granulomatous inflammation.

HOTS

H. *capsulatum* is dimorphic fungi - justify

9.5.3 Clinical Features

The disease is mostly asymptomatic. The development of symptom or symptomatic disease appears to depend on the intensity of exposure to conidia and cellular immune response of the host. The disease may be classified as follows.

1. Acute pulmonary Histoplasmosis – Fever, headache, chills, sweating, chest pain, cough and dyspnoea
2. Chronic pulmonary Histoplasmosis – Ulcerative lesions of the lips, mouth, nose, larynx and intestines
3. Cutaneous, mucocutaneous Histoplasmosis – Mucous lesions on skin, abdomen wall and thorax.
4. Disseminated Histoplasmosis – Fever, anoxia, anemia, leucopenia constant hepatosplenomegaly and multiple lymphadenopathies.

9.5.4 Laboratory Diagnosis

i. Samples

Specimens collected are sputum, bone marrow and lymph nodes, cutaneous and mucosal lesions and peripheral blood film.

a. Direct Examination

Thick and thin smears should be prepared from peripheral blood, bone marrow and stained with Calcofluor white, Giemsa or Wright stains.

The fungus is small, oval yeast like cells, 2–4 μm in diameter, within the mononuclear or polymorpho nuclear cells and occasionally in giant cells.

b. Fungal culture

The clinical samples is inoculated on Sabouraud dextrose agar (SDA) and

TN_GOVT_XII_Micro_Biology_CH09.indd 195 07-12-2021 15:25:59

Brain-heart infusion (BHI) agar with antibiotics and actidione at 25°C and 37°C. On Sabourad dextrose agar the colonies appear albino or brown. The albino type consists of white, fine aerial hyphae and brown type consists of flat colonies with light tan or dark brown in color in seven days. At 37°C the colonies grow as granular to rough, mucoid and cream-colored turning tan to brown in 14 days.

ii. Treatment

Amphotericin B is given for the treatment of disseminated and other severe forms of Histoplasmosis.

9.6 Opportunistic Mycoses

The opportunistic systemic mycoses are infections found in patients with underlying pre disposing conditions. It is produced by non pathogenic or contaminant fungi in a host, where the immunological defense mechanisms are weakened by endogenous causes like cancer, leukemia or exogenous causes like immunosuppressive therapy and AIDS. The examples of opportunistic mycoses are Candidiasis, Cryptococcosis, Aspergillosis and zygomycosis.

9.6.1 Candidiasis

Candidiasis is the commonest fungal disease found in humans affecting **mucosa, skin, nails and internal organs** of the body. It is caused by yeast like fungi called *Candida albicans*. The infection may be acute or chronic, superficial or deep and found mainly as secondary infection in individuals with immune compromised condition.



The **fungus *candida albicans*** is responsible for most **vaginal** yeast infections. Your vagina naturally contains a balanced mix of yeast, including candida, and bacteria. Certain bacteria (lactobacillus) act to prevent an **overgrowth** of yeast. But that balance can be disrupted.

Pathogenesis and Pathology

Some of the virulence factors contributing to pathogenicity are toxins, enzymes and adhesion. The organism adheres to the epithelial and endothelial cells by proteinase production. Then the yeast cells of *Candida* encounter a particular host tissue and colonization takes place at the local site or they invade deeper into the host tissue and induce various clinical symptoms.

Clinical Features

The *Candida* species are found as commensal on mucosal surfaces of the body. They cause disease as and when conditions are favourable. This yeast like fungi colonizes mucocutaneous surfaces, which can be portals of entry into deeper tissues when the host defenses are compromised. They may cause a simple lesion to event the life threatening systemic infection.

The clinical manifestations of Candidiasis are divided into two broad categories. They are:

1. Infectious Diseases

a. Mucocutaneous Involvement

i. Oral Candidiasis – Most common form of *Candida* colonizes on the oral cavity.

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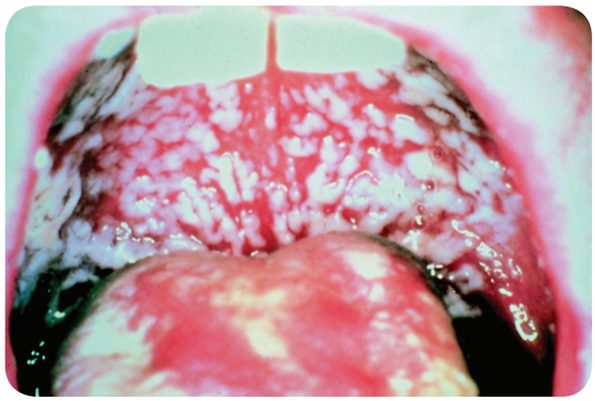

Figure 9.9: Oral candidiasis

Oral thrush is infection of the buccal mucosa, gums, tongue. Reddening of the mucous membrane gives dry, smooth metallic taste and burning at the local site (Figure 9.9).

ii. Alimentary Candidiasis – *Candida* colonizes on the oesophagus causing oesophagitis. It is mostly asymptomatic or it may cause burning pain in the epigastrium or throat.

b. Cutaneous Dermatitis

i. Diaper Dermatitis – *Candida* that colonize on the cutaneous layer causes cutaneous Candidiasis, leading to maculopapules vesicles with erythematous rash. This is common among infants and known as **Diaper rash**.

ii. Intertrigo – This is an **i**nflammatory lesion of the skin folds due to candidal infection.

c. Systemic Involvement

The *Candida* colonizes in various organs and causes various manifestations through the blood stream. Clinical features are found to be Urinary tract C**andidiasis, Candiduria, Endocarditis, Pulmonary Candidiasis, Arthritis, Osteomyelitis, Meningitis, Candidemia** and **Septicemia.**

2. Allergic Diseases

Allergic manifestation is caused due to the metabolites of *Candida*. The cutaneous allergies are **urticaria** and **eczema**, and **bronchial asthma**.

Laboratory Diagnosis

i. Samples

Specimens collected are mucous membrane from the mouth, vagina, skin and sputum based on the site of involvement.

a. Direct Examination

Gram staining LPCB, and KOH wet mount are used to visualize the yeast cells.

Presence of yeast cells approximately 4.8 µm with budding and pseudo hyphae are observed. Other stains like periodic acid - Schiff stain and Gomori's methylamine silver stain are also used to observe the fungal elements in tissue.

b. Fungal culture

The clinical specimens can be cultured on Sabouraud dextrose agar (SDA) with antibiotics and incubated at 25°C and 37°C (Figure 9.10). The colonies appear in 3–4 days as cream coloured, smooth and pasty.

Some of the species of *Candida* are *Candida albicans, Candida tropicalis, Candida krusei* and *Candida glabrata*.

ii. Special Test

Germ tube test

The culture of Candida species is treated with sheep or normal human serum and inoculated at 37°C for 2 to 4 hours. A drop of suspension is examined on the slide. The germ tubes are seen as long tube–like projections extending from

TN_GOVT_XII_Micro_Biology_CH09.indd 197 07-12-2021 15:25:59



Figure 9.10: Candida **(a)** Macroscopic Morphology **(b)** Microscopic Morphology **(c)** Germ tube

the yeast cells. The demonstration of the germ tube is known as Reynolds – Braude phenomenon.

Biochemical tests

Sugar fermentation and assimilation tests are used for the identification of Candidal species. C.*albicans* ferments Glucose and Maltose and assimilates Glucose, Maltose, Sucrose, Lactose and Galactose.

Chlamydospores formation

Candida isolates are grown on corn meal, agar (CHN) or rice starch agar (RSA) and incubated at 25°C for 2–3 days. The formation of large, thick walled terminal chlamydospores is demonstrated in C.*albicans* and C. *dubliniensis*.

iii. Treatment

1. 1% gentian violet is locally applied to the affected areas.
2. The azole creams like Clotrimazole, Miconazole, Ketoconazole and Econazole are also used.

9.6.2 Cryptococcosis

Cryptococcosis is an acute, sub acute or chronic fungal disease caused by encapsulated yeast called *Cryptococcus neoformans*. It is pathogenic to man and animals. It causes opportunistic infection, involving the lungs and disseminates to extra pulmonary sites through circulation to different body organs particularly to central nervous system causing **Meningoencephalitis**.

Infobits

What does Cryptococcus cause?

Meningitis can be caused by different germs, including bacteria, **fungi**, and viruses. Two types of fungus can cause **cryptococcal meningitis** (CM). They are called Cryptococcus neoformans (C. *neoformans*) and Cryptococcus gattii (C. *gattii*). This disease is rare in healthy people.

Pathogenesis and Pathology

Cryptococcal infection occurs through inhalation of small forms or basidiospores. The fungus may remain dormant in the lungs until the immune system weakens and then can disseminate to the central nervous system and other body sites.

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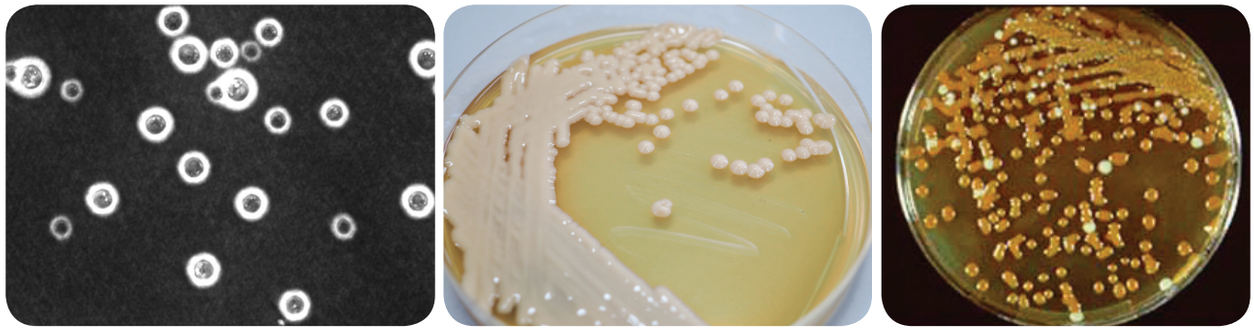

Figure 9.11: Cryptococcus **(a)** capsule demonstration **(b)** On SDA **(c)** On BSA

Clinical Features

The clinical features of Cryptococcosis depend upon the anatomical sites.

i. Pulmonary Cryptococcosis

The respiratory route is usually the portal of entry for propagules in Pulmonary Cryptococcosis that subsequently disseminate to extra pulmonary sites. The symptoms are dry cough, dull chest pain and milder or no fever with small gelatinous granules all over the lungs.

ii. CNS Cryptococcosis

This is an infection of brain and meninges leading to Meningoencephalitis. Nitrogenous source such as asparagines and creatinine present in cerebrospinal fluid enrich the yeast. The symptoms are nausea, dizziness, impaired memory, blurred vision and photophobia. The enlarged granulomatous cerebral lesions are called **cryptococcoma**.

iii. Visceral Cryptococcosis

This infection usually spreads from a primary focus to invade the optic nerve and meninges. Visual loss in patients is due to intra cranial pressure. There are two distinct patterns of visual loss namely; rapid visual loss (within 12 hrs) and slow visual loss (within weeks to months).

Laboratory Diagnosis

i. Samples

Specimens collected are mainly serum, CSF and other body fluids.

a. Direct Examination

10% Nigrosin or India ink staining, Gram staining and LPCB are used to visualize the yeast cell.

Biopsy material is stained with periodic acid - Schiff and Gomoris's methylamine silver stain to observe the fungal cells in the tissue. Round budding yeast cells with a distinct halo gelatinous capsule can be seen (Figure 9.11a). Gram positive budding yeast cells are demonstrated by Gram staining.

b. Fungal Culture

The clinical specimens can be cultured on Sabouraud dextrose agar, Bird Seed agar and incubated at 37°C. The colonies are mucoid, cream to buff - colored in SDA (Figure 9.11b), whereas brown colored due to conversion of the substrate into melanin by Phenoloxidase in BSA (Figure 9.11c).

ii. Treatment

1. Amphotericin B, Flucytosine is given together as induction and maintenance therapy.
2. Fluconazole is also recommended.

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Saccharomyces cerevisiae Fungemia: An Emerging **Infectious** Disease. Saccharomyces cerevisiae is well known in the baking and brewing industry and is also used as a probiotic in humans. However, it is a very uncommon cause of infection in humans.

Summary

This chapter dealt with the general classification of fungi in relationship with the host cells, classification of mycoses and also about the vegetative and reproductive structure, its growth and nutrition. Medically important fungi such as Dermatophytoses, Mycetoma, Histoplasmosis, Candidiasis and Cryptococcosis its pathogenesis, clinical features and laboratory diagnosis were discussed.

Evaluation

Multiple choice questions

1. _____ is the father of Medical Mycology
   a. Pasteur
   b. Raymond Jacques Sabouraud
   c. Robert koch
   d. Anton de Bary
2. _____ is an example for deep mycoses
   a. Systemic b. Opportunistic
   c. Both d. None of these
3. _____ is an example for dimorphic fungi
   a. *Histoplasma* b. *Mucor*
   c. Cryptococcus d. None of these
4. Phaeoid fungi is also called as _____
   a. Black yeast b. White yeast
   c. Moulds d. None of these
5. Tinea Barbae is also called as _____
   a. Athlete's foot b. Onchomycosis
   c. Barber's itch d. None of these
6. Tear shaped micro conidia is found in _____
   a. *T.mentagrophytes* b. *T.rubrum*
   c. *T.vercossum* d. None of these
7. _____ is called as Madura foot
   a. Piedra b. Tinea pedis
   c. Mycetoma d. None of these
8. _____ is known as Darling's disease
   a. Cryptococcosis b. Histoplasmosis
   c. Candidiasis d. None of these
9. Diaper rash is caused by _____
   a. Dermatophytes b. Histoplasmosis
   c. Candidiasis d. None of these
10. Demonstration of _____ is called as Reynolds – Braude phenomenon
    a. Arthrospore b. Chlamydospore
    c. Germ tube d. None of these

Answer the following

1. Define Mycology.
2. What is host parasitic relationship?

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3. What are toxigenic fungi?
4. Discuss about the types of mycoses?
5. Define Aeromycology?
6. What is Pseudohyphae?
7. What are Dimorphic fungi?
8. Brief note on vegetative structure of fungi.
9. Account on reproduction of fungi.
10. Short note on taxonomy of fungi?
11. What are the clinical features of dermatophytes?
12. Define Ringworm infection.
13. Differentiate dermatophytic fungi based on macroscopic and microscopic morphology
14. What is maduramycosis.
15. Short note on classification of mycetoma.
16. Define deep mycoses
17. Elaborate on clinical feature of histoplasmosis.
18. What is Opportunistic mycosis.
19. Discuss on Lab diagnosis of Candidiasis?
20. Define Germtube.
21. What is Cutaneous dermatitis?
22. Note on Meningoencephalitis.
23. What is Capsulated yeast?
24. Brief account on clinical feature of Cryptococcosis.
25. Differentiate between *Candida* and *Cryptococcus*.

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Chapter
10

Medical Virology

Learning Objectives

After studying this chapter the students will be able to,

- *Study the importance of viruses causing disease in humans.*
- *Study the classification, structure and cultivation of Viruses.*
- *Study viruses, their pathogenesis, clinical feature of viral diseases and its treatment and prophylaxis.*
- *Study the methods of collection, and processing of the sample and molecular diagnosis of viral infections.*

Chapter Outline

10.1 Evolutionary Origin of viruses

10.2 Cultivation of Viruses

10.3 Herpes Viruses

10.4 Hepatitis Viruses

10.5 Rabies Virus

10.6 Human Immuno Deficiency Virus

10.7 Arbo Virus



Viruses are **infectious agents** with size ranging from about 20 nm to 300 nm in diameter and contain only **one type of nucleic acid** (RNA or DNA) as their genome. The nucleic acid is encased in a protein shell, which may be surrounded by a lipid membrane. The entire infectious unit is termed as **virion**. Viruses are obligate intracellular parasite, they replicate only in living cells. The study of virus is called as **virology**. **Martinus Beijerinck** is known as the Father of Virology.

10.1 Evolutionary Origin of Viruses

The origin of viruses is not known, but two theories of vital origin can be summarized as follows;

i. Viruses may be **derived from (DNA or RNA)** nucleic acid components of host cells to replicate and evolve independently.

ii. Viruses may be **degenerate forms** of intracellular parasites.

Morphology

Size

Viruses are smaller than bacteria, known as filterable viruses vary widely in size.

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The **largest** among them is the **Pox virus** measuring about 300nm. The **smallest** virus is **Parvo virus** measuring about 20 nm.

Structure and Shape

The virion consists of nucleic acid surrounded by a protein coat, **the capsid**. The capsid with the enclosed **nucleic acids** is known as the nucleo capsid. The capsid is composed of a large number of **capsomers**. The functions of the capsid are to **protect** the **nucleic acid** from the deleterious agents and also to introduce viral genome into host cells by adsorbing readily to cell surfaces (Figure 10.1). Two kinds of symmetry encountered in the virus are **icosahedral** and **helical**. Virions may be **enveloped or non enveloped** (naked). The envelope or outer covering of viruses is derived from the host cell membrane when the progeny virus is released by budding. The envelope is **lipoprotein** in nature. The lipid is of host cell origin while the protein is virus coded. **Protein subunits** may be seen as **projecting spikes** on the surface of the envelope and are known as Peplomers.

Overall shape of the virus particle varies; mostly animal viruses are roughly spherical. Some are irregular and pleomorphic. The rabies virus is **bullet shaped**, Ebola virus is **filamentous** and pox viruses are **brick shaped**.

Chemical Properties

Viral protein determines the **antigenic specificity** of the virus. Some viruses contain small amounts of carbohydrates. Most Viruses do not possess any enzymes but **retro virus** has a unique enzyme, such

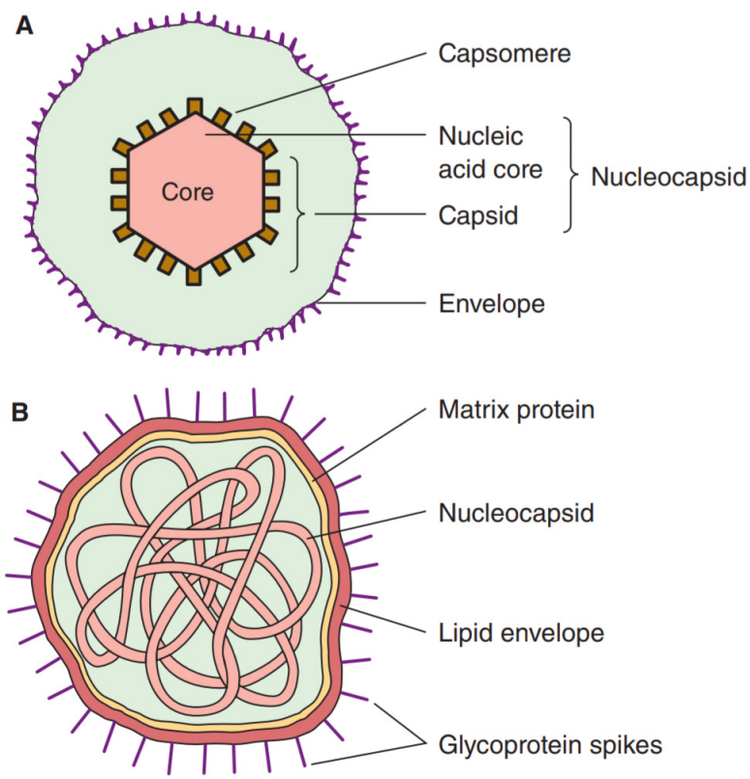

Figure 10.1: Structure of Virus

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as **RNA dependent DNA polymerase or transcriptase** which can synthesise DNA from RNA.

Resistance

Viruses are **inactivated** by sunlight, UV rays and ionizing radiations. The most active **antiviral disinfectants** are oxidizing agents such as hydrogen peroxide, potassium permanganate and hypochlorites. Organic iodine compounds are actively **virucidal**. Chlorination of drinking water kills most viruses but its efficacy is influenced by the presence of organic matter. Some viruses such as hepatitis virus, polio viruses are relatively resistant to chlorination.



Antibiotics active against bacteria are completely ineffective against viruses.

Viral Multiplication

The genetic information necessary for viral replication is contained in the viral nucleic acid, and also depends on the synthetic machinery of the host cell for replication. The Viral replication cycle can be divided into **six steps** and they are as follows, **1. Adsorption or attachment, 2. Penetration, 3. Uncoating, 4. Bio synthesis, 5. Maturation** and **6. Release.**

1. Adsorption

Virions may come into contact with cells by random collision but adsorption takes place only if there is an **affinity** between the **virus and the host**. The cell surface should contain specific receptor site for the virus to attach on to it.

2. Penetration

Bacteria possess rigid cell walls, only the viral nucleic acid is introduced intracellularly by a complex mechanism. Animal cells do not have rigid cell walls and the whole virus can enter and virus particles may be engulfed by a mechanism resembling **phagocytosis**, a process known as **'Viropexis'**. In case of the **enveloped viruses**, the viral envelope may **fuse** with the plasma membrane of the host cell and release the nucleocapsid into the cytoplasm.

3. Uncoating

Release of the viral nucleic acid from the capsid into the host cell. With most viruses, uncoating is affected by the action of lysosomal enzymes of the host cell.

4. Biosynthesis

Virus can synthesise viral nucleic acid, capsid protein and also the enzymes necessary in the various stages of viral synthesis, assembly and release. In addition certain regulator proteins are also synthesized. Most **DNA viruses** synthesise their nucleic acid in the **host cell nucleus**. Most **RNA viruses** synthesise all their components in the cytoplasm.

5. Maturation

Assembly of daughter virions follows the synthesis of viral nucleic acid and proteins. Virions assembly may take place in the host cell nucleus or cytoplasm. Herpes and adeno viruses are assembled in the **nucleus**, while picorna and pox viruses are assembled in the **cytoplasm**.

6. Release

In case of bacterial viruses, the release of progeny virions takes place by the lysis of

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the infected bacterium. However, in the case of **animal viruses**, release usually occurs **without cell lysis**. **Eclipse phase** is from the stage of penetration till the appearance of mature daughter virions. The virus cannot be demonstrated inside the host cell. The virus seems to disappear (Figure 10.2).

Viroids

Viriods are small, single stranded covalently closed circular RNA molecules existing as highly base paired rod like structure. The viroid depends on the host for replication. These are responsible for some of the transmissible plant diseases.

HOTS

Which is more dangerous – bacteria or Virus?

Prion

Prions are small proteinaceous infectious agents without genetic material. These are responsible for a number of degenerative brain diseases (Example: Creutzfeldt) and hereditary dementia.

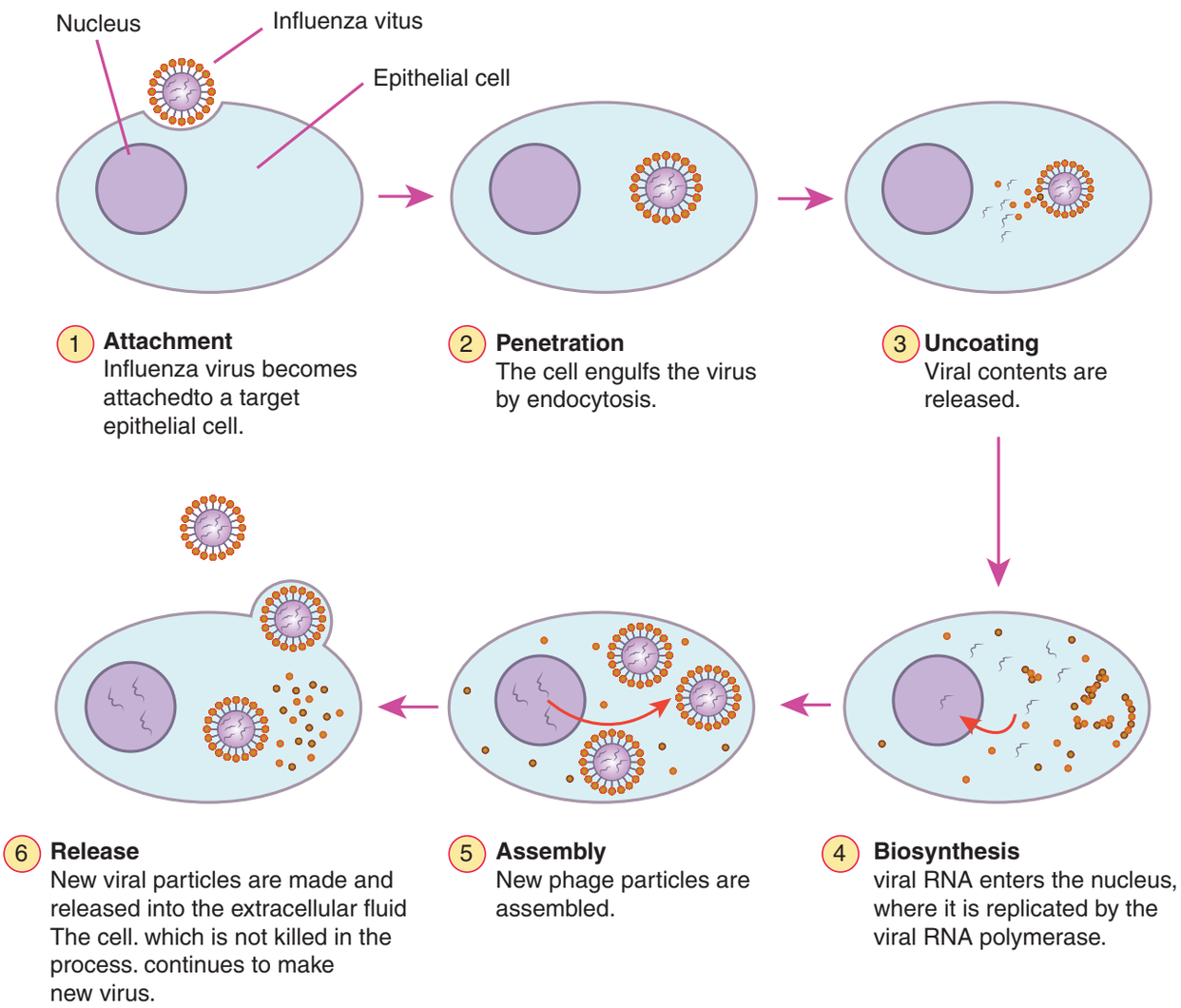

Figure 10.2: The Viral Life cycle

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10.2 Cultivation of Viruses

Viruses are **obligate intracellular** parasites; they cannot be grown on any inanimate culture medium. **Three methods** are employed for the cultivation of viruses – **inoculation into animals, embryonated eggs and tissue culture or cell culture**.

i. Animal Inoculation

The earliest method for the cultivation of viruses causing human diseases was inoculation into human volunteers. Monkeys were used for the isolation of the polio virus by **Landsteiner and Popper (1909)**. The embryonated hen's egg was first used for cultivation of viruses by **Good pasture (1931)**. The embryonated egg offers several sites for the cultivation of viruses. Non human primates provide the only method for virus cultivation. Mice are most widely employed animals in Virology.

ii. Embryonated Eggs

a. Chorioallantonic Membrane (CAM)

Inoculation on **the chorioallantonic membrane** produces visible lesions (pocks). Different viruses have different pock morphology. Example: variola or vaccinia

b. Allantonic Cavity

Inoculation on the **allantonic cavity** provides a rich yield of influenza and some paramyxo viruses.

c. Amniotic Sac

Inoculation into the **amniotic sac** is for the primary isolation of the influenza virus.

d. Yolk Sac

Inoculation into the **yolk sac** is for the cultivation of some viruses like Chlamydiae and Rickettsiae.

Allantonic inoculation is employed for growing influenza virus for vaccine production (Figure 10.3).

iii. Tissue Culture

First tissue culture in Virology was maintained by **Steinhardt and colleagues (1913)** for the vaccinia virus in fragments of rabbit cornea. Bacterial contamination was the major limitation. Different types of culture used are:

a. Organ culture

Small bits of organs can be maintained, used for the isolation of some viruses. Example: Corona virus (respiratory pathogen) cultured on tracheal ring organ culture.

b. Explant culture

Fragments of minced tissue are grown as 'explants'. This is also known as tissue culture. Example: Adeno virus cultured on Adenoid tissue explants.

iv. Cell Culture

Tissues are dissociated into the component cells by the **action of enzymes** (trypsin) or by mechanical process and are suspended in a growth medium (amino acids, vitamins, salts, glucose) **supplemented** with fetal calf serum of antibiotics and **indicator** (Phenol red). This media is dispensed in bottles, tubes or petridishes. The cells adhere to the glass surface and on incubation divides to form a confluent monolayer sheet of cells covering the

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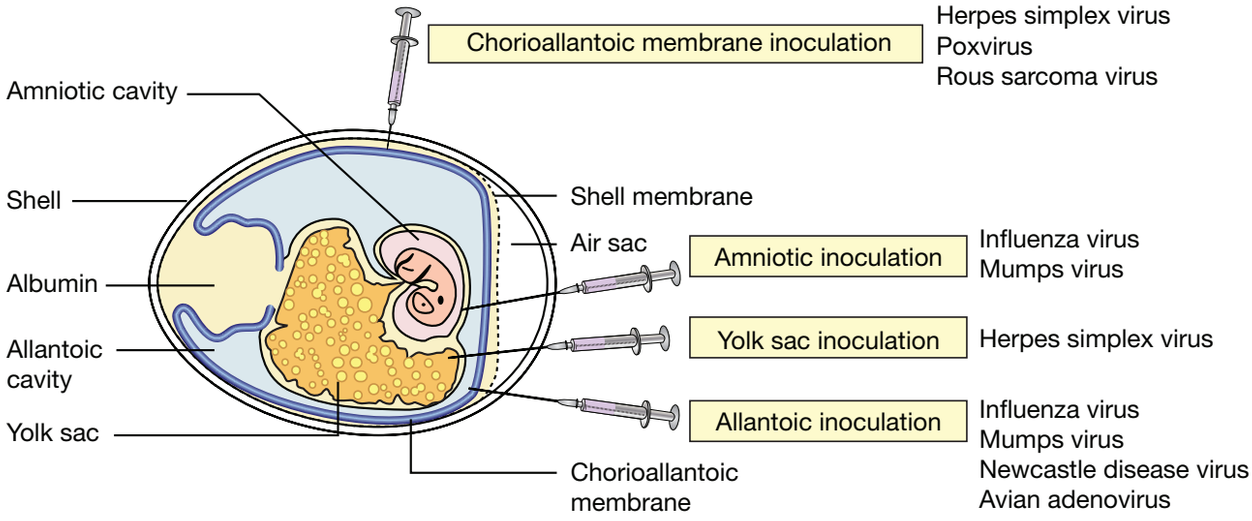

Figure 10.3: Cultivation of virus in Embryonated Egg

surface within about a week. The cell culture is classified into three types.

a Primary cell cultures

In this culture, **normal cells** are taken from the body and cultured. They are capable of only limited growth in culture. Example: Monkey kidney, Human embryonic kidney, Chick embryo cell culture.

b. Diploid cell strains

These are cells of a **single type** that retain the original **diploid chromosome number** and serotype during serial sub cultivation for limited number of times. Example: Human fibroblast.

c. Continuous cell lines

These are **single type, derived from cancer cells** that are capable of continuous serial cultivation. Example: Cells derived from cancers, such as Hela, Hep-2 and KB cell lines.

10.3 Herpes Viruses

The herpes virus family contains more than a hundred species of **enveloped DNA** viruses that affect humans and animals.

Structure

The herpes virus **capsid is icosahedral**, composed of 162 capsomers and enclosing the core containing the linear **double stranded DNA** genome. The nucleocapsid is surrounded by the lipid envelope derived from the host cell. The envelope carries **surface spikes** (Figure 10.4). **Teguments** are present in between the envelope and capsid. The **enveloped virion** measures about 200nm and the **naked virion** about 100 nm in diameter.

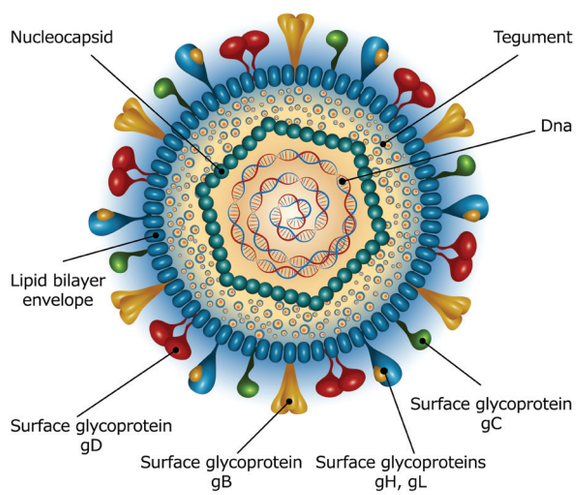

Figure 10.4: Structure of Herpes Simplex Virus

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Classification

Herpes virus belongs to the family Herpesviridae.

i. Alpha herpes viruses

They have relatively **short replicative cycle** (12–18 hours) and a variable host range. They cause latent infection in sensory ganglia. Example: Herpes simplex virus and varicella zoster virus.

ii. Beta herpes viruses

They **replicate slowly** (more than 24 hours) and have a narrow host range, grow well in fibroblasts. They cause latent infection of salivary gland and other organs. Example: Cytomegalovirus.

iii. Gamma herpes viruses

They have a **narrow host range** and replicate in lymphoblastoid cells. They are specific for either B or T lymphocytes and causes latent infection in lymphoid tissue Example: Epstein - Barr Virus.

Eight different types of herpes viruses are known whose primary hosts are humans. They have been designated as 'Human herpes virus type 1–8.

1. Herpes Simplex

The herpes simplex virus (HSV) occurs **naturally only in humans**, but it can produce experimental infection in laboratory animals. There are **two types** of the herpes simplex virus. **HSV type 1** (Human herpes virus type 1) is isolated from lesions in and around the mouth and is transmitted by direct contact or droplet spread from carrier. **HSV type 2** (Human herpes virus type 2 or **HHV type 2**) is responsible for the genital herpes infections transmitted venereally.

Pathogenesis

Herpes simplex is one of the most **common viral infection** in humans, the sources of infection are saliva, skin lesions or respiratory secretions. In type 2, transmission occurs by close contact and may be veneral in genital herpes.

The virus enters through defects in the skin or mucous membranes and multiples locally, with cell to cell spread. The **herpes lesions** are thin walled, umbilicated vesicles, the roof of which breaks down, leaving tiny superficial ulcers. They heal without scarring.

Clinical features

The clinical manifestations depend on the site of infection, age and immune status of the host and the antigenic type of the virus. They are

- Cutaneous infections
- Mucosal infections
- Ophthalmic infections
- Nervous system infections
- Visceral infections
- Genital infections

Laboratory diagnosis

Microscopy

Smears are prepared from the lesions, from the vesicles and stained with 1% aqueous solution of **toluidine blue 'O'** for 15 seconds. Multinucleated giant cells with faceted nuclei with ground glass chromatin (**Tzanck cells**) are observed.

Virus isolation

Inoculation in mice and on chick embryo CAM is insensitive. Primary human

TN_GOVT_XII_Micro_Biology_CH10.indd 208 07-12-2021 15:34:11

embryonic kidney, human amnion cells are susceptible, but **human diploid fibroblasts** are preferred. Vesicle fluid, spinal fluid, saliva and swabs may be used. Cytopathic changes may appear as early as 24–48 hrs.

Serology

Antibodies develop within a few days of infection and **rise in titre of antibodies** may be demonstrated by ELISA, neutralization or complement fixation tests.

Chemotherapy

Indoxyuridine used topically in eye and skin infection, acyclovir and vidarabine are given for deep and systemic infections.

2. Varicella Zoster

In 1889, Von Bokay had suggested that varicella (Chicken pox) and herpes zoster are **different manifestations** of the same virus infection. The virus is therefore called **Varicella zoster virus** (VZV). The chicken pox follows **primary infection** in a non immune individual, while herpes zoster is a **reaction of the latent virus** when the immunity has fallen to infective levels.

VZV is **similar** to the herpes simplex virus in its morphology. It can be grown in cultures of **human fibroblasts human amnion** or HeLa cells. Chicken pox is one of the mildest and **most common of child hood infections**. The disease may, occur at any age.



Herpes gladiatorum is **spread** through skin-to-skin contact. If you kiss someone with a herpes cold sore on their lips, you could become infected.

3. Cytomegaloviruses

Cytomegaloviruses (CMV) formerly known as **salivary gland viruses** are a group of ubiquitous herpes viruses of humans and animals. They are characterized by **enlargement of infected cells** and intranuclear inclusions. In 1926, cytomegalia presumed to be due to viral infection was reported in the salivary glands of guinea pigs and children and the viral agent was called the **'salivary gland virus'.**

Infobits

CMV is the **largest viruses** in the herpes virus family, being 150–200 nm in size.

4. Epstein – Barr Virus

A number of different viruses apparently **'Passenger Viruses'** were isolated from cultured lymphoma cells. **Epstein, Barr and Achong in 1964** observed a new type of herpes virus and named it has **'EB Virus'** affecting B lymphocytes of only human and some sub human primate **B cells have receptors** (CD21 molecules) for the virus.

The source of infection is usually the **saliva of infected persons** who shed the virus in oropharyngeal secretions. Intimate oral contact, as in kissing,

TN_GOVT_XII_Micro_Biology_CH10.indd 209 07-12-2021 15:34:11

appears to be the predominant mode of transmission. This accounts for infectious mononucleosis being called as **'The kissing disease'**.

5. Human Herpes Virus Types 6,7,8

A herpes virus, first isolated in 1986 from the **peripheral blood** of patients with lympho proliferative disease called as **human B lymphotropic virus**, renamed as HHV - 6. HHV- 7 was isolated in 1990 from **peripheral CD4 cells** of a healthy person appears to be widely distributed and transmitted through saliva.

In 1994, DNA sequences presumed to represent a new herpes virus from **tissues of Kaposi's sarcoma** from AIDS patients was named as HHV8. Later Kaposi's sarcoma was identified in persons not infected with HIV and referred to as **Kaposi's Sarcoma-associated Herpes Virus** (KSHV).

10.4 Hepatitis Viruses

The term viral hepatitis refers to a **primary infection of the liver**, hepatitis viruses consists of types A, B, C, D, E and G. Except for **type B** which is a DNA virus all the others are RNA viruses.

Two types of viral hepatitis had been recognised. **Type one** affects mainly children and young adults and transmitted by the fecal-oral route called **as infective or infectious hepatitits or type A hepatitis. Second type** transmitted mainly by receiving serum inoculation or blood transfusion named as **homologous serum jaundice, serum hepatitis transfusion hepatitis or type B hepatitis**.

Type A Hepatitis (HAV)

HAV is a 27nm non enveloped RNA virus belonging to the **picorna virus family**. It is designated as **'entero virus 72'**, HAV is recognised as new genus **'Hepatovirus'**. It can be grown in human and simian cell cultures and is the only human hepatitis virus which can be cultivated *in vitro*.

HAV transmission is by the **fecal oral route**. Infection is by ingestion. The virus multiplies in the intestinal epithelium and reaches the liver by **hematogenous spread**. Once jaundice develops, it is rarely detectable in feces. The incubation period is 2- 6 weeks. The clinical disease consists of two stages the **prodromal and the icteric stage**. The onset may be acute with fever, malaise, anorexia, nausea, vomiting and liver tenderness. These usually subside with the onset of jaundice. Recovery is slow, over a period of **4–6 weeks**. The disease is milder in children. Type A hepatitis caused by **contaminated food, water or milk**. Over crowding and poor sanitation favour its spread.

Laboratory Diagnosis

Diagnosis of type A hepatitis may be made by **demonstration of the virus** or its **antibody**. Virus can be visualized by **Immunelectron Microscopy (IEM)** in fecal extracts during the late incubation period.

IgM anti-HAV antibody appears during the late incubation period disappears after 3-4 months. **IgG** peaks in 3-4 months and persists much longer for life. ELISA kits for detection of IgM and IgG antibodies are available.

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Does HSV shorten your lifespan?

Becoming infected with **the herpes virus** seriously complicates your social, emotional and sexual life, but it is not otherwise a terribly dangerous condition to have. Having **genital herpes does** make it easier to get HIV (and thus AIDS), but otherwise, the condition is not disabling, and does not reduce lifespan.

A safe and effective formalin inactivated, **alum conjugaged vaccine** containing HAV grown in human diploid cell culture is used. Course consists of **two intra muscular injections** of the vaccine. Protection begins **4 weeks** after injection and lasts for **10 to 20 years**. No specific antiviral drug is available.

Type B Hepatitis (HBV)

HBV is a 42nm **DNA virus** with an outer envelope and an inner core 27nm in diameter. Enclosing the **viral genome** and a **DNA polymerase**. It belongs to the family Hepadna Viridae HBV is **'Hepadna Virus type 1'**. **Australia antigen** was found to be associated with serum hepatitis. It was the surface component of HBV, so named as **hepatitis B surface antigen (HBsAg)**.

3 types of particles are visualized, most abundant form is a **spherical particle**, 22nm in diameter. The second type of particle is **filamentous** or **tabular** with a diameter of 22nm both are antigenically identical. Third type of particle are fewer in number, is a **double walled spherical** structure 42 nm in diameter. This particle is the complete **hepatitis B virus**, known as **Dane particle**.

The envelope proteins expressed on the surface contains **hepatitis B surface antigen (HBsAg)**. HBsAg consists of two major polypeptides, one of which is glycosylated. The nucleocapsid or core contains **hepatitis B core antigen (HBcAg A)** (Figure 10.5)**.** Third antigen called the **hepatitis B e antigen (HBeAg)** is a soluble non particulate nucleocapsid protein.

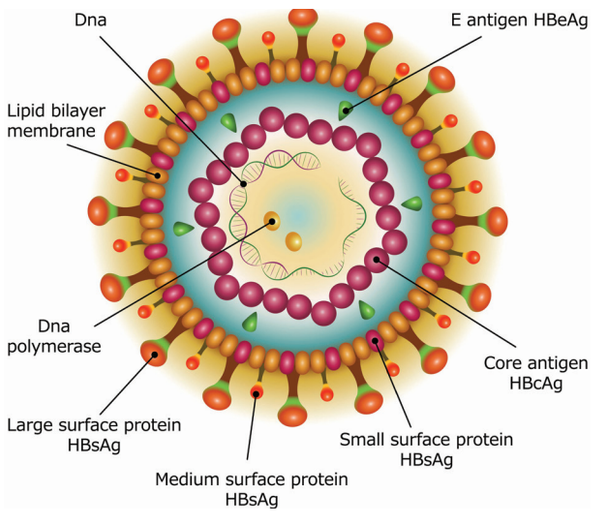

Figure 10.5: Structure of Hepatitis B Virus

The **nucleocapsid** encloses the viral genome consisting of **two linear strands of DNA** held in a circular configuration. One of the strands is **incomplete** (+ strand) DNA appears partially **double stranded** and partially **single stranded**. Associated with the + strand is a **viral DNA polymerase** (has both DNA dependent DNA polymerase and RNA dependent reverse transcriptase functions). This polymerase can **repair the gap** in the plus strand and render the **genome fully double stranded**.

Natural infection occurs **only in humans**. The virus is maintained in

TN_GOVT_XII_Micro_Biology_CH10.indd 211 07-12-2021 15:34:11

carriers whose blood contains **circulating virus** for long periods. Carriers are of two categories, the highly infectious **super carriers** and the simple carriers. Former have high titre HBsAg along with HBsAg, DNA polymerase and HBV in ciruculation. Simple carriers have low infectivity and low titre HBsAg in blood.

HBV is a **blood borne virus** and the infection is transmitted by **parenteral, sexual and perinatal models**. The virus may also be present in other **body fluids and excretions** such as saliva, breast milk, semen, vaginal secretions, urine bile and feces of these semen and saliva are known to transmit the **infection** very commonly. Transfusion of carrier blood once, the most widely known mode of infection has largely been eliminated by donor screening that is strictly enforced. Infection by direct contact with open skin lesions such as pyoderma, eczema, cuts and scratches is very common among **young children** in developing countries. Certain groups and **occupations** carry a high risk of infection. These include medical and paramedical staff of blood banks, dialysis units, barbers, sex workers.

The incubation period is long about 1- 6 months. The onset is insidious and fever is not prominent. Extra hepatic complications like arthralgia, urticaria and glomerulonephritis may occur. About 90-95% of adults with acute hepatitis B infection recover within 1-2months of onset and eliminate the virus from the body. They may be **Asymptomatic carriers** or may progress to recurrent or chronic liver disease.

Laboratory Diagnosis

Serology

Diagnosis of hepatitis B depends on the **serological demonstration** of the viral markers. **HBsAg** is the first marker to appear in **blood** after infection, being detectable. It remains in circulation throughout the symptomatic course of the disease (2- 6months). Anti HBs is the protective antibody.

HBcAg is **not demonstrable** in circulation because it's **enclosed** within the **HBsAg coat** but its antibody, anti HBc appears in serum a week or two after the appearance of HBsAg. As anti HBc remains life long, it serves as a useful indicator of prior infection with HBV.

HBeAg appears in blood concurrently with HBsAg, indicating the **high infectivity**. Molecular methods such as **DNA: DNA hybridization and PCR** at present used for HBV DNA testing are highly sensitive and quantitative.

Immunization

Both passive and active methods of immunization are available. **Active immunization** is more effective. The currently preferred vaccine is genetically engineered by cloning the **S gene for HBV** in **Baker's yeast**. A special vaccine containing all antigenic components of HBsAg (Pre-S1, Pre-S2 and s) has been developed. **No specific antiviral treatment** is available for acute HBV infection.

10.5 Rabies Virus

The Family Rhabdoviridae contains viruses that **infects mammals, reptiles, birds, fishes, insects and plants**. The disease in human being is called

TN_GOVT_XII_Micro_Biology_CH10.indd 212 07-12-2021 15:34:11

hydrophobia because the patient exhibits **fear of water**, being **incapable** of drinking though subject to **intolerable thirst**.

Pasteur established that the rabies virus was present in the **brain of infected animals**. By serial **intracerebral passage** in rabbits, he demonstrated **fixed virus** that could be **rendered immune** by a series of injections. **Vaccine was prepared** by drying pieces of **spinal card** from rabbits infected with the **fixed virus**.

Joseph Meister a nine year old boy, severely bitten by a rabid dog and in grave risk of developing rabies, was given a course of **13 inoculations** of the infected cord **vaccine** by Pasteur. The boy **survived**. This dramatic event was a **mile stone** in the development of medicine.

Morphology

The rabies virus is **bullet shaped**, with one end rounded or conical and the other planar or concave. The lipoprotein envelope, carries **knob like spikes**, composed of **glycoprotein G** responsible for pathogenesis, virulence and immunity beneath the envelope is the **matrix (M) protein layer** which may be invaginated at the planar end. The membrane may project outwards forming a **bleb**. The genome is unsegmented linear RNA (Figure 10.6).

The rabies virus isolated from natural human or animal infection is termed **'the street virus'**. Rabies has been recognized from very ancient times as a disease transmitted to humans and animals by the **bite of 'mad dogs'**. The name rabies comes from the **Latin** word rabidus, meaning **'mad'**, derived from the **Sanskrit** root rabhas, for **frenzy**.

Pathogenesis

Human infection is usually caused by the **bite of rabid dogs** or other animals. The virus present in the saliva of the animal is **deposited** in the wound (Figure 10.7). **Rarely**, infection can also occur following non-bite exposures such as licks or aerosols.

The virus appears to **multiply** in the muscles, connective tissue or nerves at the site of deposition for 48-72 hours. It penetrates the **nerve endings** and travels in the axoplasm towards the **spinal cord and brain**, at speed of about 3 mm per hour. The virus multiples and **spreads centrifugally** along the nerve trunks to various parts of the body including the salivary glands. It multiplies in the salivary glands and is **shed** in the **saliva**. The virus reaches every tissue in the body and dissemination may be interrupted at any stage by death. In humans the incubation period is usually from **1–3 months**, short as 7 days or as long as three years. The incubation period is usually short in persons bitten on the face or head and long in those bitten on the legs. This may be related to the distance the virus has to travel to reach the brain. The incubation period is generally shorter in children than in adults.

The **four stages** of the disease are as follows, **prodrome, acute encephalitic phase, coma and death**. The onset is marked by symptoms such as fever, headache, malaise, fatigue and anorexia, anxiety, agitation, irritability, nervousness, insomnia or depression. The neurological

TN_GOVT_XII_Micro_Biology_CH10.indd 213 07-12-2021 15:34:11

phase begins with hyperactivity. Attempts to drink during such painful spasms of the pharynx and larynx produce choking or gagging that patients develop a dread of even the sight or sound of water (hydrophobia).

Animal Infection

In dogs, the incubation period is **usually 3–6 weeks** but it may range from 10 days to a year. The initial signs are an **alert, troubled air and restlessness, snapping at imaginary objects, licking** or gnawing at the site of the bite.

After 2–3 days of this prodromal stage, the disease develops into either the furious or dumb types of rabies. In **furious rabies**, dog runs biting without provocation, the lower jaw droops and saliva drools from the mouth. Paralysis convulsions and death follow. **In dumb rabies,** is the paralytic form, animals lies huddled, unable to feed. About 60% of rabid dogs shed the virus in saliva. Rabid dogs usually die in 3-5days.

Laboratory Diagnosis

Human Rabies

The specimens tested are **corneal smears and skin biopsy**. Commonly used method for diagnosis is the demonstration of **rabies virus antigens** by immuno fluorescence. **Direct immunofluorescence** is done using antirabies serum tagged with fluorescein isothiocyanate.

Negri bodies in the brain, are demonstrated, Isolation of the virus by intracerebral inoculation in **mice** can be attempted from the brain, CSF, saliva and urine. The mice are examined for **signs of illness**, and their brains are examined after death.

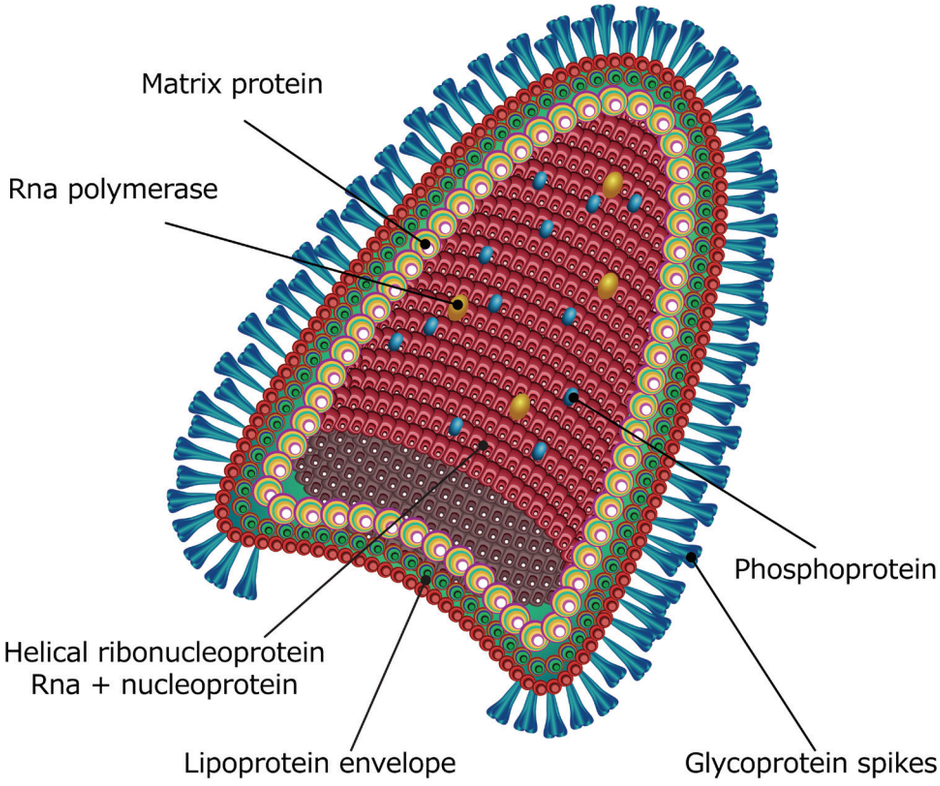

Figure 10.6: Structure of Rabies Virus

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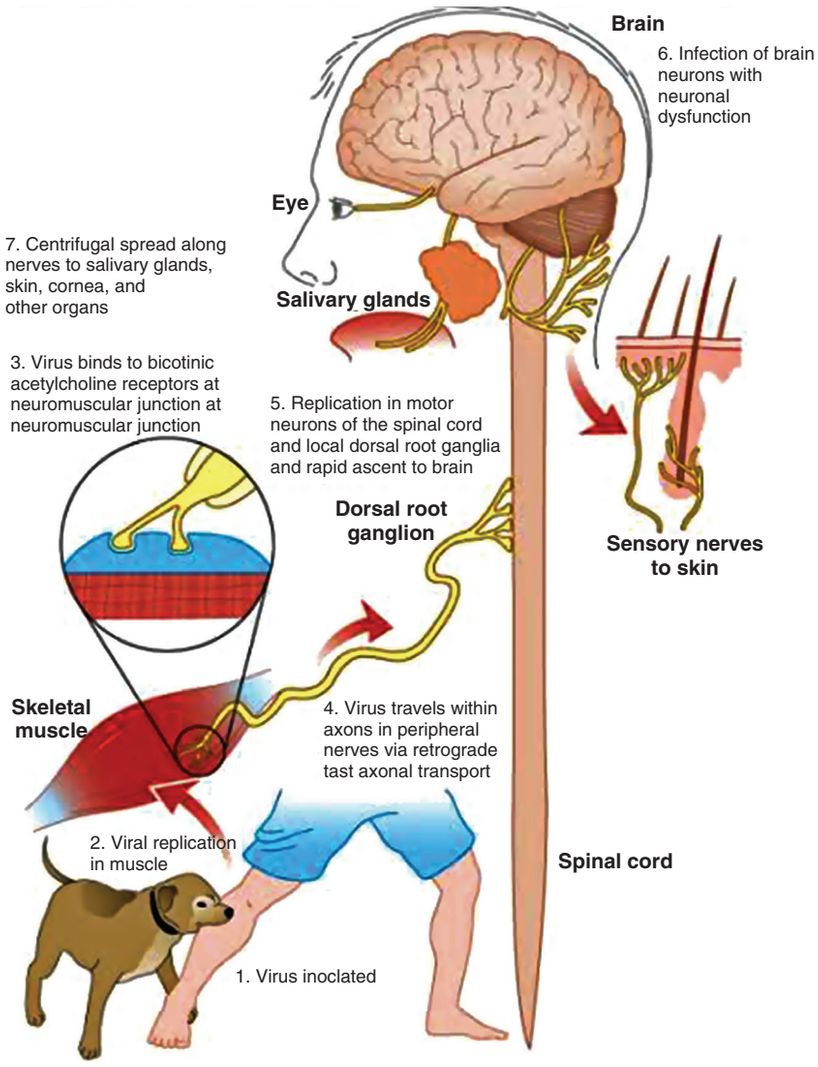

Figure 10.7: Mechanism of Rabies Infection

Animal Rabies

The **whole carcass** of the animal suspended to have died of rabies may be sent to the laboratory. The brain may be **removed** sent for biological test and microscopy respectively. The portion of brain sent should include the **hippocampus and cerebellum** as **negri bodies** are most abundant. The following tests are done in the laboratory.

1. Demonstration of rabies virus antigen by immuno fluorescence
2. Demonstration of inclusion bodies - Negri bodies are seen as intracytoplasmic, round or oval purplish pink with characteristic basophilic inner granules. Negribodies vary in size from 3.27 Mm.

Infobits

Local Treatment for rabies

- Prompt cauterization of the wounds helps to destroy the virus.
- Antirabic serum may be applied topically.
- Antitetanus measures and antibiotics to prevent sepsis.

TN_GOVT_XII_Micro_Biology_CH10.indd 215 07-12-2021 15:34:13

Antirabic Vaccines

Antirabic vaccines fall into two main categories neural and non-neural.

Neural Vaccines

Suspension's of nervous tissues of animals infected with the fixed rabies virus. Following are the modified forms.

1. **Semple Vaccine:** Vaccine developed by semple (1911). It is a 5% suspension of **sheep brain infected with fixed virus** and inactivated with phenol at 37°C leaving noresidual live virus.
2. **Beta propiolactone (BPL) Vaccine:** Beta propiolactone is used as the **inactivating agent** instead of Phenol.
3. **Infant Brain Vaccine:** The enceptalitogenic factor in brain tissue is a basic protein associated with myelin. Vaccines were developed using infant mouse, rat or rabbit brain. Infant brain vaccine is impractical in India.

Non-neural Vaccines

Non-neural vaccines includes

1. Egg Vaccines
2. Tissue Culture Vaccines
3. Subunit Vaccine

Passive Immunisation

Human rabies immune globulin **(HRIG)** is free from the danger of sensitization but should be ensured free from HIV and hepatitis viruses.

Vaccines for Animals

Antirabies immunization in animals is to be done as pre-exposure prophylaxis concentrated cell culture vaccines – inactivated virus gives good protection after a single **Intramuscular** injection. Injections are given at 12 weeks of age and repeated at 1–3 years intervals.

10.6 Human Immuno Deficiency Virus

Human Immuno Deficiency Virus (HIV), the etiological agent of AIDS, belongs to the lentivirus subgroup of the family **Retroviridae**.

Infobits

Detecting HIV sooner

Fourth generation test helps to detect HIV in blood earlier than previously recommended antibody test. It identifies the viral protein, HIV-1 P24 antigen, which appears in the blood sooner than antibodies.

Source: CDC

Structure

HIV is a spherical **enveloped Virus**, about 90-120 nm in size. The nucleo capsid has an outer icosahedral shell and an inner cone shaped core, enclosing the **ribonucleo proteins**. The genome is diploid, composed of two identical single stranded, **positive sense RNA copies**. When the virus infects a cell, the Viral RNA is transcribed by the **reverse transcriptase enzyme**, first into single stranded DNA and then to double stranded DNA (provirus) which is integrated into the host cell chromosome. The virus coded **envelope proteins** are the projecting knob like spikes which binds to the CD4 receptors on susceptible host cells (Figure 10.8).

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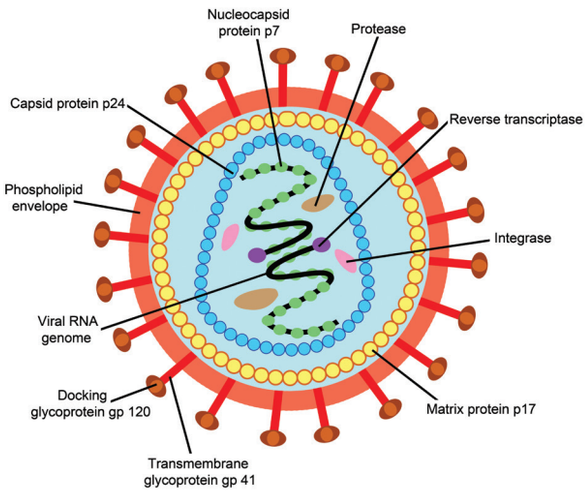

Figure 10.8: Structure of HIV

Viral Genes and Antigens

The genome of HIV contains the **three structural genes** (gag, pol and env) as well as other nonstructural and regulatory genes specific for the virus. These products of these genes, both structural and non structural act as antigens.

Genes coding for structural proteins

1. The gag gene ⟶ Determines the core and shell of the Virus. Precursor protein, p^{55} and it is cleaved into three proteins p^{15}, p^{18} and p^{24}. **Major core antigen p^{24}** can be detected in serum
2. The env gene ⟶ Determines the synthesis of envelope glycoprotein gp^{160}. Cleaved in to gp^{120} and gp^{41}
3. The pol gene ⟶ Codes for the **reverse transcriptase** and other viral enzymes such as protease and endonucleases. It's expressed as a precursor protein, which is cleaved into protein p31, p51 and p66.

Pathogenesis

Infection is transmitted when the Virus **enters the blood or tissues** of a person and comes into contact with a suitable host cell, principally the CD4 lymphocyte. The receptor for the virus is the CD4 antigen and therefore the virus may infect any cell bearing the CD4 antigen on the surface. Specific binding of the **virus to CD4 receptor** is by the envelope **glycoprotein gp^{120}**. Cell fusion is brought about by transmembrane gp^{41}. After **fusion** `with the host cell membrane, the HIV genome is uncoated and internalized into the cell. Viral reverse transcriptase mediate transcription of its RNA into double stranded DNA, which is **integrated into the genome** of the infected cell through the action of the viral enzyme integrase, causing a latent infection. The primary pathogenic mechanism in HIV infection is the damage caused to the CD4+T lymphocyte. The **T_4 cells decrease** is numbers. Infected T_4 cells do not release normal amounts of interleukin, gamma interferon and other lymphokines, this is damping effect on cell mediated immune response.

Clinical Features

AIDS is only the last stage in the wide spectrum in HIV infection.

1. Acute HIV infection

3–6 weeks of infection, persons experience low grade fever, malaise, headache, lymphadenopathy, with rash. Antibodies are usually negative at the onset of the illness but become positive during its course called **'Sero conversion illness'**.

2. Asymptomatic or latent infection

All HIV infected persons, whether or not they experience Sero conversion illness, pass through a phase of **symptomless**

TN_GOVT_XII_Micro_Biology_CH10.indd 217 07-12-2021 15:34:14

infection which may last up to several years. The infection progresses in course of time through various stages, CD4 lymphocytopenia, minor opportunistic infections, AIDS-related complex (ARC), ultimately terminating to AIDS.

3. Persistent generalized lymphadenopathy (PGL)

It is defined as the presence of **enlarged lymph nodes** at least 1cm, in diameter in two or more non contiguous extrainguinal, sites that persists for at least three months.

4. AIDS related complex (ARC)

This group includes patients with considerable **immunodeficiency**, suffering from various symptoms or minor opportunistic infections. eg. Oral candidiasis, Salmonellosis or Tuberculosis.

5. AIDS

End-stage disease, poor immune defence mechanism leading to the opportunistic infection and malignancies.

a. Commonest symptoms

Drycough, dyspnea and fever. Pheumonia may be viral (cmv) or fungal (Cryptococcus, Histoplasma).

b. Gastrointestinal system

The mouth is often involved with thrush, stomatitis, gingivitis, hairy leukoplakia. Dysphagia due to esophageal Candidiasis. Intestinal pathogen in AIDS is cryptosporidium. Other pathogens are Salmonellae, Mycobacteria, CMV or adeno viruses. 'Gay bowel syndrome' is common among the male homosexuals.

c. Central nervous system

The typical CNS opportunistic infections are toxoplasmosis and cryptococcosis. Lymphomas of the CNS are Common.

d. Malignancies

Kaposi's Sarcoma was the lesion seen in male homosexuals. The tumours commonly seen are lymphomas, both the Hodgkin and non Hodgkin types.

e. Cutaneous

Herpes lesions, Candidiasis, Dermatitis, impetigo are common cutaneous lesions.

6. Dementia

Direct **cytopathogenic damage** in the CNS. It cross the blood-brain barrier and cause encepthalopathy leading to dementia.

7. Pediatric AIDS

Viral transmission may occur to the **fetus in pregnancy**. Many of the infected children may not survive for a year. Children may also acquire the infection from blood transfusion or blood products.

Laboratory Diagnosis

Lab diagnosis of HIV infection include tests for immuno deficiency in HIV infection.

A. Immunological tests

i. Total leukocyte and lymphocyte count to demonstrate leucopenia and a lymphocyte count usually below 2000/ mm^3.

ii. Platelet count will show thrombocytopenia.

iii. Raised IgG and IgA levels.

TN_GOVT_XII_Micro_Biology_CH10.indd 218 07-12-2021 15:34:14

B. Specific tests for HIV infection

1. Antigen detection

Single massive infection, as by blood transfusion, the **virus antigens** may be detectable in blood after about two weeks. The major core antigen p24 is the virus marker in blood.

2. Polymerase Chain reaction

It is the most **senstitive** and specific test.

3. Antibody detection

Demonstration of **antibodies** is the simplest and widely employed technique. It takes 2–8 weeks to months for antibodies to appear after infection, during this period, the individual may be highly infectious. This sero negative infective stage is known as the 'window period'. Antibody can be detected by

1. ELISA
2. Western blot test.

Treatment

The treatment of AIDS include:

1. The treatment and prophylaxis of infections and tumours
2. General management
3. Immunorestrorative measures
4. Specific anti-HIV agents

Effective drugs are available, they are Zidovudine, Didanosine, Zalcitabine, Lamivudine and Protease inhibitors like Saquinavir, Ritonavir, Indinavir used as monotherapy or in various combination.

10.7 Arbo Virus

Arbo Viruses (arthropod - borne viruses) are viruses of vertebrates biologically transmitted by **hematophagous insect vectors**. They multiply in blood sucking insects and are transmitted by bite to vertebrate hosts. Arbo viruses are worldwide in distribution. Arbo viruses have been named according to the disease caused (**yellow fever**), the place of isolation of the virus (**kyasanur forest disease**) or the local name for the disease (**chikungunya**). They are classified into Toga, Flavi, Bunya, Reo and Rhabdovirus families. Arbo viruses have a very wide host range including many species of animals and birds. The most important arbo virus vectors are **mosquitoes**, followed by **ticks**.

The virus enters the body through the **bite** of the insect vector. After multiplication in the **reticuloendothelial system, viremia** of varying duration occurs, or the virus is transported to the target **organs** such as central nervous system in **encephalitis**, the liver in **yellow fever** and the capillary endothelium in **hermorrhagic fever**.

Clinical syndromes are fever with or without rash, encephalitis, hemorrhagic fever, systemic disease and yellow fever.

Diagnosis may be established by virus isolation or serology.

Samples (Blood, CSF) are inoculated intra cerebrally into sucking mice. The animal develop **fatal encephalitis**. Viruses may be isolated in tissue cultures or in eggs. Isolates are identified by hemagglutination inhibition, complement fixation, gel precipitation, immunofluorescence and ELISA. Virus isolated from insect vectors and from reservoir animal.

Toga Viruses

Toga viruses are **spherical enveloped viruses** with a diameter of 50-70nm.

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Single stranded RNA genome. The virus replicates in the **cyloplasm** of the host cell and released by budding through host cell membranes. The name Toga Virus is derived from **'toga'** meaning the Roman Mantle refers to the **viral envelope**.

The genus Alpha Virus was formerly classified as **Group A arbo viruses** which explains the name Alpha Virus. The genus Alpha Virus contains 32 species of which 13 infect humans. All are **mosquito borne**.

10.7.1 Chikungunya Virus

The virus was first isolated from human patients of ***Aedes aegypti*** mosquitoes (Figure 10.9) from Tanzania in 1952. The name Chikungunya is derived from the native word for the disease in which the patient lies **'doubled up due to severe joint pains'**. The virus first appeared in India in 1963 in Calcutta, Madras and Other areas.

The disease presents as a sudden onset of fever, Crippling joint pains, lymphadenopathy and conjunctivitis. A **maculopapular rash** in common. The fever is typically biphasic with a period of remission after 1–6 days of fever. The vector is ***Aedes aegypti***. No animal reservoir has been identified. Antibody to the virus has been demonstrated in horses, cattle and other domestic animals.

Flavi viruses

The family flaviviridae contains only one genus **flavivirus**. They are smaller than alpha viruses, being 40nm in diameter. There are over 60 arthropod borne flava viruses classified as **mosquito-borne and tick borne viruses**. Examples of mosquito borne group known as encephalitis viruses they are St. Louis encephalitis Virus, Ilheus virus, west nile virus, murray valley encephalitis virus and Japanese encephalitis. Tick borne viruses are classified in to tick borne encephalitis viruses and tick borne hemorrhagic fevers.



Figure 10.9: *Aedes aegypti*

10.7.2 Dengue

The name dengue is derived from the **'Swahili ki denga pepo'**, meaning a sudden **seizure by a demon**. Dengue fever is similar to the illness caused by chikungunya. Four types of dengue virus exist: **DEN1, DEN2, DEN3 and DEN4**.

HOTS

What is the best home remedy for dengue fever?

Dengue presents after an incubation period of 3-14 days as fever of sudden onset with headache, retrobulbarpain, conjunctival injection, pain in the back and limbs (break bone fever), lymphadenopathy and maculopapular rash. The fever is typically **biphasic** (saddle back) and lasts for 5–7 days. Dengue may be more serious forms with **hemorrhagic** manifestations (dengue

TN_GOVT_XII_Micro_Biology_CH10.indd 220 07-12-2021 15:34:15

Hemorrhagic fever) or with shock (dengue shock syndrome).

Dengue virus is transmitted from person to person by ***Aedes aegypti*** mosquitoes. The Incubation period is 8–10days. All four types of dengue virus are identified. Demonstration of circulating IgM antibody provides early diagnosis. IgM ELISA test offers reliable diagnosis. Difference between Dengue and Chikungunya is given in Table 10.2.

10.7.3 Zika Virus

Zika virus is a mosquito-borne flavivirus that was identified in Uganda in 1947 in **monkeys**. Zika spreads by daytime-active Aedes mosquitoes, such as *A. aegypti* and *A. albopictus*. The infection is known as **Zika fever** or **Zika virus disease**. Zika is related to the dengue, yellow fever, Japanese encephalitis, and West Nile viruses

Zika virus is **enveloped and icosahedral** and has a non segmented, single-stranded, positive-sense (+) **RNA genome** (Figure 10.10). A positive-sense RNA genome can be directly translated into **viral proteins**, the RNA genome encodes **seven nonstructural proteins** and **three structural proteins**. One of the structural proteins forms the envelope. The RNA genome forms a nucleocapsid along with copies of the 12-kDa capsid protein.

Viral genome replication depends on the making of double-stranded RNA from the single-stranded, positive-sense RNA (ssRNA(+)) genome followed by **transcription** and **replication** to provide viral mRNAs and **new ssRNA(+) genomes**.

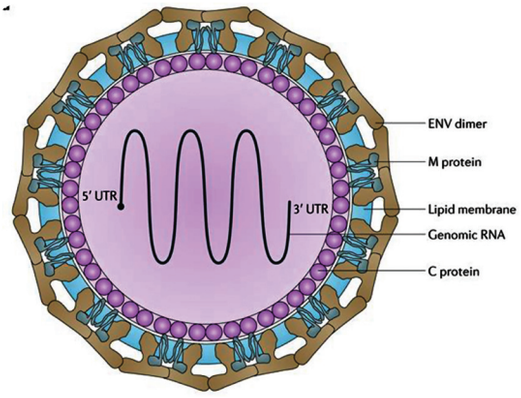

Figure 10.10: Structure of Zika Virus

Pathogenesis and Clinical features

Zika virus replicates in the mosquito's **mid gut epithelial cells** and then its salivary gland cells. After 5–10 days, the virus can be found in the mosquito's saliva. If the mosquito's saliva is inoculated into **human skin**, the virus can infect epidermal keratinocytes, skin fibroblasts in the skin and the Langerhans cells. The pathogenesis of the virus is hypothesized to continue with a **spread to lymph nodes** and the **bloodstream**.

Zika virus is primarily transmitted by the **bite of an infected mosquito** from the *Aedes* genus, mainly *Aedes aegypti*. The mosquitoes usually bite during the day, peaking during early morning and late afternoon or evening. This is the same mosquito that transmits dengue, chikungunya and yellow fever. Zika virus is also transmitted from mother to fetus during pregnancy, through sexual contact, transfusion of blood and blood products, and organ transplantation.

The incubation period of Zika virus disease is estimated to be **3–14 days**. The majority of people infected with Zika virus **do not develop symptoms**. Symptoms are generally mild including fever, rash, conjunctivitis, muscle and joint pain, malaise, and headache, and usually last

TN_GOVT_XII_Micro_Biology_CH10.indd 221 07-12-2021 15:34:15

Table 10.2: Difference between Dengue and Chikungunya

S.No	Factors	Chikungunya	Dengue
1.	Vector	*Aedes aegypti*	*Aedes aegypti*
2.	Virus	Toga viridae - alphavirus	Flavi viridae - flavivirus
3.	Incubation Period	3-7 days	4-7 days
4.	Symptoms	Chikungunya begins as an **acute febrile** illness. Pain can be severe. Other common symptoms include headache, muscle pain, joint swelling, and rash. Some patients have persistence or relapse of rheumatologic symptoms in the months following acute illness.	Dengue is an **acute febrile** illness. **Febrile Phase:** Lasts 2–7 days Fever with Headache, retro-orbital pain, joint pain, muscle and or bone pain, rash, mild bleeding (nose or gums) **Critical Phase:** Lasts 24–48 hours. Most patients improve but severe disease requiring hospitalization can occur. **Recovery Phase:** Gradual reabsorption of extravasated fluid from plasma leakage over 48–72 hours. Dieresis, hemodynamic status stabilizes,patient can temporarily become bradycardic.
5	Major symptom	Tremendous Joint pain	Bleeding and breath discomfort
6	Person at risk	Neonates exposed intrapartum, older adults, and persons with underlying medical conditions.	Some patients may develop life threatening consequences and require hospitalization. Infection with each dengue virus type confers lifetime immunity for that specific virus type.

Infobits

Which fruit is good to increase platelet count?

Pomegranate is another great fruit you can eat to increase platelets. As with all **red**fruits, the seeds of this **delicious** fruit are packed with iron, an **essential** mineral for combating low platelet count. **Pomegranate** has been used since the ancient times for its healthy and medicinal properties.

for 2–7 days. **Zika fever** (also known as Zika virus disease) is an illness caused by the Zika virus. Zika virus infection during pregnancy is a cause of **microcephaly** and other **congenital abnormalities** in the developing fetus and newborn. Zika infection in pregnancy also results in pregnancy complications such as fetal loss, stillbirth, and preterm birth.

Laboratory diagnosis

TN_GOVT_XII_Micro_Biology_CH10.indd 222 07-12-2021 15:34:15

Virus can be demonstrated from the blood or other body fluids, such as urine or semen.

Zika virus grow well in a variety of mammalian and insect cell lines. Zika virus is identified by **NAAT–** Nucleic acid Amplification test, Zika Antigen is detected by ELISA and PCR. **Zika Antibody** IgM is detected by MAC - ELISA, IgG by ELISA and by **PRNT**- plaque reduction neutralization test.

Prevention and Treatment

Protection against **mosquito bites** during the day and early evening is a key measure to prevent Zika virus infection. It is important to eliminate these mosquito breeding sites, Health authorities may also advise use of **larvicides and insecticides** to reduce mosquito populations and disease spread. There is no treatment available for Zika virus infection or its associated diseases. **No vaccine is yet available for the prevention or treatment of Zika virus infection. Development of a Zika vaccine remains an active area of research.**

Summary

This chapter dealt with the history, morphology, chemical properties, viral replication, virus classification, cultivation and detection of cytopathic effects of virus. Most important viruses such as Adeno, herpes, hepatitis, Influenza, rabies, HIV and Arbo virus, its morphology, classification, pathogenesis and its laboratory diagnosis were discussed.

Evaluation

Multiple choice questions

1. _____ is an example for smallest virus
   a. Pox virus
   b. Parvo virus
   c. Rabies virus
   d. HIV virus
2. CPE stands for _____
   a. cytoplasmic effects
   b. cytopathogenic effects
   c. cytopathic effects
   d. None of these
3. Cytomegalo viruses also called as _____
   a. Salivary gland virus
   b. Thymus gland virus
   c. Endocrine gland virus
   d. None of these
4. _____ is an example for passenger viruses
   a. HIV virus b. EB Virus
   c. Rabies virus d. None of these
5. Beta propiolactone (BPL. Vaccine is given for _____
   a. HIV virus b. Influenza Virus
   c. Rabies virus d. None of these
6. _____ is an example for mosquito-borne and tick borne viruses
   a. Dengu virus b. Flavi virus
   c. Chikungunya virus d. None of these

TN_GOVT_XII_Micro_Biology_CH10.indd 223 07-12-2021 15:34:15

Answer the following

1. What is Virology?
2. Define virion.
3. Which is the largest virus?
4. What is Nucleocapsid?
5. Brief note on Steps involved in viral multiplication.
6. What is Viropexis?
7. Define Abortive infection.
8. What are Prions?
9. Write Short note on cultivation of Virus.
10. What are Cytopathogenic Virus?
11. Classification of Herpes virus.
12. Discuss on HSV-1 and HSV-2
13. Expand VZV
14. What is EB Virus?
15. Define Dane particle
16. Genome structure of HBV
17. Give the Structure of Rabies Virus
18. What is Furious and dumb rabies?
19. Define Negri bodies
20. Discuss Vaccine for rabies
21. Note on Viral gene and antigen of HIV
22. Write about the Clinical features of HIV
23. Give the Lab diagnosis of HIV
24. Define Arbo Virus
25. Give the Symptoms of hermorrhagic fever.
26. Short note on Chikungunya virus
27. Account on Mosquito borne virus
28. Write about the Structure of Zika Virus
29. What is Zika fever

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Chapter 11

Immunology

Learning Objectives

After studying this chapter the students will be able to,

- *Understand the Antigen Antibody reactions*
- *Know the principle behind Western Blot techniques*
- *Learn about Hypersensitivity*
- *Gain knowledge about Transplantation*
- *Know Immunization/Vaccination*
- *Appreciate the Updated National Immunization Schedule chart.*

Chapter Outline

11.1 Antigen Antibody Reactions

11.2 Western Blot Techniques

11.3 Hypersensitivity

11.4 Transplantation

11.5 Immunization/Vaccination

11.6 Updated National Immunization Schedule Chart

The immune system refers to a collection of cells and proteins that function to protect the skin, respiratory passages, intestinal tract and other areas from foreign antigens, such as microbes (organisms such as bacteria, fungi, and parasites), viruses, cancer cells, and toxins. The immune system works by recognising the difference between one's own body cells and alien cells, allowing it to destroy anything that could be potentially harmful. Immune deficiency diseases decrease the body's ability to fight invaders, causing vulnerability to infections. In the previous year, we have elaborately discussed with the main components and function of the immune system. This chapter deals with the role of Immune system in both health and disease.

11.1 Antigen Antibody Reactions

The interaction between antigen and antibody is called antigen-antibody reactions. It is abbreviated as Ag-Ab reaction. This reaction is the basis of humoral immunity. The antigen and the antibody react to form immune complex.

Ag + Ab ---------- Ag – Ab complex

The reaction between antigen and antibody is highly specific. It is compared to the lock and key system. The part of the antigen that combines with the antibody is called epitope or antigenic determinant. The part of antibody which combines with the antigen is called paratope or antigen

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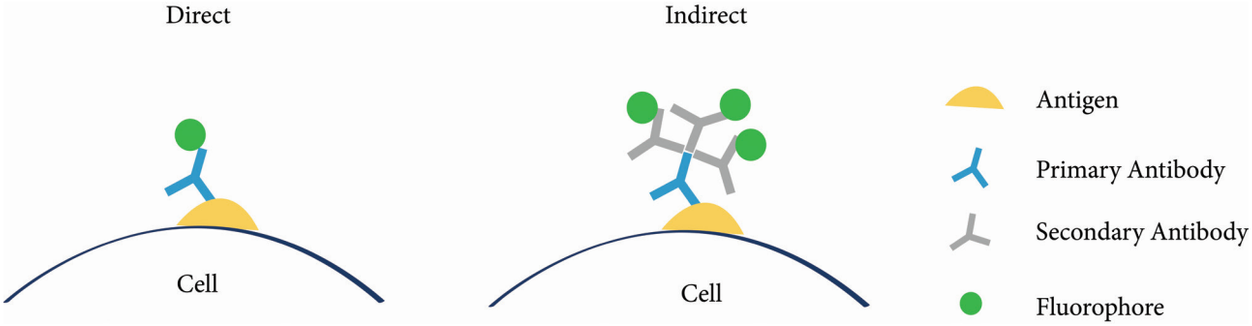

Figure 11.1: Methods in Immunofluorescence

determining site. Most of the antibodies have two binding sites and IgM has 5–10 binding sites.

Immunofluorescence

When antibodies are mixed with the fluorescent dyes such as fluorescein or rhodamine, they emit radiation. This phenomenon of emitting radiation by antibodies labelled with fluorescent dye is called immuno fluorescence. This reaction is well observed under fluorescent microscope. It is used to locate and identify antigens in tissues.

Types of Immunofluorescence

- Direct method
- Indirect method

Direct Method

In this method, the antibody labelled with fluorescent dye is directly applied on the tissue section. The labelled antibody binds with specific antigen. This can be observed under the fluorescent microscope.

Indirect Method

In this method, unlabelled antibodies are directly applied on the tissue sections which bind with the specific antigens. Then the antibody labelled with the fluorescent dye is added to the tissue. Anti-antibody specifically binds with already added or linked unlabelled antibody (Figure 11.1).

ELISA (Enzyme Linked Immuno Sorbent Assay)

ELISA (Enzyme-Linked Immuno Sorbent Assay) is a plate-based assay technique designed for detecting and quantifying substances such as peptides, proteins, antibodies and hormones. It is also known as Enzyme Immuno Assay (EIA).

In 1971, after the descriptions of Peter Perlmann and Eva Engvall at Stockholm University in Sweden, ELISA has become the system of choice when assaying soluble antigens and antibodies. All assays for antibody production depend upon the measurement of interaction of elicited antibody with antigen.

Principle

The **principle of ELISA** is very simple. The test is generally conducted in micro titre plates. (Figure 11.2 Micro titre plate).

If the antigen is to be detected the antibody is fixed in the micro titre plate and vice versa. Test sample is added in the microtitre plate, if there is presence of Ag or Ab in the test sample, there will

TN_GOVT_XII_Micro_Biology_CH11.indd 226 07-12-2021 15:38:10

be Ag-Ab reactions (with immobilized Ab or Ag). Later enzyme labelled antibody is added in the reaction mixture, which will combine with either test antigen or Fc portion of test antibody.

The enzyme system consists of:

1. **An enzyme**: Horse Radish Peroxidase(HRP), alkaline phosphatase which is labelled or linked, to a specific antibody.
2. **A specific substrate**:
   - O-Phenyl-diamine-dihydrochloride for peroxidase
   - P nitrophenyl Phosphate- for Alkaline Phosphatase

Substrate is added after the antigen-antibody reaction. The enzyme hydrolyses the substrate to give a yellow colour compound in case of alkaline phosphatase (Figure 11.3). The intensity of the colour is proportional to the amount of antibody or antigen present in the test sample, which can be quantified using ELISA reader (Figure 11.4 ELISA reader)

Types

There are **four kinds** of ELISA assay tests. They are: Direct ELISA, Indirect ELISA, Sandwich ELISA and Competitive ELISA (Figure 11.5).



Figure 11.2: Micro titre plate



Figure 11.4: ELISA Reader

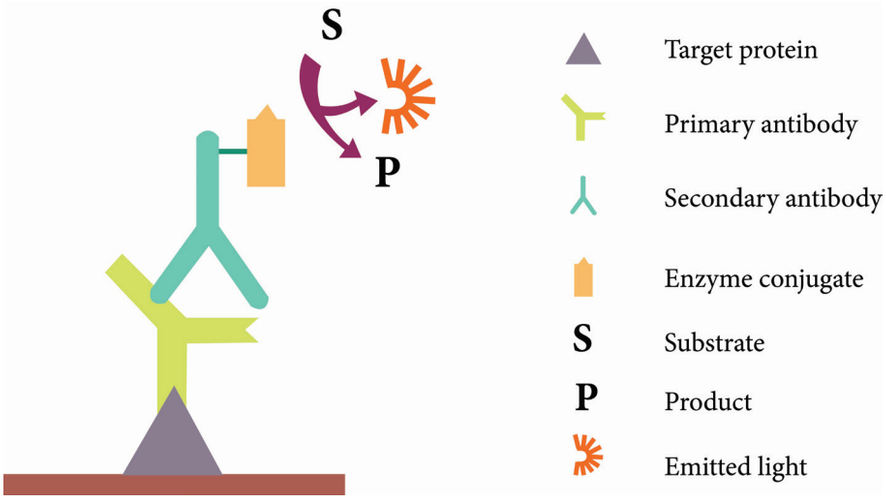

Figure 11.3: Basic steps in ELISA

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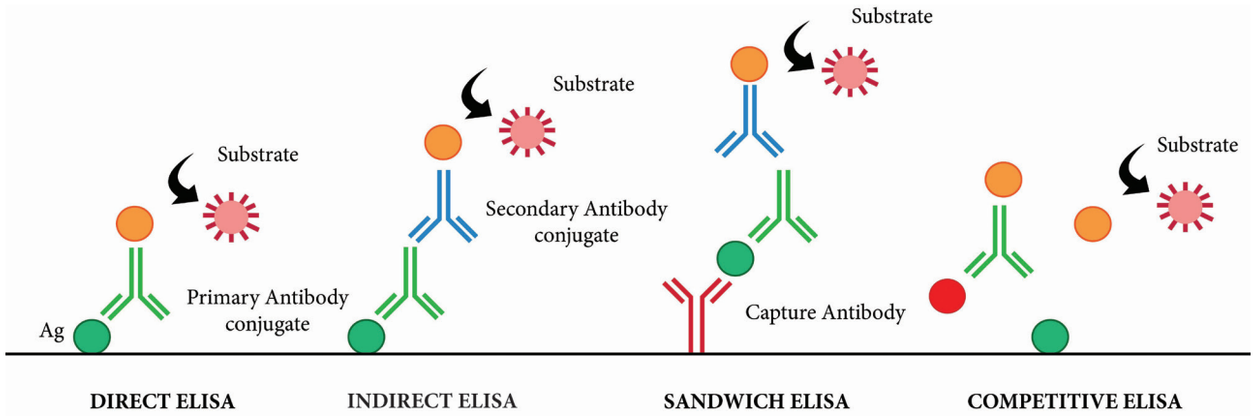

Figure 11.5: Types of ELISA

i. Direct ELISA

An antigen is immobilized in the well of an ELISA plate. The antigen is then detected by an antibody directly conjugated to an enzyme such as HRP. Direct ELISA detection is much faster than other ELISA techniques as fewer steps are required. The assay is also less prone to error since fewer reagents and steps are needed, i.e. no potentially cross-reacting secondary antibody needed. Finally, the direct ELISA technique is typically used when the immune response to an antigen needs to be analyzed.

ii. Indirect ELISA

Indirect ELISA is used to detect antibody. A known antigen is coated on the micro titre plate. If the patient's serum contains antibody specific to the antigen, the antibody will bind to the antigen. After incubation the wells are washed and the enzyme labelled anti Human Gamma Globulin (HGG) is added to the well. Anti-HGG can react with antigen antibody complex. The substrate for the enzyme is added finally which is hydrolysed by the enzyme which develops a colour.

iii. Sandwich ELISA

Sandwich ELISA is used to detect antigen. A known antibody is coated on the micro titre plate. A test antigen is added to each well and allowed to react with the bound antibody.

If the patient's serum contains antigen specific to the antibody, the antigen will bind to the antibody. Specifically bound antigen and antibody will remain in the wells even after washing. The second antibody is added and allowed to react with bound antigen. Substrate is added to measure colour reaction.

iv. Competitive ELISA

It is used for the detection of antigens. Antibody is first incubated with a sample-containing antigen. The antigen and antibody complex is added to the antigen coated microtitre well. If more antigen present in the sample, the less free antibody will be available to bind to the antigen coated well. Addition of an enzyme conjugated secondary antibody specific to the primary antibody can be used to determine the amount of primary antibody bound to the well. It is a quantitative test for the antigen detection.

Application

An ELISA test may be used to diagnose: HIV, Lyme disease, pernicious anaemia, Rocky Mountain spotted fever, rotavirus, squamous cell carcinoma, syphilis,

TN_GOVT_XII_Micro_Biology_CH11.indd 228 07-12-2021 15:38:12

toxoplasmosis, varicella-zoster virus, which causes chickenpox and Zika virus.

11.2 Western Blot techniques

Macromolecules immobilized or fixed on nitrocellulose membrane i.e., blotted can be subjected to a variety of analytical techniques more easily. Southern blotting was the first blotting technique developed which made the analysis and recording of DNA easy. Later the technique was extended for analysis of RNA and proteins and they have acquired the jargon terms Northern and Western Blotting respectively. Western blotting is also known as immunoblotting because it uses antibodies to detect the protein. Western blotting is a quantitative test to determine the amount of protein in sample.

Principle

Western blotting technique is used for the identification of a particular protein from the mixture of a proteins. In this method, the proteins are first extracted from the sample. Extracted proteins are subjected to Poly Acryl - amide Gel Electrophoresis (PAGE). Transfer of proteins from poly acryl amide to the nitrocellulose paper is achieved by applying electric field. When radio labelled specific antibody is added on such membrane it binds to the specific complementary protein. Finally the proteins on the membrane can be detected by staining or through ELISA technique.

Steps

Step I: Extraction of Protein

The most common protein sample used for Western blotting is cell lysate. The protein from the cell is generally extracted by mechanical means or by adding chemicals which can lyse the cell. The extraction step is termed as **tissue preparation**. Protease inhibitor is used to prevent the denaturing of proteins. Using spectroscopy the concentration of the protein sample is analysed and diluted in loading buffer containing glycerol. This will help the sample to sink in the well. Bromothymol blue is used as tracking dye and is used to monitor the movement of the sample.

Step II: Gel electrophoresis

The protein sample is loaded in well of SDS-PAGE (Sodium dodecyl sulfate-poly-acryl amide gel electrophoresis). The proteins are separated on the basis of electric charge, isoelectric point, molecular weight, or combination of all these. Proteins are negatively charged, so they move toward positive (anode) pole as electric current is applied. Smaller proteins move faster than the larger proteins.

Step III: Blotting

Blotting refers to the transfer of the protein from the gel to the nitrocellulose paper by capillary action. Electro blotting is done nowadays to speed up the process. In electro-blotting nitrocellulose membrane is sandwich between gel and cassette of filter paper and then electric current is passed through the gel causing transfer of protein to the membrane.

Step IV: Blocking

The nitrocellulose membrane is non-specifically saturated or masked by using casein or Bovine serum albumin (BSA) before adding the primary antibody. This

TN_GOVT_XII_Micro_Biology_CH11.indd 229 07-12-2021 15:38:12

blocking step is very important in western blotting as antibodies are also proteins and they are likely to bind to the nitrocellulose paper.

Step V: Treatment with primary and secondary antibody

The primary antibody is specific to desired protein so it forms Ag-Ab complex. The secondary antibody is enzyme labelled and is against primary antibody (anti-antibody) so it can bind with Ag-Ab complex. Alkaline phosphatase or Horseradish peroxidase (HRP) is labelled with secondary antibody.

Step VI: Treatment with suitable substrate

Finally, the reaction mixture is incubated with specific substrate. The enzyme convert the substrate to give visible coloured product, so band of colour can be visualized in the membrane (Figure 11.6).

Application

1. The size and concentration of protein in given sample is determined by western blotting.
2. It is used in the detection of antibody against virus or bacteria in serum and helps in the disease diagnosis.
3. Western blotting technique is the confirmatory test for HIV. It detects anti HIV antibody in patient's serum.
4. Useful to detect defective proteins.

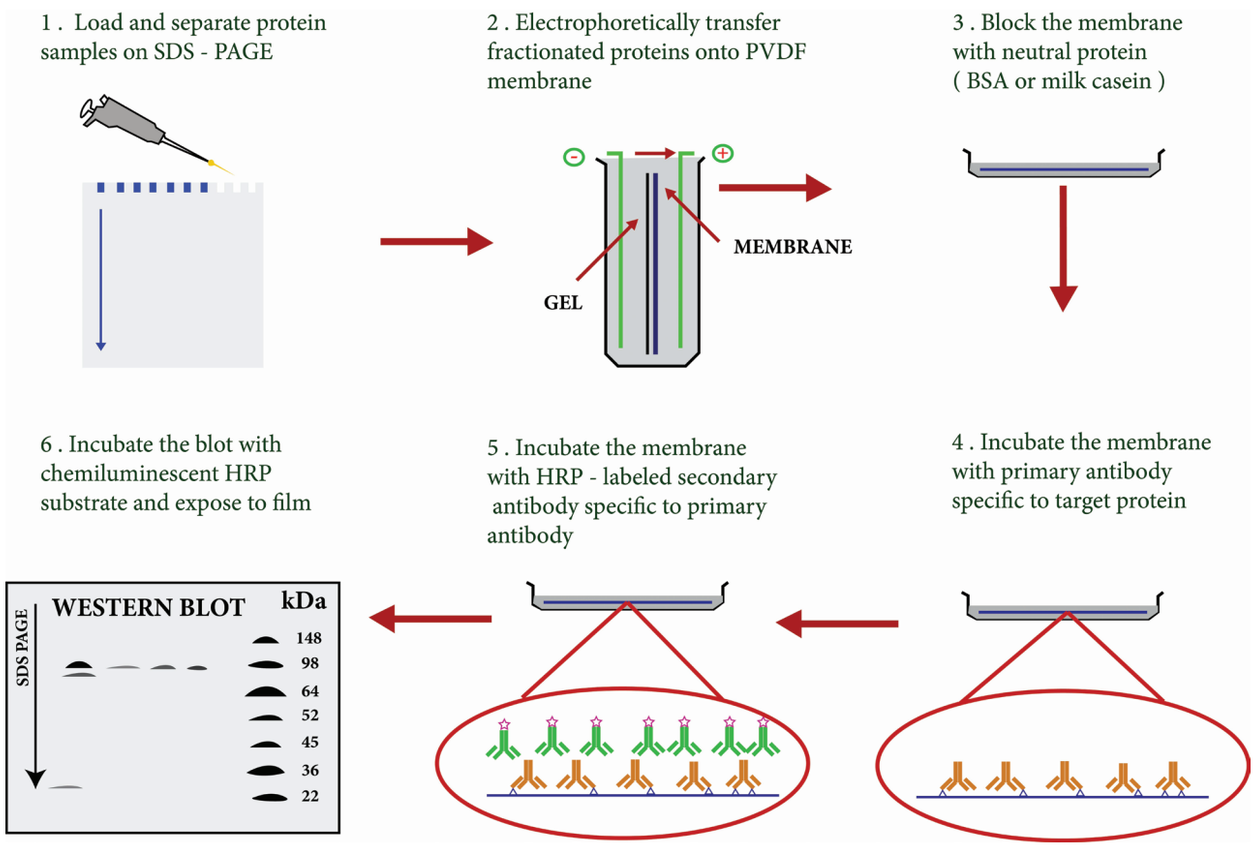

Figure 11.6: Western blot technique

TN_GOVT_XII_Micro_Biology_CH11.indd 230 07-12-2021 15:38:13

11.3 Hypersensitivity

Hypersensitivity is defined as the exaggerated immunological response leading to severe symptoms and even death in a sensitized individual when exposed for the second time. It is commonly termed as allergy. The substances causing allergic/ hypersensitivity is known as allergens. Example:. Drugs, food stuffs, infectious microorganisms, blood transfusion and contact chemicals.

Classification of Hypersensitivity (Coombs and Gell Classification)

Type I: Immediate (Atopic or anaphylactic) Hypersensitivity

Type II: Antibody–dependent Hypersensitivity

Type III: Immune complex mediated Hypersensitivity

Type IV: Cell mediated or delayed Hypersensitivity

Type I: Immediate (Atopic or anaphylactic) Hypersensitivity

This type of hypersensitivity is an allergic reaction provoked by the re-exposure to a specific antigen. The antigen can make its entry through ingestion, inhalation, injection or direct contact. The reaction may involve skin, eyes, nasopharynx and gastrointestinal tract. The reaction is mediated by IgE antibodies (Figure 11.7). IgE has very high affinity for its receptor on mast cells and basophils. Cross linking of IgE receptor is important in mast cell trigerring. Mast cell degranulation is preceded by increased Ca^{++} influx. Basophils and mast cells release pharmacologically active substances such as histamines and tryptase. This causes inflammatory response. The response is immediate (within seconds to minutes). Hence, it is termed as immediate hypersensitivity. The reaction is either local or systemic.

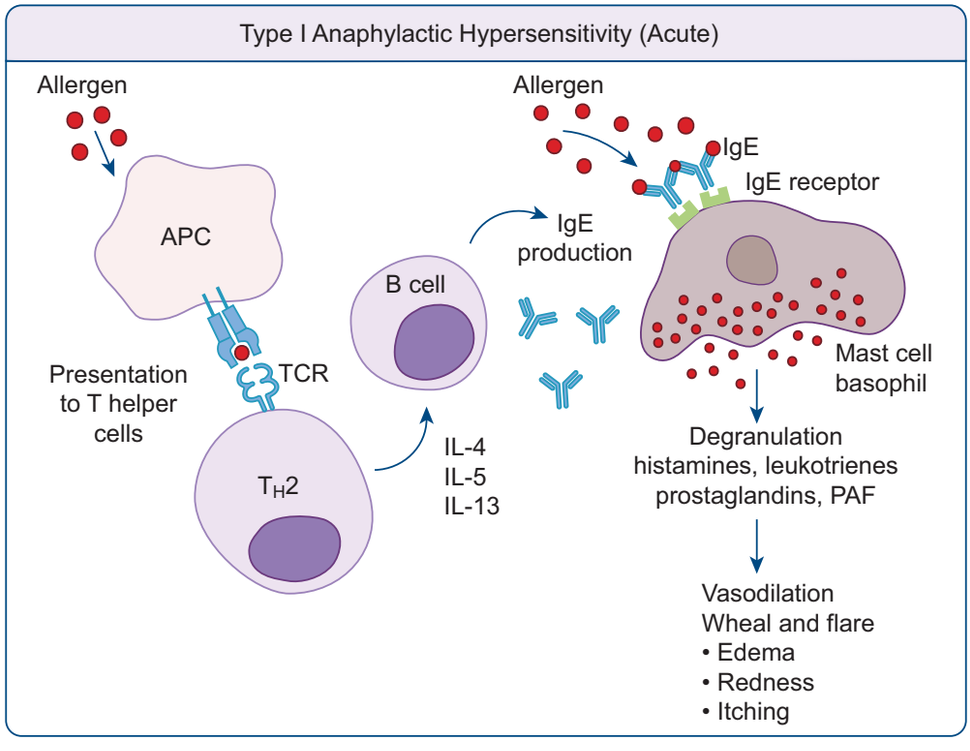

Figure 11.7: Type I hypersensitivity

TN_GOVT_XII_Micro_Biology_CH11.indd 231 07-12-2021 15:38:14

Hay Fever

Allergic rhinitis is commonly known as hay fever. Allergic rhinitis develops when the body's immune system becomes sensitized and overreacts to something in the environment like pollen grains, strong odour of perfumes, dust etc. that typically causes no problem in most people. When a sensitive person inhales an allergen the body's immune system may react with the symptoms such as sneezing, cough and puffy swollen eyelids.

Type II Hypersensitivity: Antibody dependent hypersensitivity

In this type of hypersensitivity reactions the antibodies produced by the immune response binds to antigens on the patient's own cell surfaces. It is also known as cytotoxic hypersensitivity and may affect variety of organs or tissues. Ig G and Ig M antibodies bind to these antigens and form complexes. This inturn activates the classical complement pathway and eliminates the cells presenting the foreign antigen. The reaction takes hours to day (Figure 11.8).

Drug induced haemolytic anaemia

Certain drugs such as penicillin, cephalosporin and streptomycin can absorb non-specifically to protein on surface of RBC forming complex similar to hapten-carrier complex. In some patients these complex induce formation of antibodies, which binds to drugs on RBC and induce complement mediated lysis of RBC and thus produce progressive anaemia. This drug induced haemolytic anaemia is an example of Type II hypersensitivity reaction.

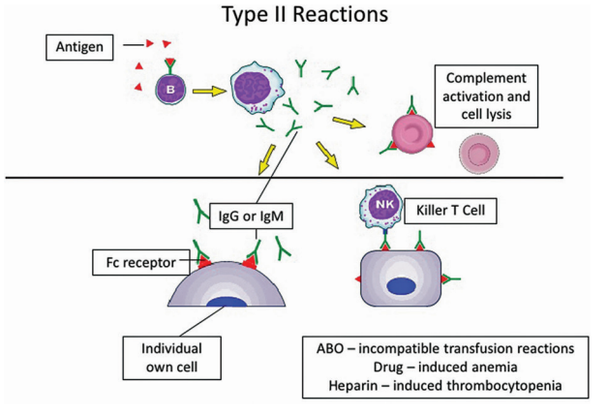

Figure 11.8: Type II hypersensitivity

Type III Hypersensitivity: Immune complex mediated hypersensitivity

When a huge amount of antigen enters into the body, the body produces higher concentrations of antibodies. These antigens and antibodies combine together to form insoluble complex called immune complex. These complexes are not completely removed by macrophages. These get attached to minute capillaries of tissues and organs such as kidneys, lung and skin (Figure 11.9). These antigen-antibody complexes activate the classical complement pathway leading to vasodilation. The complement proteins and antigen-antibody complexes attract leucocytes to the area. The leukocytes discharge their killing agents and promote massive inflammation. This can lead to tissue death and haemorrhage.

Arthus reaction

It was first observed by Arthus. It is a local immune complex reaction occurring in the skin. Horse serum and egg albumin are the antigens that induce the arthus reaction. It is characterized by erythema, induration, oedema, haemorrhage and

TN_GOVT_XII_Micro_Biology_CH11.indd 232 07-12-2021 15:38:14

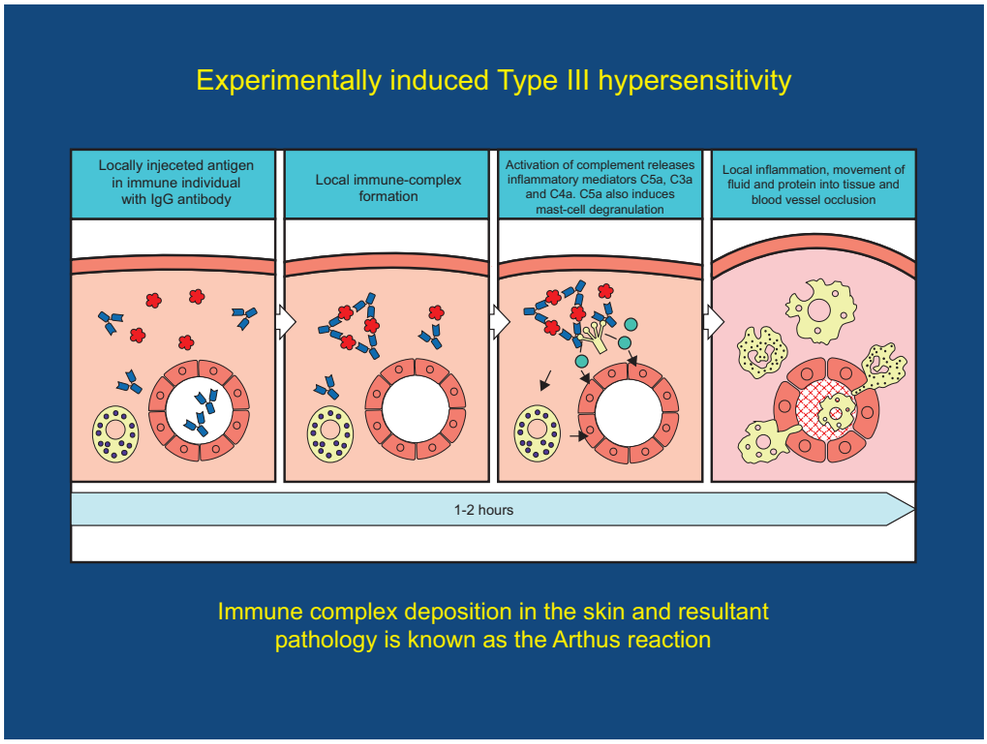

Figure 11.9: Type III hypersensitivity

necrosis. This reaction occurs when antibody is found in excess. It appears in 2–8 hours after injection and persists for about 12–24 hours (Table 11.1).

Type IV hypersensitivity: Cell Mediated Delayed Hypersensitivity

It is often called as delayed hypersensitivity reaction as the reaction takes two to three days to develop. Type IV hypersensitivity is involved in the pathogenesis of many autoimmune and infectious diseases such as tuberculosis and leprosy. T lymphocytes, monocytes and macrophages are involved in the reaction. Cytotoxic T Cells cause direct damage whereas the T helper cells secrete cytokines and activate monocytes

Table 11.1: Difference between Immediate Hypersensitivity and Delayed Hypersensitivity

Sn. No.	Immediate Hypersensitivity	Delayed Hypersensitivity
1.	It appears and disappears rapidly	It appears slowly and last longer.
2.	It is induced by antigens or haptens by any route	Induced by infection, injection of antigen intra dermally or with adjuvants of by skin contact.
3.	The reaction is antibody mediated B-cell response	The reaction is T-cell mediated response.
4.	Passive transfer is possible with serum	Cannot be transferred with serum but can be transferred by lymphocytes
5.	Desensitization is easy, but does not last long	Desensitization is difficult but long lasting.

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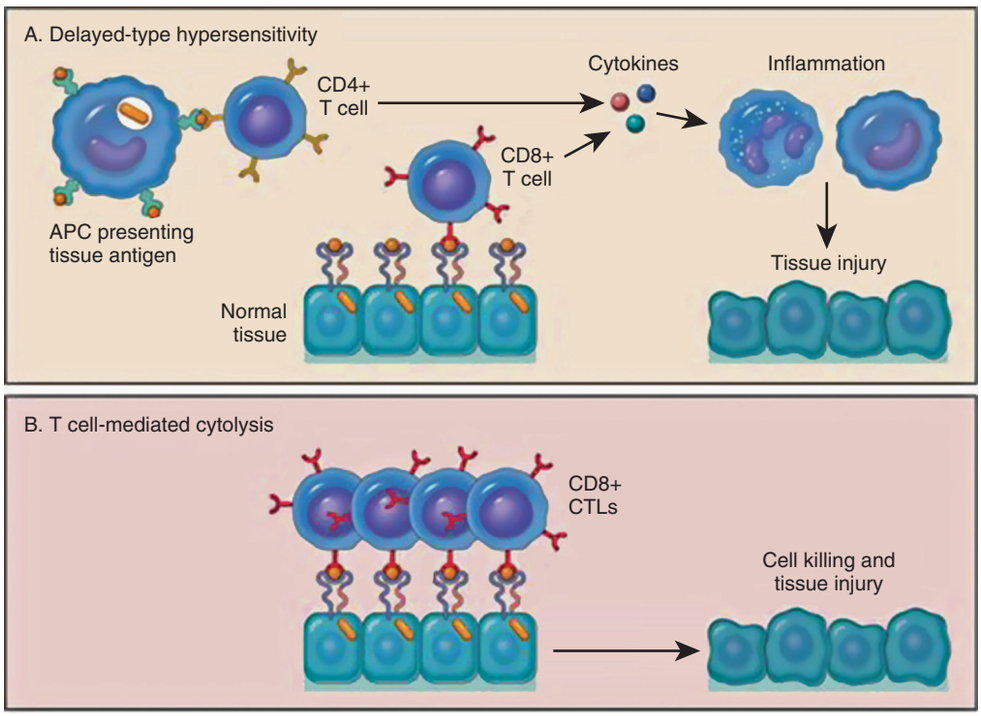

Figure 11.10: Type IV delayed hypersensitivity

and macrophages and cause the bulk damage (Figure 11.10).

Tuberculin reaction (Mantoux Reaction)

When a small dose of tuberculin is injected intra dermally in an individual already having tubercle bacilli, the reaction occurs. It is due to the interaction of sensitized T cell and tubercle bacterium. The reaction is manifested on the skin very late only after 48–72 hours.

11.4 Transplantation

Transfer of living cells, tissues or organs from one part of the body to another or from one individual to another is known as transplantation.

A tissue or organ that is removed from one site and placed to another site usually in a same or different individual is called graft. The individual who provides the graft is called donor and the individual who receives the graft is called host or recipient.

If the graft is placed into its normal anatomic location, the procedure is called orthotopic transplantation. If the graft is placed in a different site it is called heterotopic transplantation.

Transplantation is the only form of treatment for most end-stage organ failure. In clinical practice, transplantation is used to overcome a functional and anatomic deficit in the recipient. Transplantation of kidneys, hearts, livers, lungs, pancreas and bone marrow are widely done today.

Methods of Transplantation

- Auto grafting: The transfer of self tissue from one body site to another in the same individual
- Allografting: The transfer of organs or tissues from human to human
- Xenografting: The transfer of tissue from one species to another (Figure 11.11).

TN_GOVT_XII_Micro_Biology_CH11.indd 234 07-12-2021 15:38:15

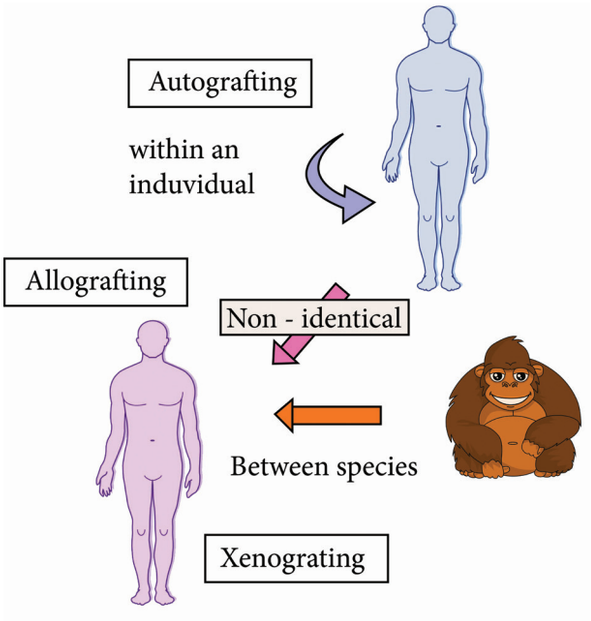

Figure 11.11: Types of Grafting

Graft Acceptance

When transplantation is made between genetically identical individuals the graft survives and lives as healthy as it is in the original places. When the graft tissue remains alive, it is said to be accepted and the process is called graft acceptance.

Graft Rejection

When transplantation is made between genetically distinct individual the graft tissue dies and decays. When the graft tissue dies, the graft is said to be rejected and the process is called graft rejection. It is of two types. They are

i. Host Verses Graft Reaction

ii. Graft Verses Host Rejection.

Host Verses Graft Reaction (HVG)

The graft tissue antigens induce an immune response in the host. This type of immune response is called host versus graft reaction.

Allograft Rejection

Types of allograft rejection

- Acute rejection–Quick graft rejection. It is due to stimulation of thymocytes and B lymphocytes
- Hyperacute rejection–It is a very quick rejection. It is due to pre-existing humoral antibodies in the serum of the host as a result of presensitization with previous grafts.
- Insidious rejection–It is a secret rejection due to deposition of immune complex on the tissues like glomerulus membrane that can be demonstrated in kidney by immune fluorescence.

Mechanism of Allograft Rejection

Immunological contact

When tissue is implanted as graft, its antigen can pass into local lymph nodes of the host. The graft antigens then make contact with the lymphocytes of the host. Production of sensitized T cells and cytotoxic antibodies are produced in the host. This brings about graft rejection.

First set rejection

When the graft is made between genetically different individuals, the graft gets blood supply from the host and it appears to be normal for the first 3 days. But on the 5th day, sensitized T cells, macrophages and a few plasma cells invade the graft. Inflammation starts in the graft. This leads to necrosis. It is similar to the primary immune response to an antigen.

Second set rejection

When a graft is implanted in an individual who has already rejected a graft is second set rejection. This is similar to the secondary immune response of our body.

TN_GOVT_XII_Micro_Biology_CH11.indd 235 07-12-2021 15:38:16

Cell mediated cytotoxic reaction

The 1st set of rejection of allograft is brought about mainly by CMI response. In this process the cells involved in the cytotoxic mediated immunity involves. On stimulation of these cells interferon causes the lysis of the graft.

Antibody mediated cytotoxic reaction

The 2nd set rejection of graft is brought about mainly by HMI response. This is one of the hyperacute rejection brought about by the antibodies. Complement, macrophages, mast cells, platelets, B cells bring about this reaction.

Graft versus Host Rejection (GVH)

Sometimes the graft tissue elicits an immune response against the host antigens. This immune response is called graft versus host reaction. It occurs when:

- Graft remains inside the host and the host should not reject the graft.
- The graft should have immune competent T cells.
- The transplantation antigens of the host should be different from that of the graft.



A transplanted heart usually beats slightly faster than normal because the heart nerves are cut during surgery.

Mechanism of the graft rejection

The graft lymphocytes aggregate in the host lymphoid organs and are stimulated by the lymphocytes of the host. The stimulated lymphocytes produce lymphokines. Lymphocytes in turn activate the host T cell. Activated T cell further activates the B cells. The stimulated B cell reacts with the self antigen and causes the damage.

How to prevent graft rejection?

Before transplantation the following things should be done to avoid graft rejection.

- Perform blood grouping and Rh grouping
- HLA typing should be done
- Immuno suppressive drugs should be administered
- Suitable donor should be chosen

11.5 Immunization/Vaccination

Father of Immunology is Edward Jenner. He produced the vaccine for small pox from cow pox virus. Vaccine is a substance that is introduced into the body to prevent the disease produced by certain pathogens. Vaccines consist of dead pathogens or live but attenuated (artificially weakened) organisms.

Immunization programmes and the development of new vaccines play an important role in protecting individuals against illness. Vaccination works by safely exposing individuals to a specific pathogenic microbe, artificially increasing their immunity to it.

Vaccines are made from

- Live micro-organisms that have been 'treated' so that they are weakened (attenuated) and are unable to cause disease.
- Dead micro-organisms.
- Some part or product of the micro-organism that can produce an immune response.

TN_GOVT_XII_Micro_Biology_CH11.indd 236 07-12-2021 15:38:16

Vaccine Types

- **Live attenuated vaccines:** These vaccines contain modified strains of a pathogen that have been weakened but are able to multiply within the body and remain antigenic enough to induce a strong immune response. Example: Oral Polio vaccine
- **Heterologous vaccine:** These are a group of live attenuated vaccines produced from the strains that are pathogenic in animals and not in humans. It is a vaccine that confers protective immunity against a pathogen that shares cross-reacting antigens with the microorganisms in the vaccine. Example: Cow pox virus that protects against small pox in humans.
- **Killed inactivated vaccines:** These groups of vaccine are produced either by killing or inactivating the bacteria or virus by chemical treatment or heat. Example:; Polio virus
- **Sub unit vaccine:** The antigenic determinant / epitope (the very specific part of the microbe) is used to prepare the vaccine.
- **DNA Vaccines**: When the genes for microbe's antigens are introduced into the body some cells will take up the DNA. The DNA then instructs those cells to make the antigen molecules. The cells secrete the antigens and display them on their surfaces. The body's own cells become vaccine generating factories.

Routes of Administration

- Deep subcutaneous or intramuscular route – most vaccines
- Oral route – Oral BCG vaccine
- Intradermal route – BCG vaccine
- Scarification – Small pox vaccine
- Intranasal route – Live attenuated influenza virus

Types of Immunization

Immunization is of two types:

1. Passive Immunization
2. Active Immunization

1. Passive Immunization

- Passive immunization is produced without challenging the immune system of the body. It is done by administration of serum or gamma globulins from a person who is already immunized to a non- immune person.
- Passive immunization is the administration of preformed antibodies either intravenously or intramuscularly.
- It is used to provide rapid protection in certain infections such as diphtheria or tetanus or in the event of accidental exposure to certain pathogens such as hepatitis B.
- It is also used to provide protection in immune compromised individuals.

Passive natural immunization - acquired from the mother before and after birth. Before birth, immunity is transferred from mother to the fetus in the form of maternal antibodies through placenta. After birth, the antibodies (Ig A) are transferred through breast milk (Table 11.2).

TN_GOVT_XII_Micro_Biology_CH11.indd 237 07-12-2021 15:38:16

Table 11.2: Passive Immunization

Infection	Source of Antiserum	Indications
Tetanus	Immune human; horse	Post exposure (plus vaccine)
Diptheria	Horse	Post-exposure
Gas gangrene	Horse	Post-exposure
Botulism	Horse	Post-exposure
Varicella-Zoster	Immune human	Post-exposure in immunodeficiency
Rabies	Immune human	Post exposure (plus vaccine)
Hepatitis B	Immune human	Post-exposure prophylaxis
Hepatitis A	Pooled human Ig	Prophylaxis
Measles	Immune human	Prophylaxis
Snakebite	Horse	Post-bite
Some autoimmune disease	Pooled human ig	Acute thrombocytopenia and neutropenia

Passive artificial immunization - developed by injecting previously prepared antibodies using serum from humans or animals. This type of immunity is useful for providing immediate protection against acute infections like tetanus, measles etc.

2. Active Immunization

Active immunization is the administration of vaccines containing microbial products with or without adjuvants in order to obtain long term immunological protection against the offending microbe.

At present the normal route of vaccination in most instances is either intramuscular or subcutaneous.

Oral immunization is the method of choice for polio and *Salmonella typhi* vaccines. However, there is an increasing awareness that this route of immunization may be the best for most immunizations since nearly all infectious agents gain entrance through the mucosal surfaces.

Active natural immunization involves activation of immune system in the body to produce antibodies. It is achieved in both clinical and subclinical infections.

Active artificial immunization is achieved by the administration of vaccines or toxoids.

Antigen preparations

Most vaccines consist of attenuated organisms, killed organisms, inactivated toxins, or sub cellular fragments and more recently genes for antigens in viral 'vectors', and DNA itself. Thus, vaccines must be capable of targeting the immune system appropriately i.e. cellular/or humoral mechanisms (Table 11.3).

TN_GOVT_XII_Micro_Biology_CH11.indd 238 07-12-2021 15:38:16

Table 11.3: Antigen Preparations Used in Vaccines

Type of antigen	Examples	
	Viruses	Bacteria
Normal heterologous organism	Vaccinia (Cowpox)	
Living attenuated organism	Measles	BCG
	Mumps	Typhoid (New)
	Rubella	
	Polio (Sabin)	
	Yellow fever	
	Varicella-Zoster	
Whole killed organism	Rabies	Pertussis
	Poli (Salk)	Typhoid
	Influenza	Cholera
Inactivated toxin (toxoid)		Diphtheria
		Tetanus
		Cholera (New)
Capsular polysaccharide		*Meningococcus*
		Pneumococcus
		Haemophilus
		Typhoid (New)
Surface antigen	Hepatitis B	

Adjuvants

Nonliving vaccines, especially those consisting of small molecules require the inclusion of agents to enhance their effectiveness.

These adjuvants include microbial, synthetic and endogenous preparations having adjuvant activity, but at present only aluminium or calcium salts are generally used in humans.

Adjuvants should enable antigens to be slowly released, preserve antigen integrity, target antigen presenting cells and induce cytotoxic lymphocytes.

11.6 Updated National Immunization Schedule Chart

Immunization/vaccination produce a response in the body that is similar to the body's response to a natural infection (Table 11.4). Immunization or vaccines can therefore protect the body from a disease before the disease has a chance to cause illness. Immunization has helped to reduce the impact of communicable disease on health and well being. Some diseases have been well controlled and other has been eliminated from some parts of the world because of vaccination. Stopping vaccination may lead to epidemic.

Summary

The reaction between antigen and antibody is highly specific. It is compared to the lock and key system. **ELISA** (Enzyme-Linked Immuno Sorbent Assay) is a plate-based assay technique designed for detecting and quantifying substances such as peptides,

TN_GOVT_XII_Micro_Biology_CH11.indd 239 07-12-2021 15:38:16

Table 11.4: National immunization schedule

Sn. No.	Vaccine	Due age	Route
1.	BCG	At birth	Intra dermal
2.	Hepatitis B-Birth dose	At birth	Intra muscular
3.	OPV-O	At birth	Oral
4.	OPV 1, 2 & 3	At 6 weeks, 10 weeks & 14 weeks	Oral
5.	Pentavalent 1, 2 & 3 (Diphtheria + Pertuss is + Tetanus + Hepatitis B + Hib)	At 6 weeks, 10 weeks & 14 weeks	Intra muscular
6.	Inactivated polio vaccine	At 6 & 14 weeks	Intra muscular
7.	Rotavirus (where applicable)	At 6 weeks, 10 weeks & 14 weeks	Oral
8.	Pneumococcal conjugate vaccine (where applicable)	At 6 weeks & 14 weeks. At 9 completed months – booster	Intra muscular
9.	Measles/Rubella 1st dose	At 9 completed months – 12 months	Subcutaneous
10.	DPT Booster-1	16–24 months	Intra muscular
11.	Measles/Rubella 2nd dose	16–29 months	Subcutaneous
12.	OPV Booster	16–24 months	Oral
13.	DPT Booster – 2	5–6 years	Intra muscular
14.	TT	10 years & 16 years	Intra muscular

proteins, antibodies and hormones. There are **four kinds** of ELISA assay tests. They are: Direct ELISA, Indirect ELISA, Sandwich ELISA and Competitive ELISA. Western blotting technique is used for the identification of particular protein from the mixture of proteins. The most common protein sample used for Western blotting is cell lysate. Blotting refers to the transfer of the protein from the gel to the nitrocellulose paper by capillary action. The substances causing allergic/hypersensitivity is known as allergens. Allergic rhinitis develops when the body's immune system becomes sensitized and overreacts to something in the environment like pollen grains, strong odour of perfumes, dust etc. Certain drugs such as penicillin, cephalosporin and streptomycin can absorb non-specifically to protein on surface of RBC forming complex similar to hapten-carrier complex.

Transfer of living cells, tissues or organs from one part of the body to another or from one individual to another is known as transplantation. The graft tissue antigens induce an immune response in the host. This type of immune response is called host versus graft reaction. The ultimate goal of any immunization program is the eradication of the disease. Active natural immunization involves activation of immune system in the body to produce antibodies. It is achieved in both clinical and subclinical infections Immunization has helped to reduce the impact of communicable disease on health and well being.

TN_GOVT_XII_Micro_Biology_CH11.indd 240 07-12-2021 15:38:16

Evaluation

Multiple choice questions

1. Antibody reacts with _____ to give agglutination.
   a. Particulate antigen
   b. Hapten and antigen
   c. Antibody and soluble antigen
   d. Carrier and antibody
2. Anaphylaxis refers to
   a. Immediate hypersensitivity
   b. Hyposensitivity
   c. Delayed hypersensitivity
   d. Auto sensitivity
3. Atopy occurs due to
   a. House dust b. Egg
   c. Pollen d. all the above
4. In type II reaction, _____ is involved.
   a. IgG antibody
   b. IgG and IgM antibodies
   c. IgM antibody
   d. IgE antibody
5. _____ acts as an ACP.
   a. Macrophage
   b. RBC
   c. T cells
   d. Mucosal cells
6. Phagocytosis is enhanced by _____ process.
   a. Pinocytosis
   b. Opsonisation
   c. Endocytosis
   d. None
7. _____ produce antibodies.
   a. T cells
   b. B cells
   c. Ts cells
   d. Plasma cells
8. Sabin is _____ vaccine.
   a. Injection
   b. Recombination
   c. Oral
   d. Subunit
9. _____ is an injectable polio vaccine.
   a. Salk
   b. TAB
   c. Sabin
   d. BCG

Answer the following

1. What do you understand by the term antigen presentation?
2. Define: Pathogenicity.
3. Match the following:
   a. MMR - Subunit vaccine
   b. Salk - Triple vaccine
   c. HBV - Recombinant vaccine
   d. Sabin - Killed vaccine
   e. Influenzae - Live vaccine
4. What is meant by attenuation?
5. Describe toxin with examples.
6. Match:
   a. Mast cell - Myelomaprotein
   b. Primary immune - IgG
   c. Secondary immune response - IgM
   d. Secretory antibody - IgA
   e. Plasma cell tumor - IgE
7. Write a note on Lymphocytes.
8. Define the following:
   a. Immunity
   b. Innate immunity
   c. Acquired Immunity
   d. Active immunity
   e. Passive immunity

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Chapter
12

Microbial Genetics

Learning Objectives

After studying this chapter the students will be able to,

- *Define gene, genome, genetic code, genotype, phenotype, mutagen, wildtype*
- *Describe transcription and translation*
- *Classify mutations and its types and Understand how mutants are formed*
- *Know the mode of action of physical and chemical mutagens*
- *Identify the purpose of and outline the procedure for Ames test*
- *Compare the gene transfer mechanisms*
- *Know the types of cloning vectors used in genetic engineering*
- *Describe how plasmids and bacteriophages are used to transfer foreign DNA*
- *Explain the role of restriction enzymes in recombinant DNA technology*
- *Know the types of restriction enzymes*
- *Understand agarose gel electrophoresis and PCR techniques*
- *Explain RAPD and RFLP*

Chapter Outline

12.1 Concept of Gene

12.2 Transcription

12.3 Genetic Code

12.4 Translation

12.5 Types of Mutation

12.6 Formation of Mutants

12.7 Transfer of Genetic Material

12.8 Recombinant DNA Technology

12.9 Vectors – Types and Characteristics

12.10 Restriction Enzymes

12.11 Techniques in Genetic Engineering

12.1 Concept of Gene

The fundamental unit of information in living systems is the **gene**. **Genome** is the set of all genes and genetic signals of a cell. The information contained in genes is converted to molecules that determine the metabolism, structure and form of microorganisms. Gene is expressed through a sequence of events. A gene can be defined biochemically as a segment of DNA (or, in a few cases, RNA) that encodes the information required to produce a functional biological product. The final product is usually a protein. Not all genes are involved in protein synthesis; some code instead for rRNA and tRNA.

The central dogma of molecular biology, comprises the three major processes (Figure 12.1). The first is **replication**, the

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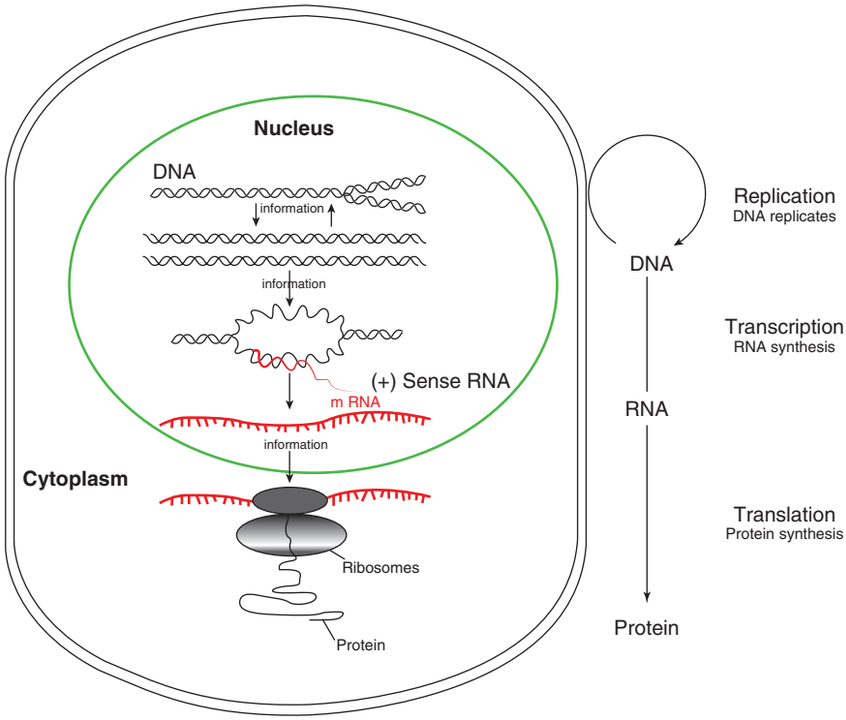

Figure 12.1: Central dogma of molecular biology

copying of parental DNA to form daughter DNA molecules with identical nucleotide sequences. The information contained in the base sequence of DNA is copied into protein molecule through an RNA molecule. The second is **transcription**, production of mRNA from DNA. It is the process by which the segment corresponding to a particular gene is selected and an RNA molecule is synthesized. The third is **translation**, The production of an amino acid sequence from an RNA base sequence. The genetic message encoded in messenger RNA (mRNA) is translated on the ribosomes into a polypeptide with a particular sequence of amino acids. The order of amino acid in a polypeptide chain is determined by DNA base sequence.

12.2 Transcription

An important feature of RNA synthesis is that even though the DNA molecule being copied is double stranded, in any particular region of DNA only one strand serves as a template. The DNA strand copied into RNA molecule is called CODING OR SENSE STRAND.

The synthesis of RNA consists of five discrete stage (Figure 12.2):

1. Promoter recognition: RNA polymerase binds to DNA within a specific base sequence (20–200 bases long) called a promoter. The sequence TATAAT (or a nearly identical sequence) often called a pribnow box or – 10 region is found as part of all prokaryotic promoters.

   The RNA polymerase of the bacterium *E.coli* consists of five protein subunits. Four of the subunits comprise the core enzyme (catalyzes the joining of the nucleoside triphosphates to the RNA) and fifth subunit, the σ subunit (required for promoter binding).

2. Local unwinding of DNA occurs and RNA polymerase forms an open promoter complex.

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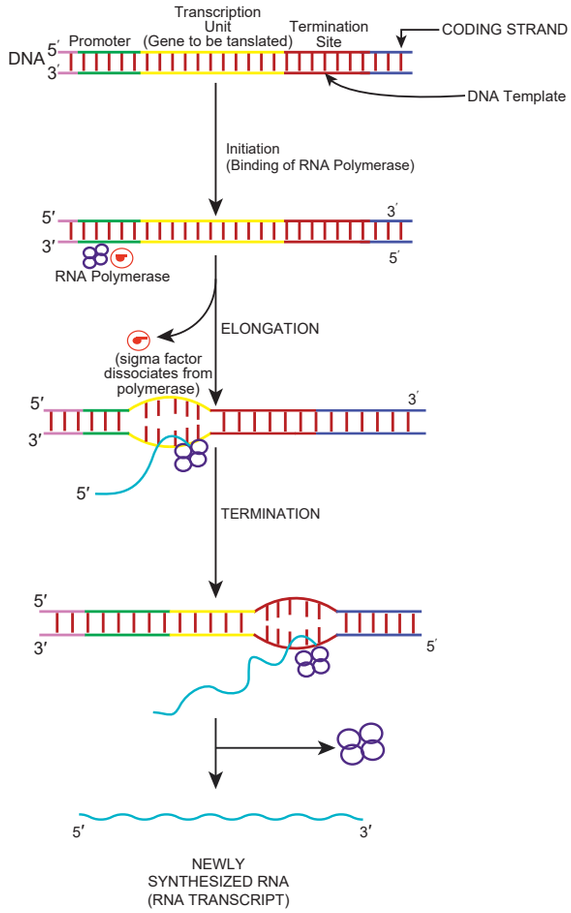

Figure 12.2: Major events in transcription

3. The first nucleoside triphosphate is placed at polymerization start site (near to the initial binding site) and synthesis begins.
4. RNA polymerase then moves along the DNA, adding ribonucleotides, to the growing RNA chain.
5. RNA polymerase reaches chain termination sequence and both the newly synthesized RNA and the polymerase are released. Two kinds of termination events are known those that are self – terminating (dependent on the base sequence only) and those that require the presence of the termination protein Rho.

Initiation of a second round of transcription need not await completion of the first, for the promoter becomes available once RNA polymerase has polymerized 50–60 nucleotides. In bacteria most mRNA molecules are degraded within a few minutes after synthesis. This degradation enables cells to dispense with molecules that are no longer needed.

In prokaryotes mRNA molecules commonly contain information for the amino acid sequences of several different polypeptide chains. In this case, such a molecule is called polycistronic mRNA. Cistron is a term used to mean a base sequence encoding a single polypeptide chain. The genes contained in polycistronic mRNA molecule (Figure 12.3) often encode the different portions of a metabolic pathway. For example, in *E. coli* the ten enzymes needed to synthesize histidine are encoded in one mRNA molecule.

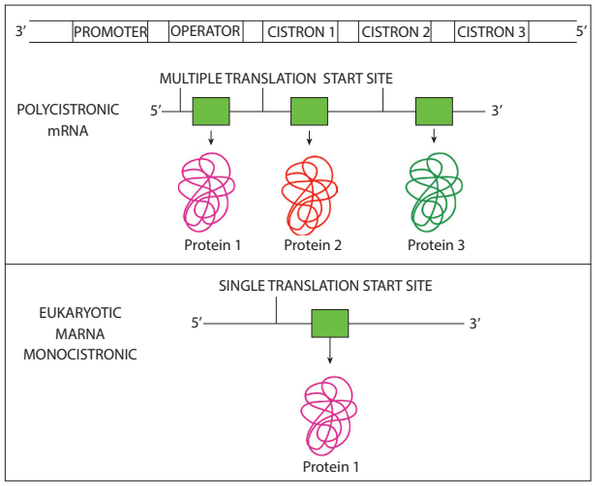

Figure 12.3: Polycistronic mRNA

In prokaryotes the immediate product of transcription (called the primary transcript) is mRNA, in contrast in eukaryotes the primary transcript must be converted to mRNA. This conversion called RNA processing consists of two types of events- modification of termini and excision of untranslated sequences (non-coding sequence or introns) embedded

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within coding sequences (exons). Introns excision and the joining of exons to form an mRNA molecule is called RNA splicing. The introns are present in almost all eukaryotic transcripts but are rare in the free – living unicellular eukaryotes such as yeast. Some bacterial genes do contain introns.

Synthesis of rRNA and tRNA

Ribosomal RNA and tRNA are also transcribed from genes. The production of these molecules is not as direct as synthesis of bacterial mRNA. The main difference is that these RNA molecules are excised from large primary transcripts. Highly specific RNA excise rRNA and tRNA from these large transcripts, and other enzymes produce the modified bases in tRNA.

12.3 Genetic Code

A tRNA molecule "reads" the base sequence of mRNA. The language read by the tRNA molecules is called the genetic code, which is a set of relations between sequences of three adjacent bases on an mRNA molecule and particular amino acids. (A RNA base sequence (a set of 3 bases) corresponding to a particular amino acid is called a codon). The genetic code is the set of all codons. Only four bases in DNA serve to specify 20 amino acids in proteins, so some combination of bases is needed for each amino acid. Before the genetic code was elucidated, it was reasoned that if all codons were assumed to have the same number of bases, then each codon would have to contain at least three bases. Codons consisting of pairs of bases would be insufficient because four bases can form only $4^2 = 16$ pairs, and there are 20 amino acid. Triplets of bases would suffice because, these can form $4^3 = 64$ triplets. In fact, the genetic code is a triplet code, and all 64 possible codons carry information of some sort. Several different codons designate the same amino acid. Furthermore, in translating mRNA molecules the codons do not overlap but are used sequentially. The same genetic code is used by almost all biological systems and hence is said to be universal (exceptions are mitochondria and a few unusual microorganisms). The codons are by convention written with the 5′ end at the left. The complete code is shown in Table 12.1.

Features of the Code:

- Sixtyone codons correspond to amino acids. Four codons are signals. These are the three stop codons – UAA, UAG, UGA – and the one start codons, AUG. The start codons (initiation codon) also specifies the amino acid methionine. In rare cases, certain other codon (E.g. GUG) initiate translation. No normal tRNA molecule has an anticodon (a sequence of three bases on tRNA that can base – pair with a codon sequence in the mRNA) complementary to any of the stop codons UAG, UAA or UGA, which is why these codons are stop signals.
- The code is highly redundant i.e. more than one codons code for an amino acid. Only tryptophan and methionine are specified by one codon. The synonymous codons usually differ only in third base (except for serine, leucine and arginine).

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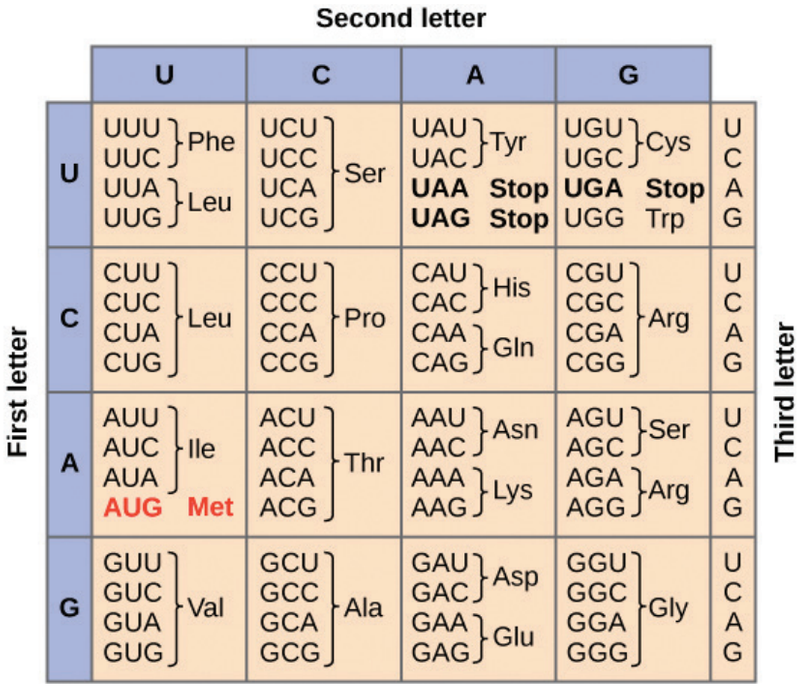

The 20 amino acids and their abbreviations	
Amino acid	3-letter abbreviation
Alanine	Ala
Arginine	Arg
Asparagine	Asn
Aspatic acid	Asp
Aspartic acid & Asparagine	Asx
Cysteine	Cys
Glutamine	Gln
Glutamic acid	Glu
Glutaine or Glutamic acid	Glx
Glycine	Gly
Histidine	His
Isoleucine	IIe
Leucine	Leu
Lysine	Lys
Methionine	Met

Table 12.1: Genetic code

HOTS

1. How many of the 64 codons can be made from the three nucleotides A, U, and C?
2. If codons were four bases long, how many codons would exist in a genetic code?

12.4 Translation

RNA is translated from the 5′ end of the molecule toward the 3′ end. Polypeptides are synthesized from the amino terminus toward the carboxyl terminus, by adding amino acids one by one to the carboxyl end.

Not all the base sequences in an mRNA is translated into amino acid sequences of polypeptides. Initiation of polypeptide synthesis may begin hundreds of nucleotides from the 5′ – P terminus of the RNA. The section of untranslated RNA before the region encoding the first polypeptide chain is called a **leader**, which in some cases contains regulatory sequences that influence the rate of protein synthesis. The major events in translation are (Figure 12.4).

1. An mRNA binds to the surface of a protein synthesizing particle, the **Ribosome**.
2. The tRNA – amino acid complexes (made by the aminoacyl tRNA synthetases) bind sequentially, one by one, to the mRNA molecule that is attached to the ribosome.

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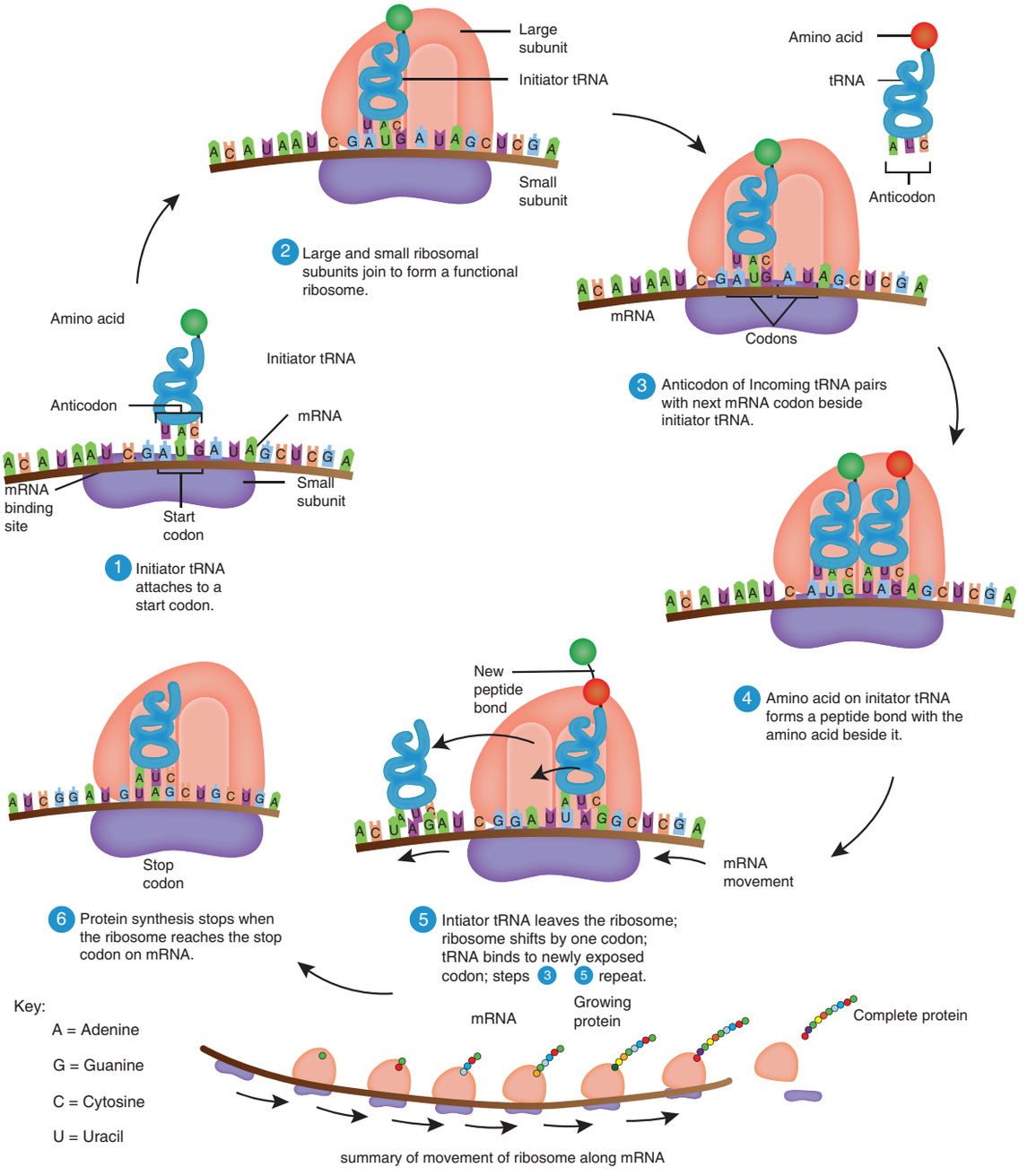

Figure 12.4: Major events in Translation

3. Peptide bonds are made between successively aligned amino acids.
4. Finally the chemical bond between the tRNA and its attached amino acids is broken and the completed protein is removed.

- The 3′ terminal of the tRNA molecule (Figure 12.5) is covalently linked to the amino acid corresponding to the particular mRNA codon
- When an amino acid has become attached to a tRNA molecule, the tRNA is said to be acylated or charged
- An important feature of initiation of polypeptide synthesis in both prokaryotes and eukaryotes is the use of a **specific initiating tRNA molecule**. In prokaryotes this tRNA molecule is acylated with the modified amino acid N – formyl methionine (fMet). This

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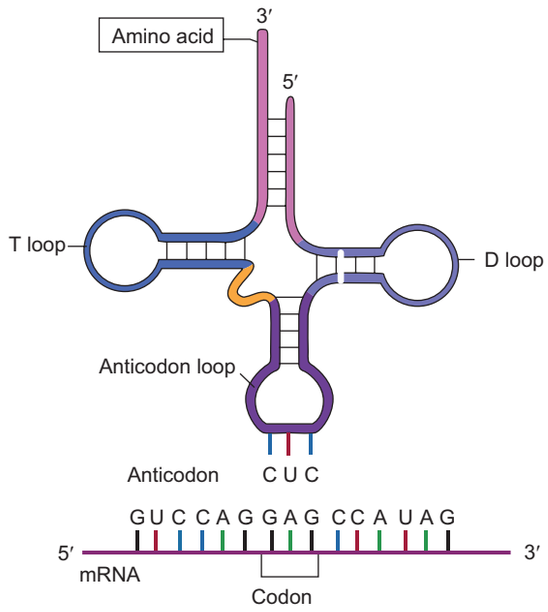

Figure 12.5: tRNA

tRNA is often designated **tRNA^{fMet}**. Both tRNA^{fMet} and tRNA^{Met} recognize the codon AUG, but only tRNA^{fMet} is used for initiation. All prokaryotic proteins while being synthesized have fMet at the amino terminus. However, this amino acid is frequently deformylated or removed later.

- The usual form of translation unit is a polyribosome or polysome wherein an mRNA is covered with ribosomes.

Redundancy and the Wobble Hypothesis

The identity of the third base of a codon appears to be unimportant. (The first base in a codon is at the 5′ end and the third base is at the 3′ end). Wobble refers to the less stringent requirement for base pairing at the third position of the codon than at the first two positions. That is the first two bases must follow Watson and Crick base pairing rule (A with U, or G with C), but the third base pair can be of a different type (for example, G with U). The Wobble hypothesis explains the pattern of redundancy in the code in that certain anticodons (For example, those containing U and G in the first position of the anticodons) can pair with several codons during translation (Figure 12.6).

12.5 Types of Mutations

The base sequence of DNA determines the amino acid sequence of a protein. The chemical and physical properties of each protein are determined by its amino acid sequence, so a single amino acid change is capable of altering the activity of, or even completely inactivating, a protein. **Genotype** refers to the genetic composition

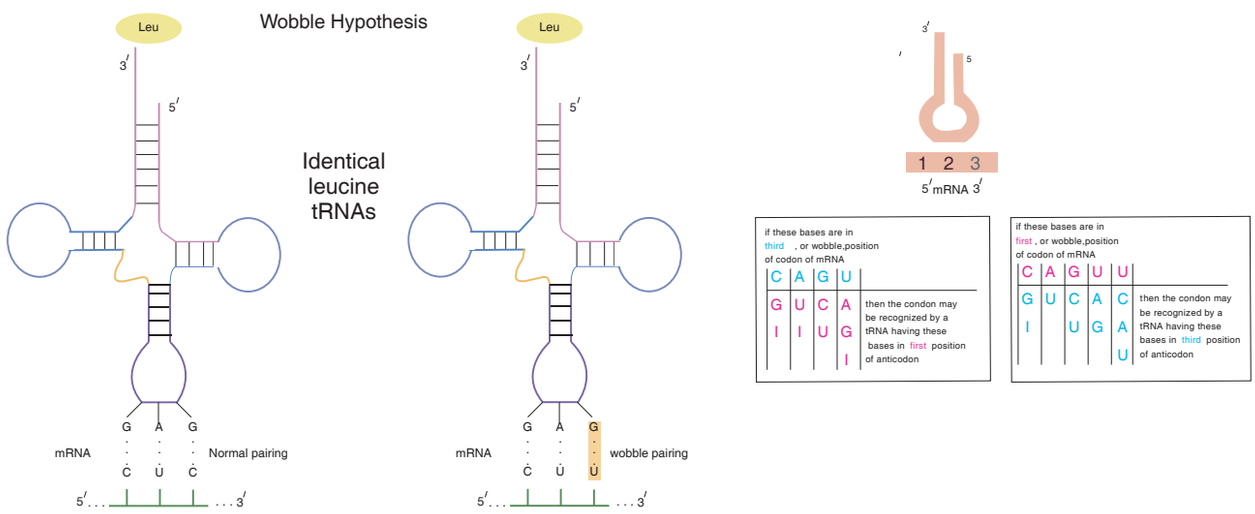

Figure 12.6: Wobble hypothesis

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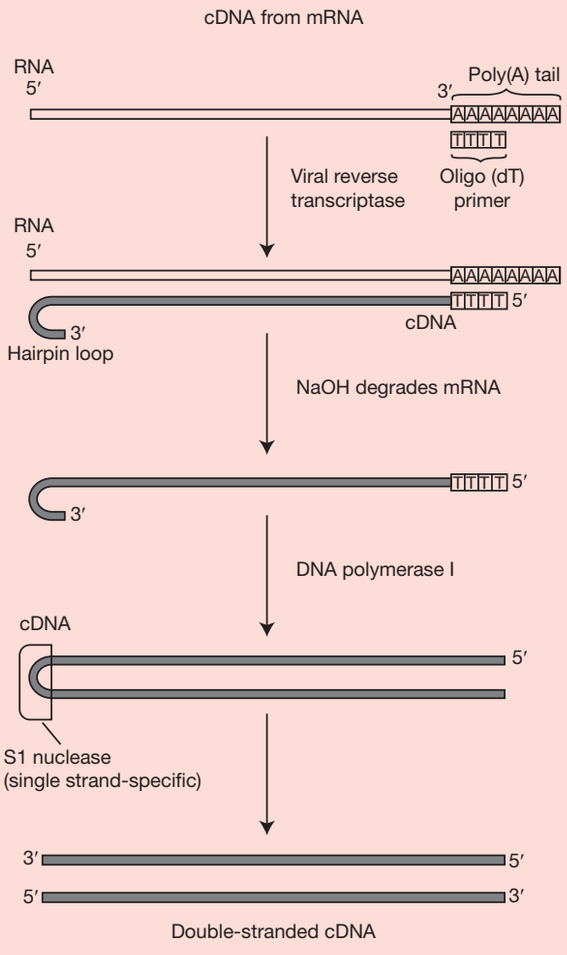
In 1970 Howard Temin and David Baltimore independently discovered the enzyme **reverse transcriptase** that retroviruses use to produce DNA copies of their RNA genome. This enzyme can be used to construct DNA copy, called **complementary DNA** (cDNA), of any RNA as shown in figure below . Thus genes or major portions of the gene can be synthesized from mRNA.



of an organism. **Phenotype** is an observable property of organism. The functional form of a gene is called **Wildtype** because presumably this is the form found in nature.

Mutation is the process by which the sequence of base pairs in a DNA molecule is altered.The alteration can be a single base pair substitution, insertion or deletion.

Mutations can be divided into two general categories:

1. **Base–pair substitution mutation** involves a change in the DNA such that one base pair is replaced by another.
   - A mutation from one purine – pyrimidine base pair to the other purine –pyrimidine base pair is a transition mutation (Figure 12.7a). E.g. AT to GC, CG to TA.
   - A mutation from a purine - pyrimdine base pair to a pyrimidine – purine base pair is a transversion mutation (Figure 12.7b). E.g. AT to TA, CG to GC.
2. **Base pair insertion or deletions** involves the addition or deletion of one base pair. If one or more base pairs are added to or deleted from a protein coding gene, the reading frame of an mRNA can change downstream of the mutation. An addition or deletion of one base pair, for example, shifts the mRNA's downstream reading frame by one base, so that incorrect amino acids are added to the polypeptide chain after the mutation site. This type of mutation, called a frame shift mutation (Figure 12.8) usually results in a nonfunctional protein.

Frame shift mutations:

- May generate new stop codons, resulting in a shortened protein.
- May result in a read through of the normal stop codon, resulting in longer than normal proteins
- Or may result in a complete alteration of the amino acid sequence of a protein.

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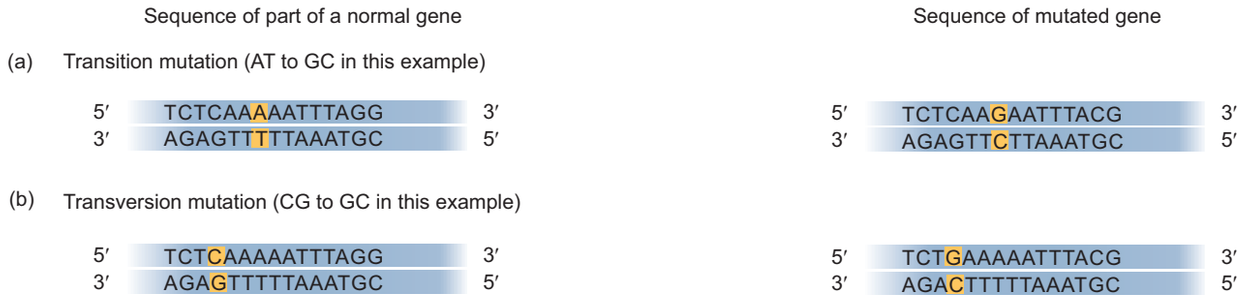

Figure 12.7: (a) Transition mutations **(b)** transversion mutations

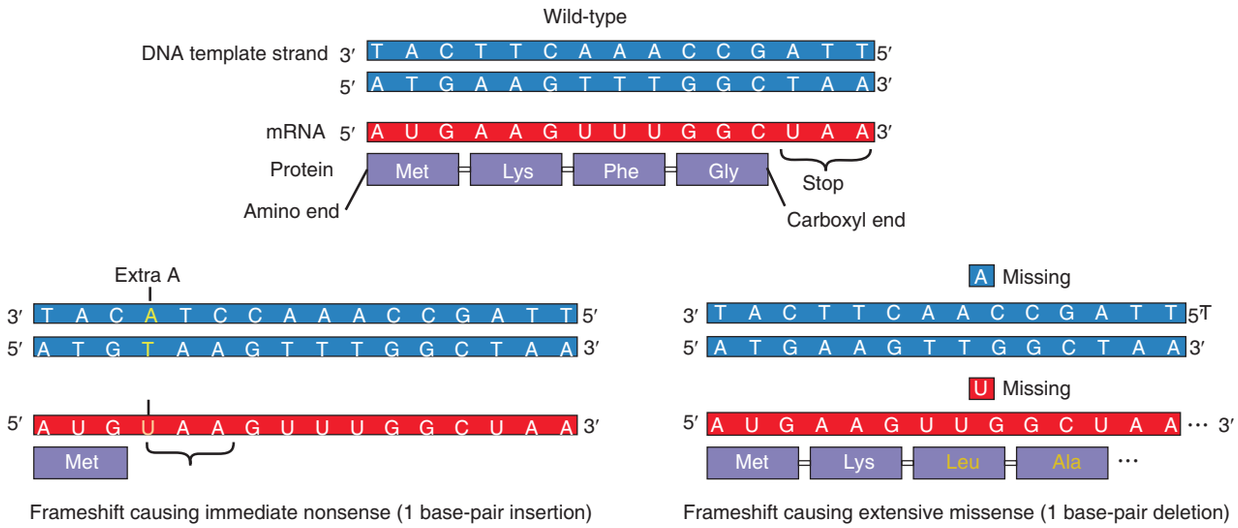

Figure 12.8: Frameshift mutations

Point mutations are single base changes, that do not affect the reading frame, that is, the mutation only makes a single change in a single codon, and everything else is undisturbed.

Mutations can also be defined according to their effects on amino acid sequences in proteins. They are

1. A missense mutation (Figure 12.9a) is a gene mutation in which a base – pair change in the DNA changes a codon in an mRNA so that a different amino acid is inserted into the polypeptide.
2. A neutral mutation (Figure 12.9b) is a subset of missense mutations in which the new codon codes for a different amino acid that is chemically equivalent to the original and therefore does not affect the proteins function. Consequently, the phenotype does not change.
3. A silent mutation (Figure 12.9c) is also a subset of missense mutations that occurs when a base – pair change in a gene alters a codon in the mRNA such that the same amino acid is inserted in the protein. In this case, the protein obviously has a wild type function.
4. A nonsense mutation (Figure 12.9d) is a gene mutation in which a base – pair change in the DNA, changes a codon in an mRNA to a stop (nonsense) codon (UAG, UAA or UGA). Nonsense mutation cause premature chain termination so instead of complete polypeptides, shorter than normal polypeptide fragments (often nonfunctional) are formed.

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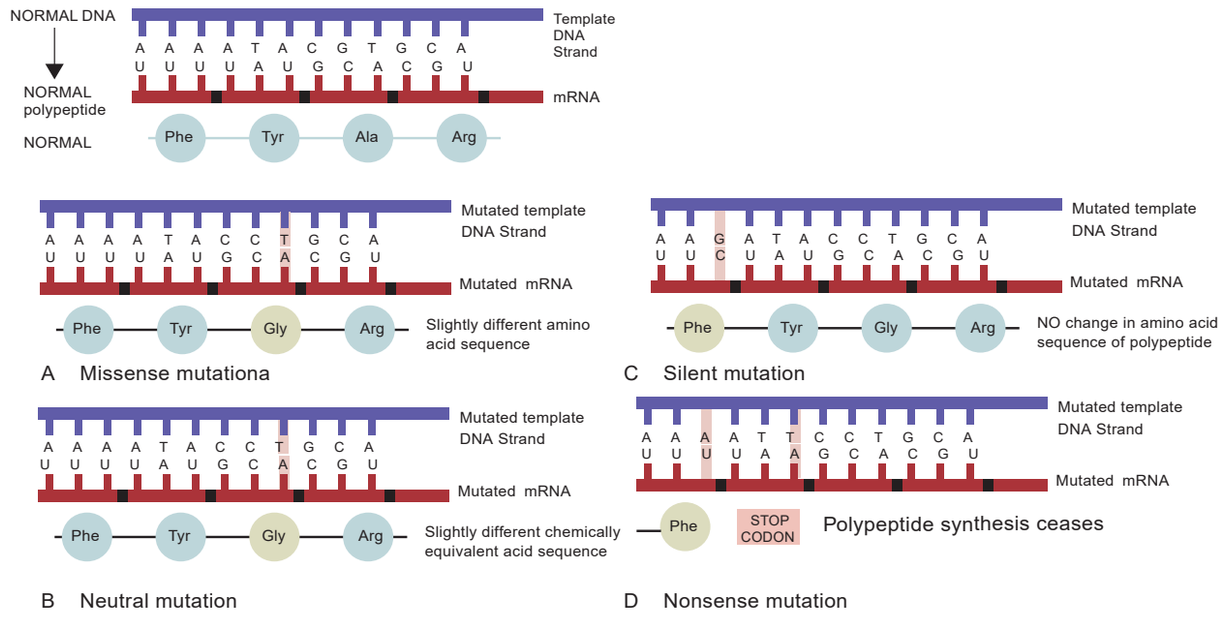

Figure 12.9: **(a)** Missense **(b)** neutral **(c)** silent **(d)** nonsense mutation respectively

Forward mutations change the genotype from wild type to mutant and reverse mutations (or reversions or back mutations) change the genotype from mutant to wild type or to partially wild type. An organism which has reverted is a Revertant. The effects of mutation may be diminished or abolished by a suppressor mutation. Suppressor mutation is a mutation at a different site from that of the original mutation. A suppressor mutation masks or compensates for the effects of the initial mutation, but it does not reverse the original mutation.

12.6 Formation of Mutants

The term mutant refers to an organism in which either the base sequence of DNA or the phenotype has been changed. A mutant is an organism whose genotype differs from that found in nature. The process of formation of mutant organism is called mutagenesis. In nature and in the laboratory, mutations sometimes arise spontaneously, without any help from the experimenter. This is called spontaneous mutagenesis. The two mechanisms that are most important for spontaneous mutagenesis are

1. Errors occurring during replication and
2. Spontaneous alteration of bases.

Mutations can also be induced experimentally by application of mutagens. Mutagens are agents that cause mutations.

Mutagens and their Mode of Action

Physical Mutagens

UV radiation: UV light causes mutations because the purine and pyrimidine bases in DNA absorb light strongly in the ultraviolet range (254 to 260 nm). At this wavelength, UV light induces point mutations primarily by causing photochemical changes in the DNA. One of the effects of UV radiation on DNA is the formation of abnormal chemical bonds between adjacent pyrimidine

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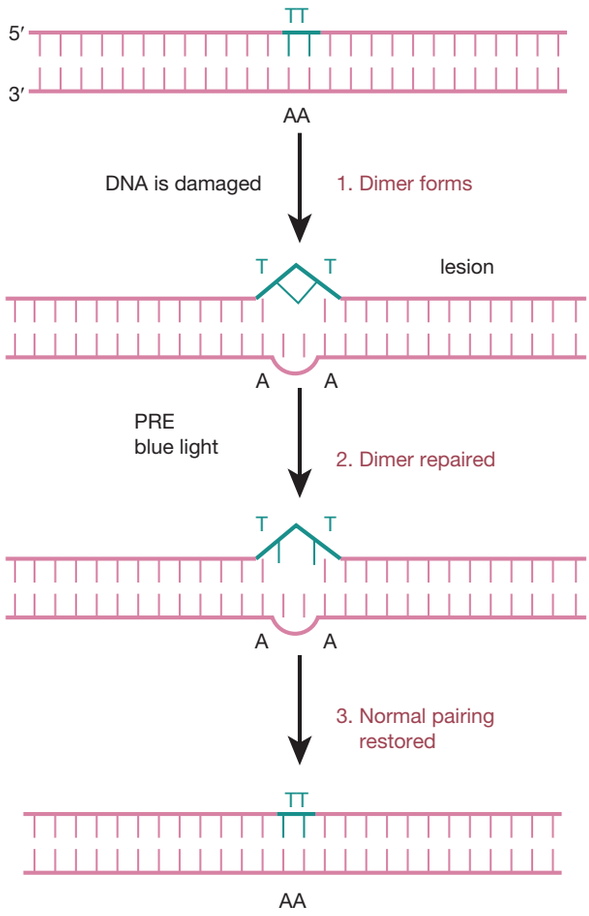

Figure 12.10: UV induced DNA damage

molecules in the same strand, or between pyrimidines on the opposite strands, of the double helix. This bonding is induced mostly between adjacent thymines, forming what are called thymine dimers (Figure 12.10), usually designated TT. This unusual pairing produces a bulge in the DNA strand and disrupts the normal pairing of T's (thymines) with corresponding A's(adenines) on the opposite strand. If UV induced genetic damage is not repaired, mutations or cell death may result.

Chemical Mutagens

Chemical mutagens include both naturally occurring chemicals and synthetic substances. These mutagens can be grouped into different classes on the basis of their mechanism of action. They are

i. **Base analogs** are bases that are similar to the bases normally found in DNA. E.g. 5 – bromouracil (5-BU). TA to CG (Figure 12.11).

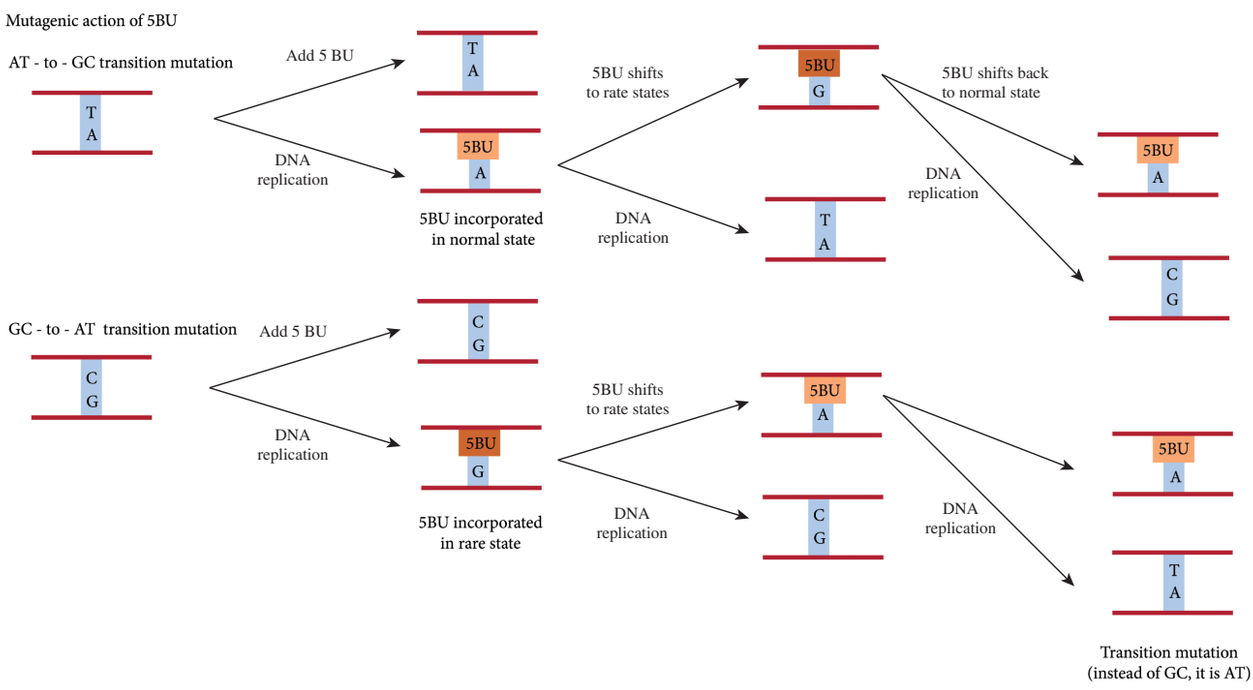

Figure 12.11: Mutagenic effects of 5-BU

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Not all base analogs are mutagens. For example, AZT (Azidothymidine), one of the approved drugs given to patients with AIDS, is an analog of thymidine, but it is not a mutagen, because it does not cause base pair changes.

ii. **Base Modifying Agents** are chemical that act as mutagens by modifying the chemical structure and properties of bases. The three types of mutagens that work in this way are

1. A deaminating agent e.g.: Nitrous acid removes amino groups (- NH_2) from the bases guanine, cytosine, and adenine.
2. Hydroxylamine (NH_2 OH) is a hydroxylating mutagen that react specifically with cytosine, modifying it by adding a hydroxyl group (OH) so that it can pair solely with adenine instead of with guanine.
3. Alkylating agents like methymethane sulfonate (MMS) introduces alkyl groups onto the bases at a number of location.

iii. Intercalating agents

Acridine, proflavin, ethidium bromide are a few examples of intercalating agents. These insert (intercalate) themselves between adjacent bases in one or both strands of the DNA double helix. Intercalating agents can cause either additions or deletions.

Original base	Mutagen	Modified base Pairing partner	Predicted transition
Cytosine	Nitrous acid (H_2NO) →	Uracil ═ Adenine	CG TA
Cytosine	Hydroxylamine (NH_2OH) →	Hydroxyl amino cytosine ═ Adenine	CG TA
Guanine	Methylmethane sulfonate(MMS) (alkylating agent) →	O-Methylguanine ═ Thymine	CG TA

Figure 12.12: Action of three base modifying agents.

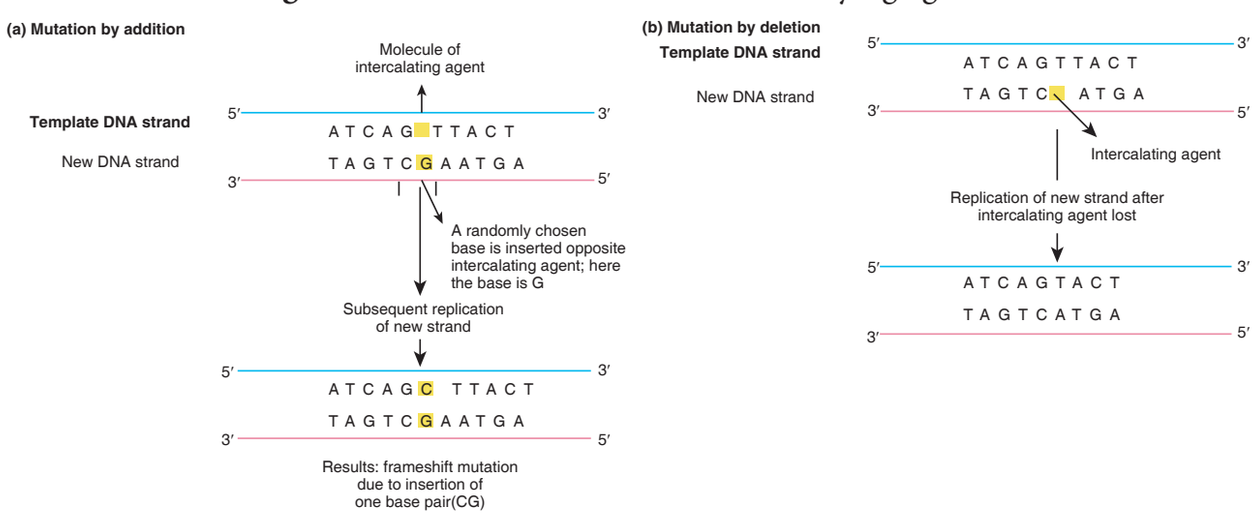

Figure 12.13: Mutations due to intercalating agents

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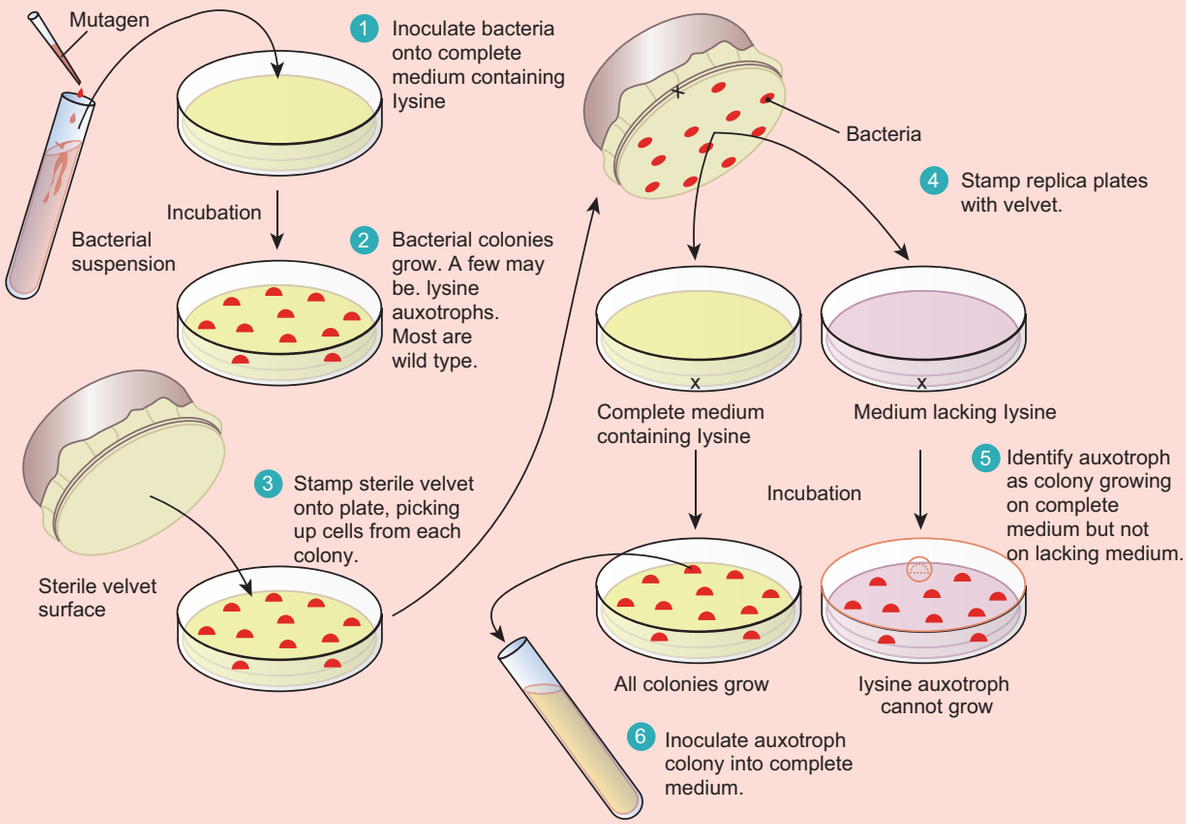
Isolation and detection of Mutants

Once mutations are induced, then, they must be detected if they are to be studied. Selection and screening procedures historically have helped geneticists isolate mutants of interest from a heterogenous mixture in a mutagenized population. When isolating mutants of a particular organism, one must know the normal or wild type characteristics so as to recognize an altered phenotype. A suitable detection system for the mutant phenotype under study also is needed. Detection systems in bacteria and other haploid organisms are straightforward because any new allele should be seen immediately, even if it is recessive mutation. The detection of mutants can be direct and complex. For example, the replica plating technique is used to detect auxotrophic mutants (mutants which are deficient in synthesizing a particular biochemical compound). **Replica plating technique** distinguishes between mutant and wild type strain based on their ability to grow in the absence of a particular biosynthetic end product Figure below. A lysine auxotroph, for instance, will grow on lysine supplemented media but not on a medium lacking an adequate supply of lysine because it cannot synthesize this amino acid.



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The Ames Test: A Screen for Potential Carcinogens

Everyday we are exposed to a wide variety of chemicals in our environment, such as drugs, cosmetics, food additives, pesticides, and industrial compounds. Many of these chemicals can have mutagenic effects, including genetic diseases and cancer. Some banned chemical warfare agents (e.g. mustard gas) also are mutagens.

A number of chemicals (subclass of mutagens) induce mutations that result in tumorous or cancerous growth. These chemical agents are called chemical carcinogens. Directly testing the chemicals for their ability to cause tumors in animals is time consuming and expensive. However, the fact that most chemical carcinogens are mutagens led Bruce Ames to develop a simple, inexpensive, indirect assay for mutagens. In general Ames test is an indicator of whether the chemical is a mutagen. The Ames test assays the ability of chemicals to revert mutant strains of the bacterium *Salmonella typhimurium* to wild type. The mutant strain of *S.typhimurium* is auxotrophic to histidine (his^-), that is it requires histidine for its growth and cannot grow in the absence of histidine. The mutant strain is grown in a histidine deficient medium containing the chemical to be tested. A control plate is also set up which does not contain the chemical. After incubation the control plates may have few colonies resulting from spontaneous reversion of the his- strain. Compared to the control plates if there are increased number of colonies on test plate, it indicates that the chemical has reverted the mutant strain back to wild type. This chemical is likely to be a carcinogen. Figure 12.14 shows steps in Ames test.

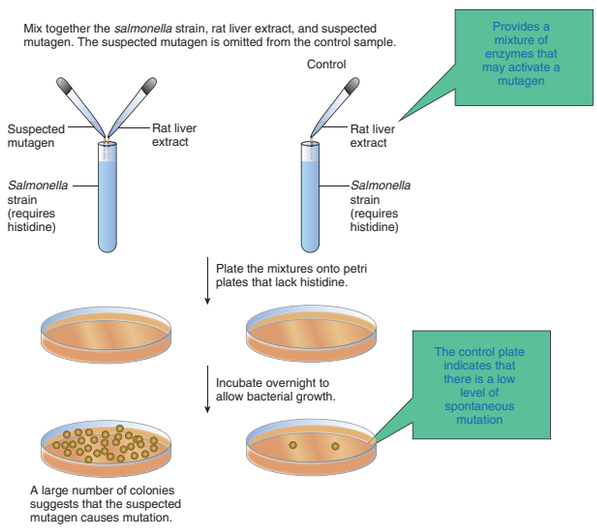

Figure 12.14: Steps in Ames test

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DNA Repair

Both prokaryotes and eukaryotes have a number of repair systems that deal with different kinds of DNA damage. All the systems use enzymes to make correction. Without this repair systems lesions would accumulate and be lethal to the cell or organism. Not all lesions are repaired, and mutations do appear, but at low frequencies. At high doses of mutagens, repair systems are unable to correct all of the damage, and cell death may result. We can group repair systems into different categories on the basis of the way they operate. Some systems correct damaged areas by reversing the damage. This type of repair is called direct correction or direct reversal. Other systems excise the damaged areas and then repair the gap by new DNA synthesis. Some of the DNA repair systems are

- Mismatch repair by DNA polymerase proofreading
- Repair of UV induced pyrimidine dimers- Photo reactivation or Light repair
- Base excision repair
- Nucleotide excision repair

TN_GOVT_XII_Micro_Biology_CH12.indd 255 07-12-2021 15:43:40



With recombinant DNA technology it is possible to mutate a gene at specific positions in the test tube by SITE SPECIFIC MUTAGENESIS and then introduce the mutated gene back into the cell and investigate the phenotypic changes produced by the mutation in vivo. Such techniques enable geneticists to study, for example, genes with unknown function and specific sequences involved in regulating a gene's expression.

12.7 Transfer of Genetic Material

Normally, genes and the characteristics they code for are passed down from parent to progeny. This is called vertical gene transfer. Bacteria and some lower eukaryotes are unique in that they can pass DNA from one cell of the same generation to another. The exchange of genes between two cells of the same generation is referred to as horizontal gene transfer. Mechanisms like transformation, transduction and conjugation takes place naturally and may bring about genetic variation and genetic recombination. These gene transfer mechanisms are also employed in genetic engineering to introduce desired gene into the cells. Introducing a foreign gene or recombinant DNA into the cells is one of the techniques used in genetic engineering. The success of cloning depends on the efficiency of gene transfer process. The most commonly employed gene transfer methods are transformation, conjugation, transduction, electroporation, lipofection and direct transfer of DNA. The choice of the method depends on the type of host cell (bacteria, fungi, plant, animal). Figure 12.15 shows methods of DNA transfer.

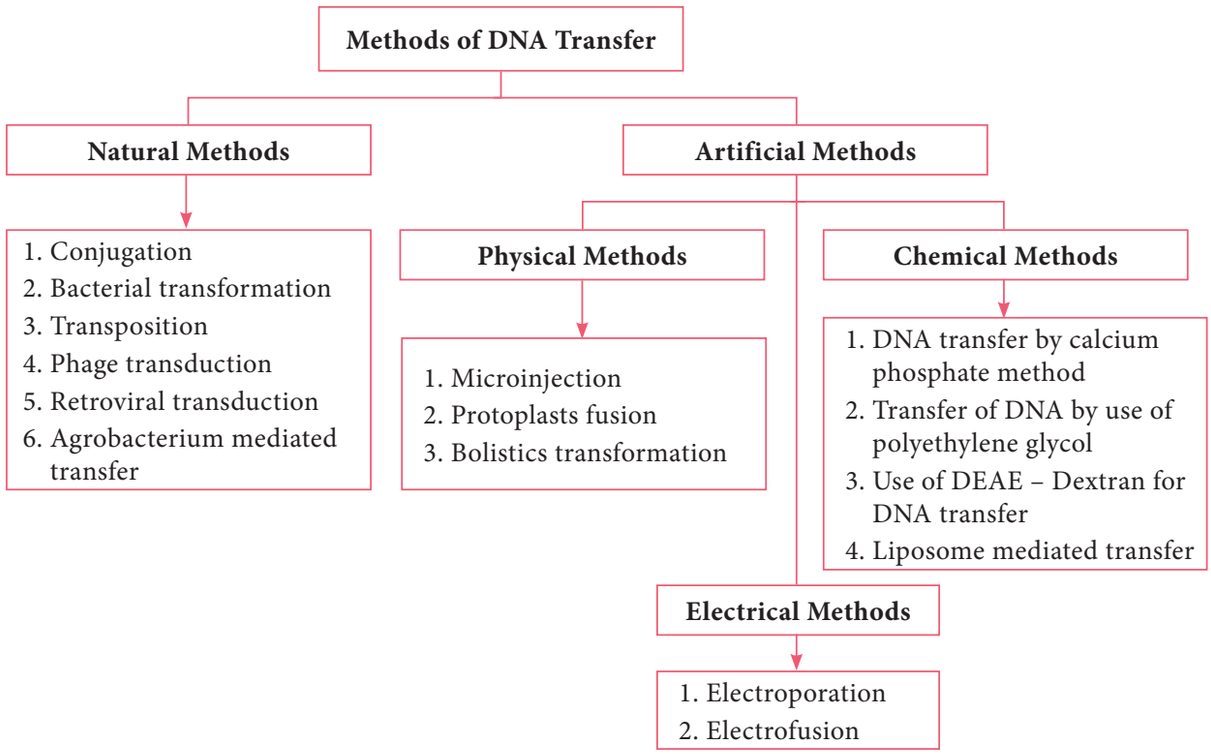

Figure 12.15: Methods of DNA transfer

Note: The term Transfection is used for the transfer of DNA into eukaryotic cells by various physical or chemical means.

TN_GOVT_XII_Micro_Biology_CH12.indd 256 07-12-2021 15:43:40

12.7.1 Transformation

Transformation is genetic alteration of a cell resulting from the direct uptake, incorporation and expression of exogenous genetic material (exogenous DNA) from its surroundings. Transformation occurs naturally in some species of bacteria, but it can also take place by artificial means in other cells. For transformation to happen, bacteria must be in a state of competence. Competence refers to the state of being able to take up exogenous DNA from the environment. There are two forms of competence: natural and artificial. Transformation works best with DNA from closely-related species. The naturally-competent bacteria carry sets of genes that provide the protein machinery to bring DNA across the cell membrane(s).

There are some differences in the mechanisms of DNA uptake by gram positive and gram negative cells. However, they share some common features that involve related proteins. The DNA first binds to the surface of the competent cells on a DNA receptor, and passes through the cytoplasmic membrane via DNA translocase. Only single stranded DNA may pass through, one strand is therefore degraded by nucleases in the process, and the translocated single-stranded DNA may then be integrated into the bacterial chromosomes. Figure 12.16 shows mechanism of transformation.

Artificial competence can be induced in laboratory by procedures that involve making the cell passively permeable to DNA. Typically, the cells are incubated in a solution containing divalent cations; most commonly, calcium chloride solution under cold condition, which is then exposed to a pulse of heat shock. Electroporation is another method of promoting competence. Using this method, the cells are briefly shocked with an electric field of 10–20 kV/cm which is thought to create holes in the cell membrane through which the plasmid DNA may enter. After the electric shock, the holes are rapidly closed by the cell's membrane-repair mechanisms.

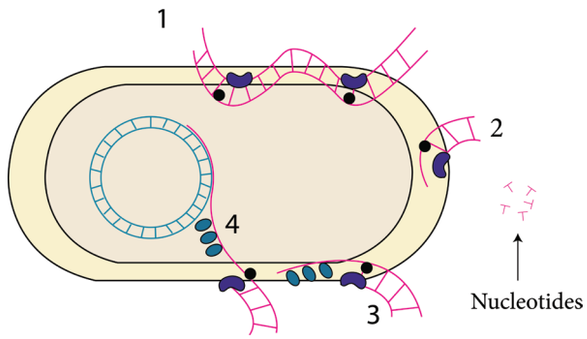

Figure 12.16: Mechanism of transformation **1.** Binding of DNA; **2.** Degradation of one strand; **3.** Entry of ssDNA; **4.** Integration into host chromosome.

12.7.2 Conjugation

The initial evidence for bacterial conjugation, came from an experiment performed by Joshua Lederberg and Edward L Tatum in 1946. Later in 1950, Bernard Davis gave evidence that physical contact of the cells was necessary for conjugation. During conjugation, two live bacteria (a donor and a recipient) come together, join by cytoplasmic bridges (e.g. pilus) and transfer single stranded DNA (from donor to recipient).

Inside the recipient cell, the new DNA may integrate with the chromosome (rather rare) or may remain free (as is the case with plasmids). Conjugation can occur among the cells from different genera of bacteria, while transformation takes place among the cells of a bacterial genus.

TN_GOVT_XII_Micro_Biology_CH12.indd 257 07-12-2021 15:43:41

A plasmid called the fertility or F factor plays a major role in conjugation. The F factor is about 100 kilobases long and bears genes responsible for cell attachment and plasmid transfer between specific bacterial strains during conjugation. F factor is made up of

a. tra region (tra operon / transfer genes): genes coding the F pilus and DNA transfer,

b. Insertion sequence: genes assisting plasmid integration into host cell chromosome.

Thus, the F factor is an **episome** - a genetic material that can exist outside the bacterial chromosome or be integrated into it.

During $F^+ \times F^-$ mating or conjugation (Figure 12.17a) the F factor replicates by the rolling circle mechanism and a copy moves to the recipient. The channel for DNA transfer could be either the hollow F pilus or a special conjugation bridge formed upon contact. The entering strand is copied to produce double – stranded DNA.

F factor can integrate into the bacterial chromosome at several different locations by recombination between homologous insertion sequences present on both the plasmid and host chromosomes. The integration of F factor into bacterial chromosome results in formation of HFR (High Frequency Recombination) cell. When integrated, the Fplasmid's tra operon is still functional; the plasmid can direct the synthesis of pili, carry out rolling circle replication, and transfer genetic material to an F^- recipient cell. An HFR cell is so called because it exhibits a very high efficiency of chromosomal gene transfer in comparison with F^+ cells. In F^+ cells the independent F factor rarely transfer chromosomal genes hence the recombination frequency is low. Figure 12.17b shows formation of HFR cell. When an HFR cell is mated with F^- cell the F^- recipient does not become F^+ unless the whole chromosome is transferred as explained in Figure 12.17c. The connection usually breaks before this process is finished. Thus, complete F factor usually is not transferred, and the recipient remains F^-.

Because the F plasmid is an episome, it can leave (deintegrate) the bacterial chromosome. Sometimes during this process, the plasmid makes an error in excision and picks up a portion of the chromosomal material to form an F′ plasmid. Figure 12.17d shows formation of F′. During F′XF^- conjugation (Figure 12.17e) the recipient becomes F′ and is a partially diploid since it has two set of the genes carried by the plasmid.

The natural phenomenon of conjugation is now exploited for gene transfer and Recombinant DNA technology. In general, the plasmids lack conjugative functions and therefore, they are not as such capable of transferring DNA to the recipient cells. However, some plasmids with conjugative properties can be prepared and used.

12.7.3 Transduction

Transduction is the transfer of bacterial genes from one bacteria to other by viruses. Example: Bacteriophage (Bacterial viruses). To understand the

TN_GOVT_XII_Micro_Biology_CH12.indd 258 07-12-2021 15:43:41

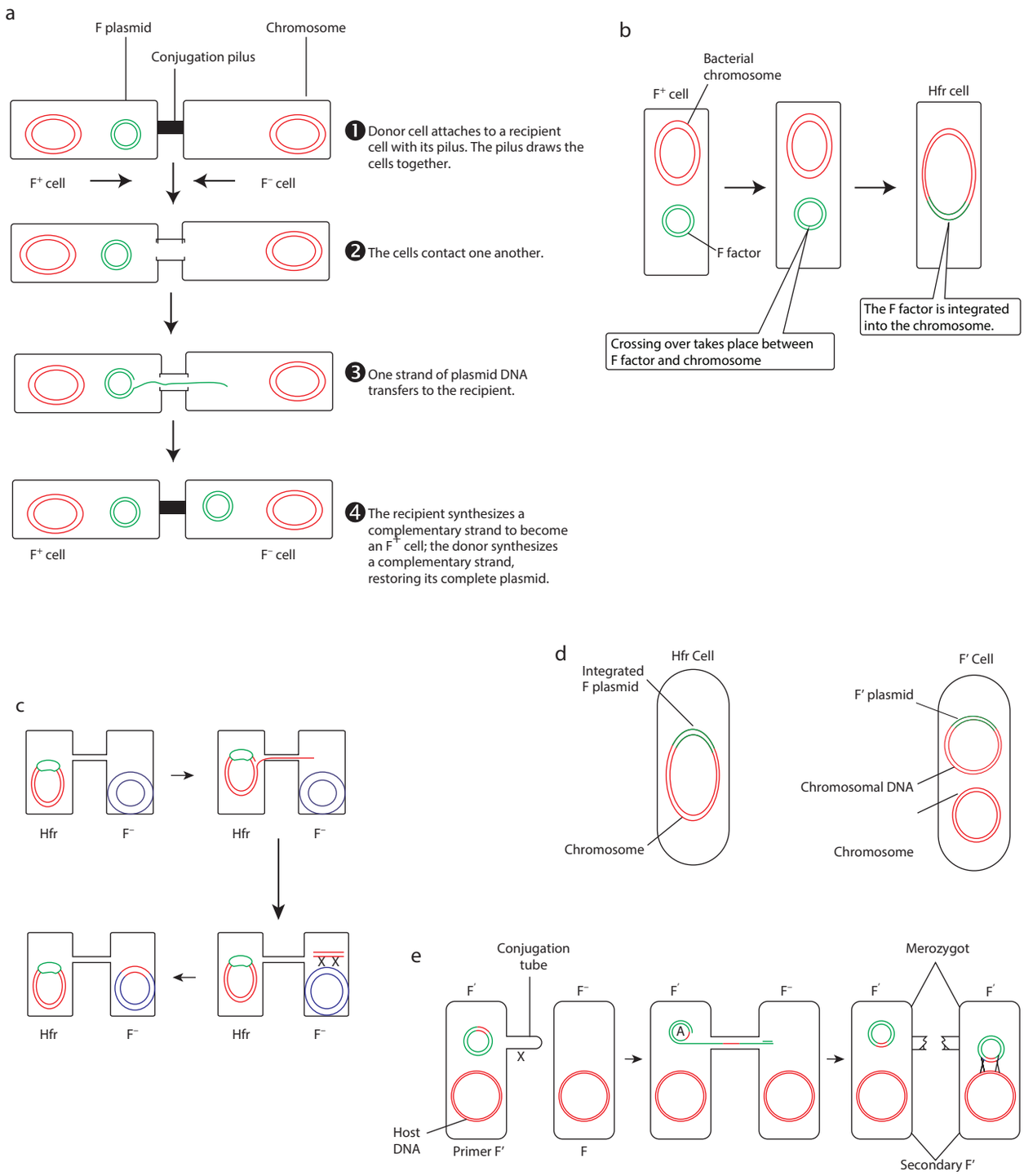

Figure 12.17: Mechanism of conjugation **(a)** $F^+ \times F^-$ **(b)** HFR cell Formation **(c)** HFR $\times$ F^- **(d)** F′ formation **(e)** F′ $\times$ F^-

TN_GOVT_XII_Micro_Biology_CH12.indd 259 07-12-2021 15:43:41

role of bacteriophage in gene transfer, the lifecycle of bacteriophage is described below briefly.

After infecting the host cell, a bacteriophage (phage for short) often takes control and forces the host to make many copies of the virus. Eventually the host bacterium bursts or lyses and releases new phages. This reproductive cycle is called a lytic cycle because it ends in lysis of the host.

The lytic cycle (Figure 12.18) has four phases.

1. Attachment - Virus particle attaches to a specific receptor site on the bacterial surface.
2. Penetration - the genetic material, which is often double stranded DNA, then enters the cell.
3. Biosynthesis - After adsorption and penetration, the virus chromosome forces the bacterium to make viral components-viral nucleic acids and proteins.
4. Assembly - Phages are assembled from the virus components. Phage nucleic acid is packed within the virus's protein coat.
5. Release - mature viruses are released by cell lysis.

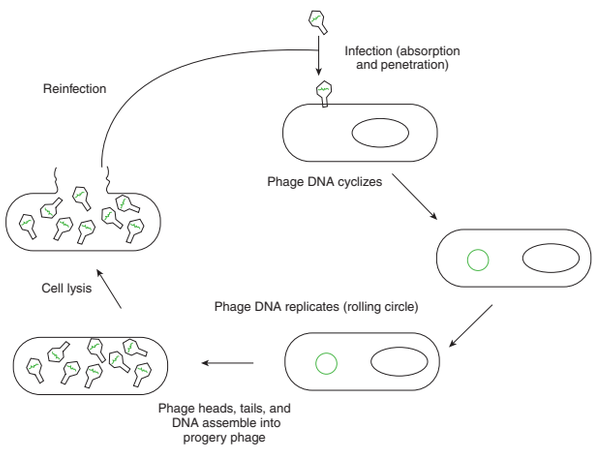

Figure 12.18: Lytic cycle

Bacterial viruses that reproduce using a lytic cycle often are called **virulent bacteriophages** (e.g. T phages) because they destroy the host cell. The genome of many DNA phages such as the lambda phage, after adsorption and penetration do not take control of its host and does not destroy the host. Instead the viral genome remains within the host cell and is reproduced along with the bacterial chromosome. The infected bacteria may multiply for long periods while appearing perfectly normal. Each of these infected bacteria can produce phages and lyses under appropriate environmental conditions. This relationship between phage and its host is called lysogeny (Figure 12.19).

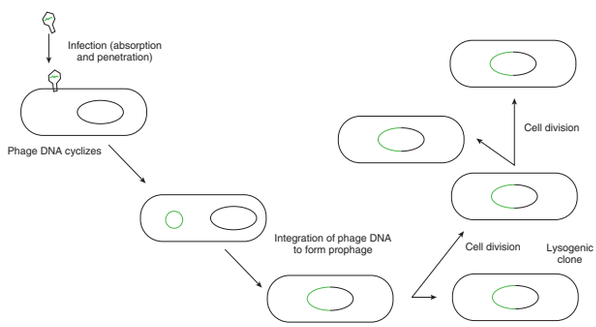

Figure 12.19: Lysogeny

Bacteria that can produce phage particles under some conditions are said to be **lysogens** or **lysogenic bacteria**. Phages which are able to establish lysogeny are called **temperate phages**.

The latent form of virus genome that remains within the host without destroying the host is called the prophage.

The prophage usually is integrated into the bacterial genome. Sometimes phage reproduction is triggered in a lysogenized culture by exposure to UV radiation or other factors. The lysosomes are then destroyed and new phages released – This phenomenon is called induction (Figure 12.20).

TN_GOVT_XII_Micro_Biology_CH12.indd 260 07-12-2021 15:43:41

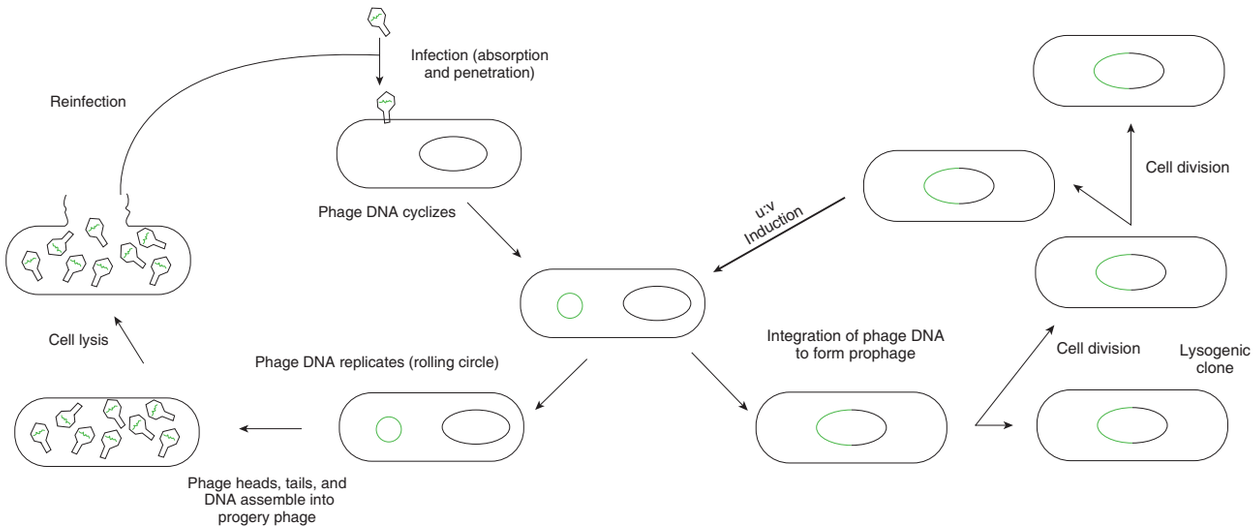

Figure 12.20: Induction of lysogen

Sometimes, bacterial genes are incorporated into a phage capsid because of errors made during the virus life cycle. The virus containing these genes then infects them into another bacterium, resulting in the transfer of genes from one bacterium to the other. Transduction may be the most common mechanism for gene exchange and recombination in bacteria.

There are two very different kinds of transduction.

1. Generalized transduction
2. Specialized transduction

Generalized transduction (Figure 12.21a) occurs during the lytic cycle of virulent and temperate phages. During the assembly stage, when the viral chromosomes are packaged into protein capsids, random fragments of the partially degraded bacterial chromosome also may be packaged by mistake. The resulting virus particles often injects the DNA into another bacterial cell but does not initiate a lytic cycle. Thus in generalized transduction any part of the bacterial chromosome can be transferred. Once the DNA has been injected it may integrate into the recipient cell's chromosome to preserve the transferred genes. About 70 to 90% of the transferred DNA is not integrated but is often able to survive and express itself. However, if the transferred DNA is degraded gene transfer is unsuccessful.

Specialized Transduction (Figure 12.21b) is also called restricted transduction in which only specific portions of the bacterial genome is carried by the phage. When a prophage is induced to leave the host chromosome, exicision is sometimes carried out improperly. The resulting phage genome contains portions of the bacterial chromosome next to the integration site. When this phage infects another bacterium, it transfers the bacterial genes from the donor bacterium along with phage DNA. Here only the bacterial genes that are close to the site of prophage are transferred. So, this transduction is called specialized.

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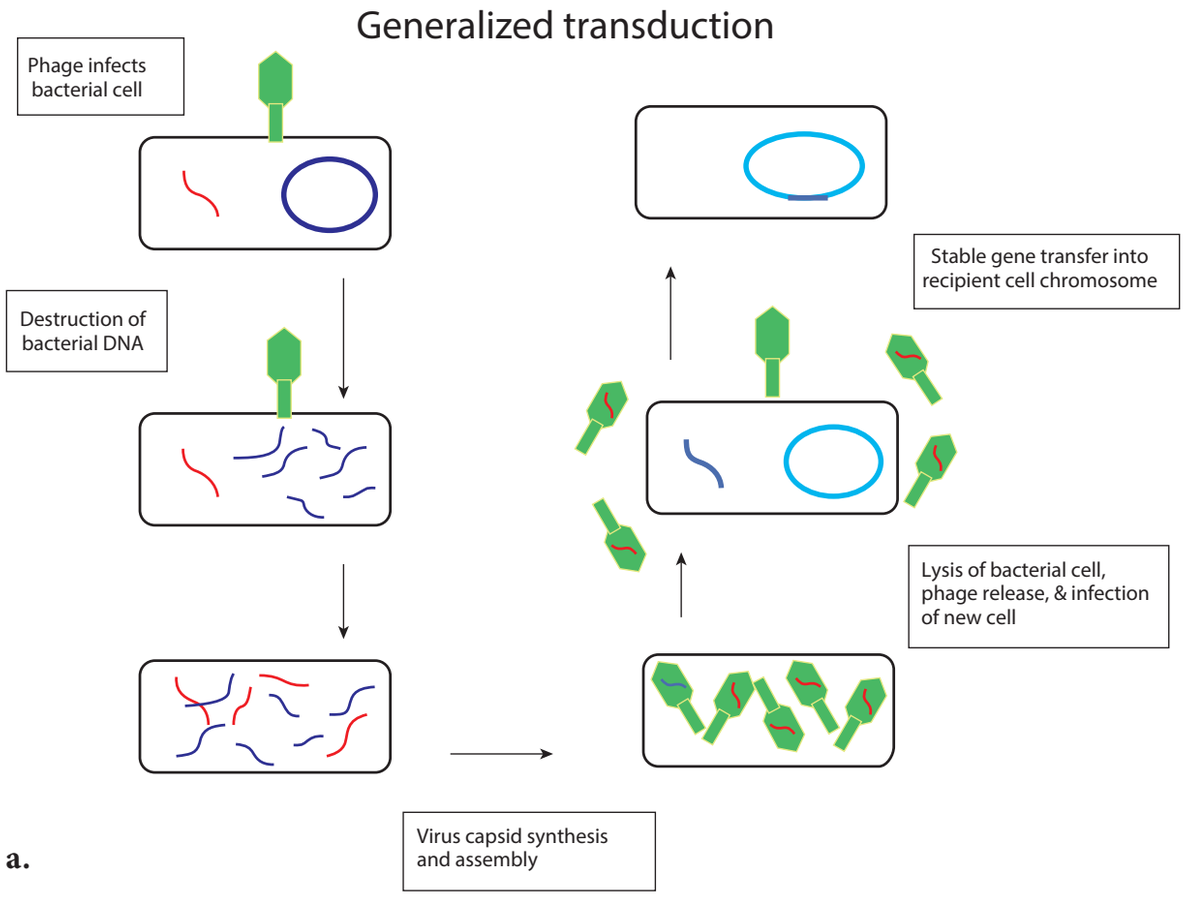


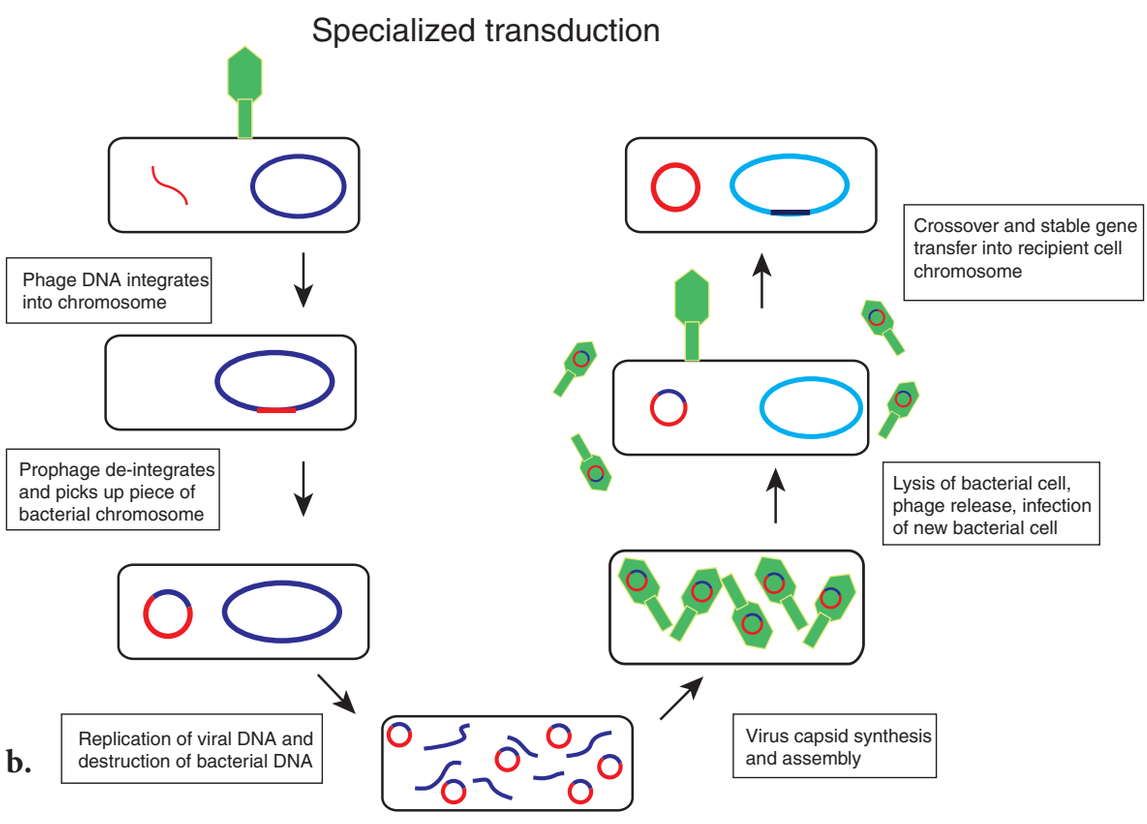


Figure 12.21: **(a)** Generalized Transduction **(b)** Specialized Transduction

TN_GOVT_XII_Micro_Biology_CH12.indd 262 07-12-2021 15:43:41

Infobits

The chromosomes of bacteria, viruses, and eukaryotic cells contain pieces of DNA that move around the genome. Such movement is called transposition. DNA segments that carry the genes required for this process and consequently move about chromosomes are transposable elements or transposons. Transposons are also called jumping genes because they can jump from one DNA to another, resulting in mutation of the cell. They were first discovered in 1951 by Barbara McClintock whose significant discovery was ignored by scientific community for many years. She was awarded the Nobel Prize in 1983.

12.8 Recombinant DNA Technology

One of the practical applications of microbial genetics and the technology arising from it is the recombinant DNA technology. The deliberate modification of an organism's genetic information by directly changing its nucleic acid genome is called genetic engineering and is accomplished by a collection of methods known as recombinant DNA technology. Recombinant DNA technology opens up totally new areas of research and applied biology. Thus, it is an essential part of biotechnology, which is now experiencing a stage of exceptionally rapid growth and development. In general sense, **recombination** is the process in which one or more nucleic acids molecules are rearranged or combined to produce a new nucleotide sequence. Usually genetic material from two parents is combined to produce a recombinant chromosome with a new, different genotype. Recombination results in a new arrangement of genes or parts of genes and normally is accompanied by a phenotypic change.

There are many diverse and complex techniques involved in gene manipulation. However, the basic principles of recombinant DNA technology are reasonably simple, and broadly involve the following stages (Figure 12.22).

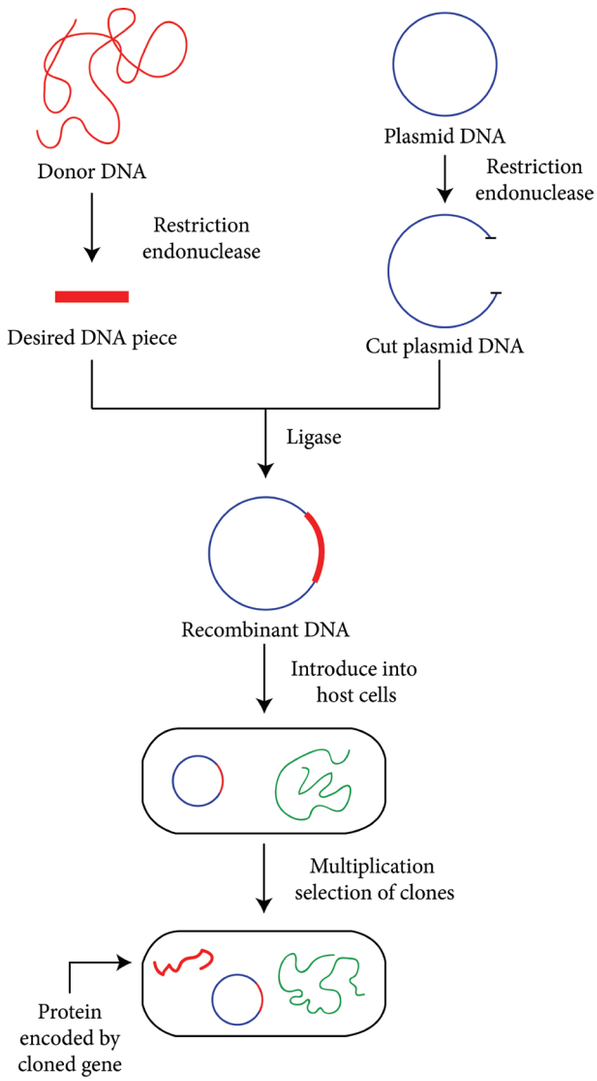


Figure 12.22: Basic principles of recombinant DNA technology

1. Isolation of DNA from the source (Donor)
2. Generation of DNA fragments and selection of the desired piece of DNA
3. Insertion of the selected DNA into a cloning vector (Example: a plasmid) to

TN_GOVT_XII_Micro_Biology_CH12.indd 263 07-12-2021 15:43:42

create a recombinant DNA or chimeric DNA.

4. Introduction of the recombinant vectors into host cells (Example: bacteria)
5. Multiplication and selection of clones containing the recombinant molecules
6. Expression of the gene to produce the desired product.

Cloning in the molecular biology sense (as opposed to cloning whole organisms) is the making of many copies of a segment of DNA, such as a gene. Cloning makes it possible to generate large amounts of pure DNA, such as genes, which can then be manipulated in various ways, including mapping, sequencing, mutating and transforming cells. An overview of cloning strategies in recombinant DNA technology is shown in Figure 12.23.

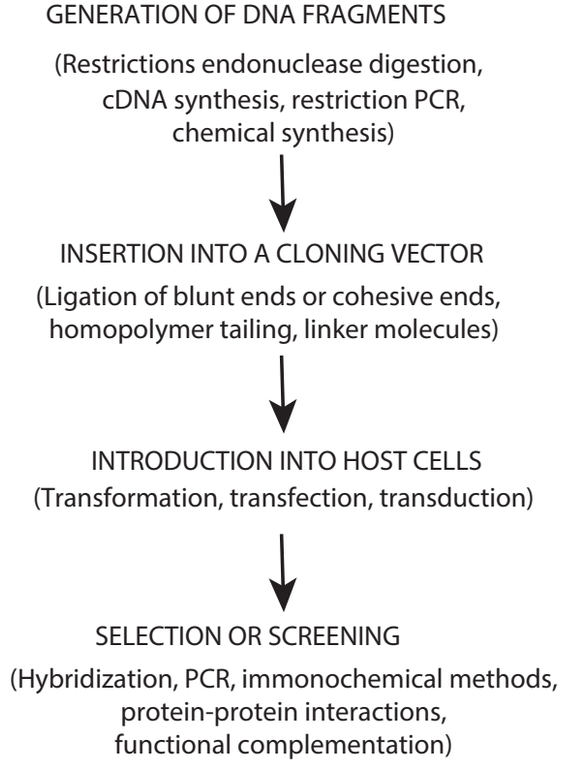


Figure 12.23: An overview of cloning strategies in recombinant DNA technology

12.9 Vectors, Types and Characteristics

Vectors are the DNA molecules, which carry a foreign DNA fragment to be cloned. They are cloning vehicles, examples of which are Plasmids, Bacteriophages, cosmids, phagemids and artificial chromosomes. The vector types differ in the molecular properties they have and in the maximum size of DNA that can be cloned into each.

Characteristics of an ideal vector.

1. Should be small in size
2. Should contain one or more restriction site
3. Should be self replicating
4. Should contain an origin of replication sequence (ori)
5. Should possess genetic markers (to detect the presence of vectors in recipient cells)

Plasmid Cloning Vectors

Bacterial plasmids are extra chromosomal elements that replicate autonomously in cells. Their DNA is circular and double stranded and carries sequences required for plasmid replication (ori sequence) and for the plasmid's other functions. (Note: A few bacteria contain linear plasmids. Example: *Streptomyces species*, *Borellia burgdorferi*). The size of plasmids varies from 1to 500 kb. Plasmids were the first cloning vectors. DNA fragments of about 570kb are efficiently cloned in plasmid cloning vectors. Plasmids are the easiest to work with. They are easy to isolate and purify, and they can be reintroduced into a bacteria by transformation. Naturally occurring plasmid vectors rarely possess all the characteristics of an ideal vector. Hence plasmid cloning vectors are

TN_GOVT_XII_Micro_Biology_CH12.indd 264 07-12-2021 15:43:42

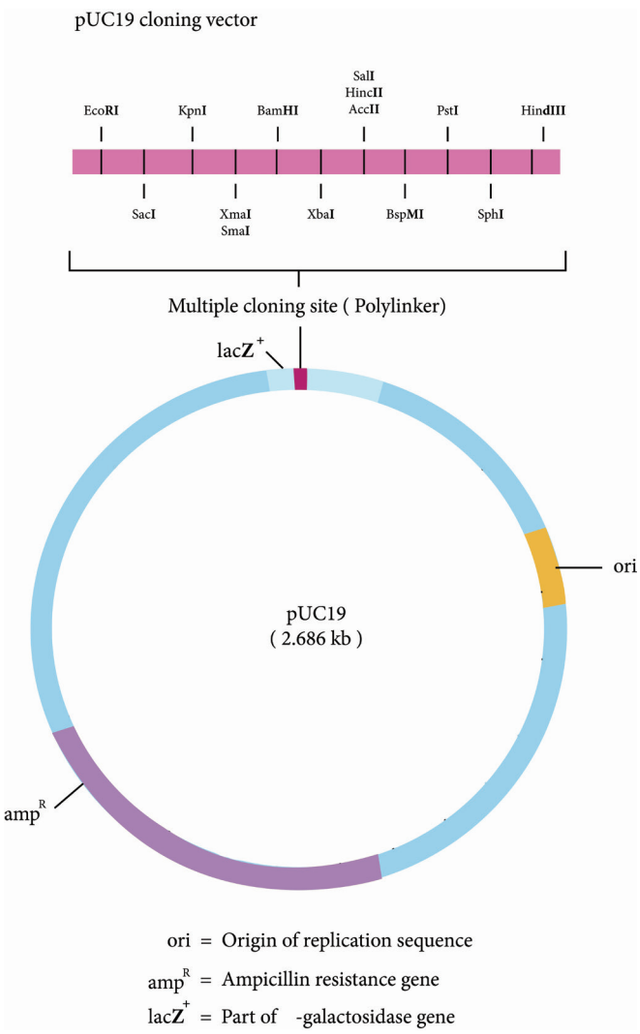
derivatives of natural plasmids and are "engineered" to have features useful for cloning DNA.

Examples of plasmid cloning vectors : pBR 322 (plasmid discovered by Bolivar and Rodriguez 322) and pUC 19 (plasmid from University of California). Herbert Boyer and Stanley Cohen in 1973 showed it was possible to transplant DNA segments from a frog into a strain of *Escherichia coli* using pSC101, a genetically modified plasmid, as the vector. The work laid the foundation for the birth of Genetech, the first company dedicated to commercialization of recombinant DNA.

Figure 12.24a and 12.24b shows genetic maps of plasmid cloning vectors PUC19 and PBR322 respectively.

Plasmid cloning vector PUC 19 has 2,686 –bp and has following features:

1. It has a high copy number; so many copies of a cloned piece of DNA can be generated readily.
2. It has amp R (ampicillin resistant) selective marker
3. It has a number of unique restriction sites clustered in one region, called a multiple cloning site (MCS) or polylinker
4. The MCS is inserted into part of the E.coli β – galactosidase (lac Z^+) gene. Figure 12.25 illustrates how a piece of DNA can be inserted into a plasmid cloning vector such as pUC19



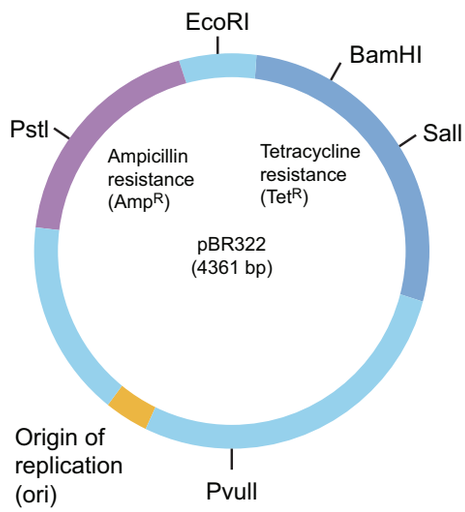


Figure 12.24: (a) PUC19 **(b)** PBR322

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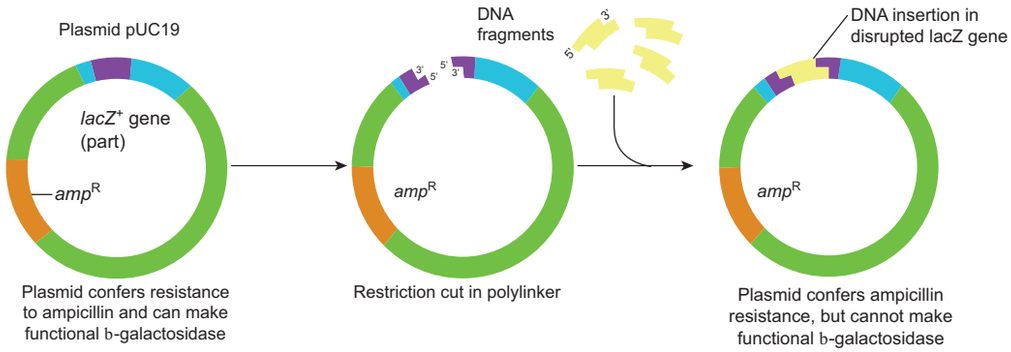

Figure 12.25: Insertion of a piece of DNA into the plasmid cloning vector pUC19 to produce a recombinant DNA molecule

Bacteriophage as Cloning Vectors

They are viruses that replicate within the bacteria. A phage can be employed as vector since a foreign DNA can be spliced into phage DNA, without causing harm to phage genes. The phage will reproduce (replicate the foreign DNA) when it infects bacterial cell. Both single and double stranded phage vectors have been employed in recombinant DNA technology. Derivatives of phage can carry fragments up to about 45 kb in length. Example PI bacteriophage and phage λ.

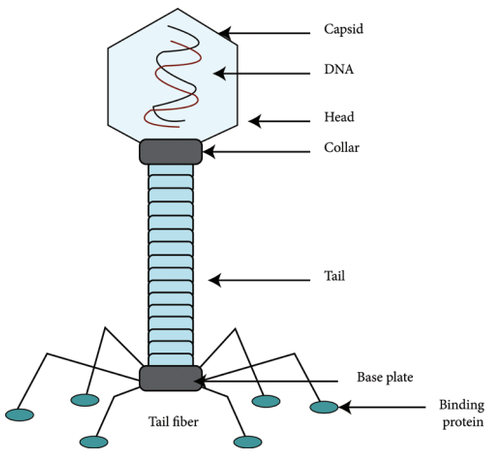

Figure 12.26: Structure of phage λ

The main advantage of using phage vectors is that foreign DNA can be packed into the phage (invitro packaging), the latter in turn can be injected into the host cell very effectively (Note: no transformation is required). Figure 12.28 shows how a λ phage is used for cloning.

Infobits

Terms associated with plasmids:

1. Low copy number plasmids are plasmids that occur low in number in each cell.
2. High copy number are plasmids that occur high in number in each cell.
3. Conjugative plasmids carry a set of transfer genes (tra genes) that facilities bacterial conjugation.
4. Non – conjugative plasmidsare plasmids that do not possess transfer genes.
5. Stringent plasmids are plasmids that are present in a limited number (1–2 per cell).
6. Relaxed plasmids are plasmids that occur in large number in each cell.
7. F plasmids possess genes for their own transfer from one cell to another
8. R plasmids carry genes resistance to antibiotics.

TN_GOVT_XII_Micro_Biology_CH12.indd 266 07-12-2021 15:43:45

Cosmids: Cosmids are the vectors possessing the characteristics of both plasmid and bacteriophage. The advantage with cosmids is that they carry larger fragments of foreign DNA (35–45 kb) compared to plasmids.

Phagemids: Phagemids are the combination of plasmid and phage and can function as either plasmid or phage. Since they posses functional origins of replication of both plasmid and phage λ they can be propagated (as plasmid or phage) in appropriate E.coli.

Artificial chromosome Vectors: Artificial chromosomes are cloning vectors that can accommodate very large pieces of DNA, producing recombinant DNA molecules resembling small chromosomes. Example: Yeast Artificial Chromosome (YAC), Bacterial Artificial Chromosomes (BACs)

Plasmid shuttle Vectors: The plasmid vectors that are specifically designed to replicate in two or more different host organisms(say in E.coli and yeast) are referred to as shuttle vectors. The origins of replication for two hosts are combined in one plasmid.

Expression vectors: An expression vector is a cloning vector containing the

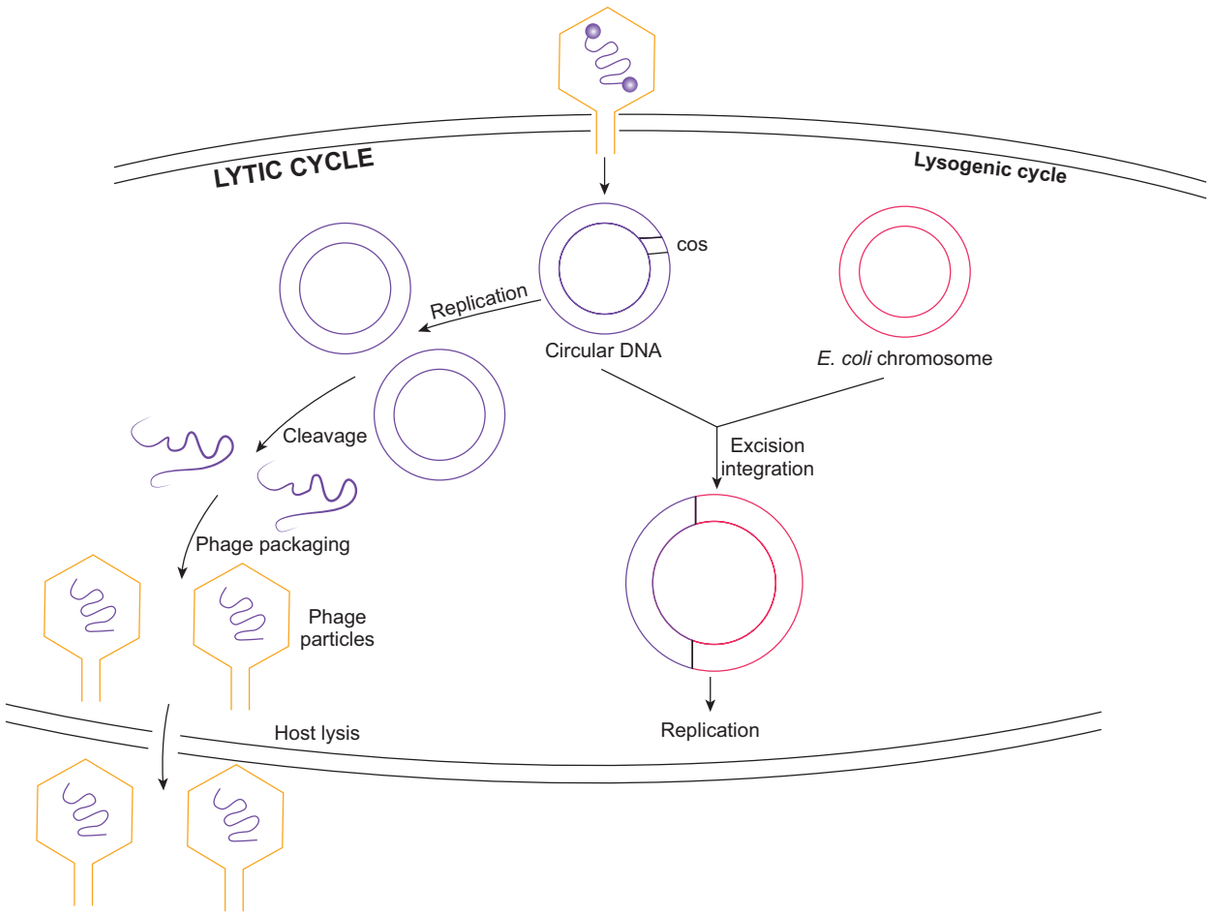

Figure 12.27: Life cycle of phage λ

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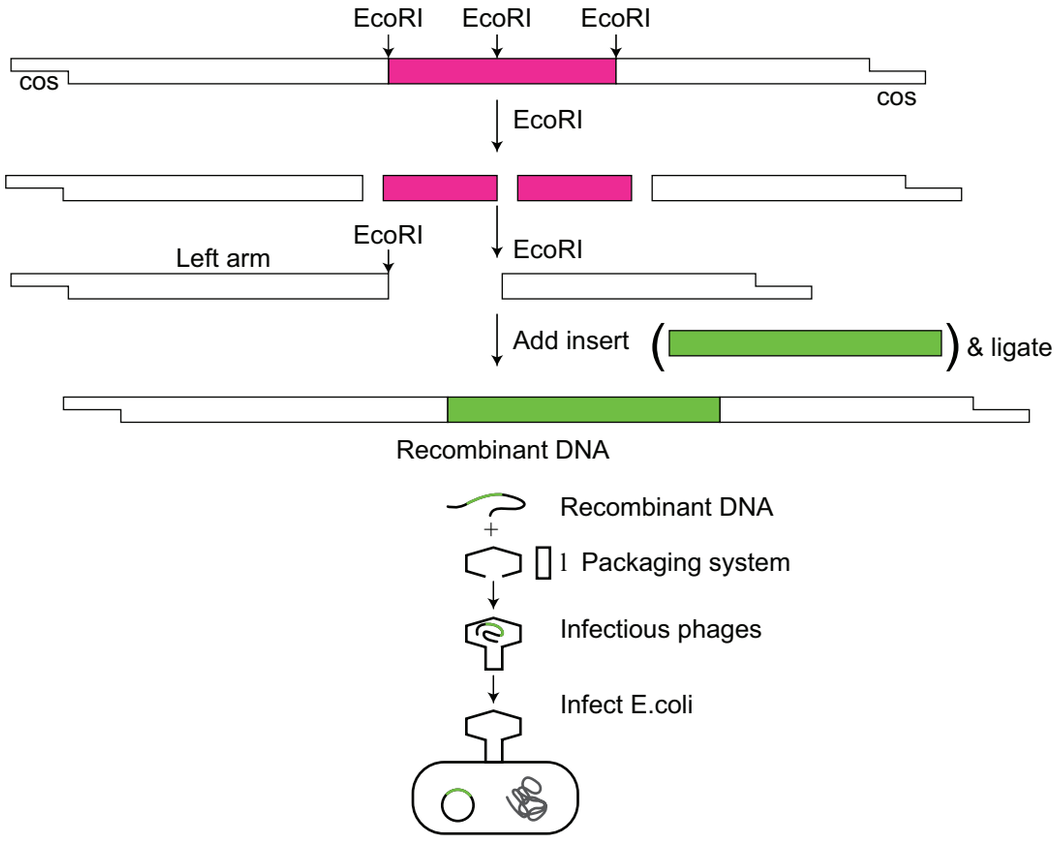

Figure 12.28: Cloning using a λ phage

regulatory sequences (promoter sequence) necessary to allow the transcription and translation of a cloned gene or genes.

12.10 Restriction Enzymes

In 1960s Swiss microbiologist Werner Arber and American microbiologists Hamilthon Othanel Smith and Daniel Nathans discovered restriction enzymes. The discovery, for which the three men shared the 1978 Nobel Prize for Physiology or Medicine. Restriction enzymes or restriction endonucleases are one of the most important groups of enzymes for the manipulation of DNA. It is one of the important molecular tools used by a genetic engineer. These are the bacterial enzymes that recognize a specific base sequence in a DNA molecule (from any source) and make two cuts one in each strand generating 3′ – OH and 5′ – P termini. They were first discovered in E.*coli*. E.*coli* produces the restriction enzyme to cut the viral DNA and protect itself. The host E.coli DNA is protected by its own restriction enzyme since its methylated. Since these enzymes restrict the viral replication the word restriction is added to these enzymes. Hind II was the first discovered restriction endonuclease.

The site where the DNA is cut by a restriction enzyme is called recognition sequence. Restriction endonucleases can specifically recognize DNA with a particular sequence of 4-8 nucleotides and cleave. Each recognition sequence has two fold rotational symmetry i.e. the same nucleotide sequence occurs on both strands of DNA which run in opposite direction. Such sequences are referred to as palindromes, since they read similar in both directions (forwards and backwards). Majority

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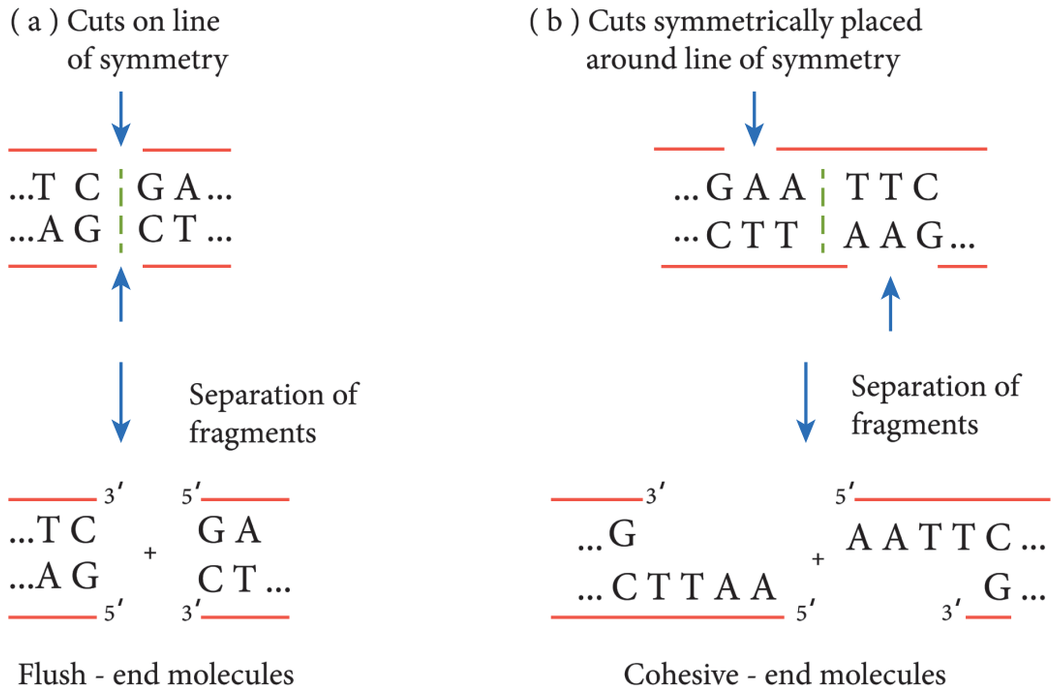

Figure 12.29: Two types of cuts made by restriction enzymes

Type	No of Enzyme and sub units	Cleavage site	Examples	Bacterial source
I	One with 3 sub units for recognition cleavage and methylation	1000 bp from recognition site	EcoK1	*Escherichia coli*
			Cfr A1	*Citrobacter fruendii*
II	Two different enzymes to cleave or modify the recognition sequence	Same as recognition or close to recognition site	Eco R1	*Escherichia coli*
			Alu I	*Arthrobacter luteus*
III	One with 2 subunits	24- 26 bp from recognition site	Hinf III	*Haemophilus influenzae*
			Pst II	*Providencia stuarti*

Table 12.2: Types and features of restriction enzyme

of restriction endonucleases (particularly type II) cut DNA at defined sites within recognition sequence. Type II restriction enzymes make two single – stand breaks) one break in each strand. There are two distinct arrangements of these breaks 1. both breaks at the center of symmetry (generating flush or blunt ends) or 2. breaks that are symmetrically placed around the line of symmetry generating cohesive ends. Figure 12.29 shows two types of cuts made by restriction enzymes. The arrow indicates the cleavage site. The dashed line is the center of symmetry of the sequence (Table 12.2).

Application of Recombinant DNA Technology

a. Production of medically useful proteins such as somatostain, insulin, human growth hormone and interferon. It decreases the dependency on human tissues and solves problem of limited production.

TN_GOVT_XII_Micro_Biology_CH12.indd 269 07-12-2021 15:43:45

b. Development of synthetic vaccines for instance, vaccines for malaria and rabies a recombinant hepatitis vaccine is already commercially available.

c. Gene therapy

d. Diagnosis of infection diseases.

e. To manufacture industrially important products like enzymes using bacteria, fungi and cultured mammalian cells.

12.11 Techniques in Genetic Engineering

There are several techniques used in recombinant DNA technology or gene manipulation. The most frequently used methods are agarose gel electrophoresis, isolation and purification of nucleic acids, nucleic acid blotting techniques, DNA sequencing, chemical synthesis of DNA, gene transfer methods, polymerase chain reaction, construction of gene library, radiolabeling of nucleic acids etc, few of them are discussed here.

12.11.1 Agarose Gel Electrophoresis

Electrophoresis refers to the movement of charged molecules in an electric field. The negatively charged molecules move towards the positive electrode while the positively charged molecules migrate towards the negative electrode. Gel electrophoresis is a routinely used analytical technique for the separation and purification of specific DNA fragments. The gel is composed of either polyacrylamide or agarose. Polyacrylamide gel electrophoresis (PAGE) is used for the separation of smaller DNA fragments while agarose electrophoresis is convenient for the separation of DNA fragments ranging in size from 100 base pairs to 20 kilobase pairs.



A genomic library is a collection of clones that contains at least one copy of every DNA sequence in an organism's genome. Like libraries with books, genomic libraries are a great source of information; in this case, the information is about the genome. Specific sequences in cDNA libraries and genomic libraries can be identified via a number of approaches, including the use of specific antibodies, cDNA probes and oligonucleotide probes

Human artificial chromosome (HAC)-based vectors offer a promising system for delivery and expression of full-length human genes of any size into human cells, and a tool for determining human chromosome function. It does not have the problem of limited cloning capacity of other vectors, and it also avoids possible insertional mutagenesis caused by integration into host chromosomes by viral vector.

Gel electrophoresis can also be used for the separation of RNA molecules. A diagramatic view of the agarose gel electrophoresis unit is shown in Figure 12.30a.

Steps

1. Gel is set with wells on one end.
2. The gel is placed in an electrophoresis apparatus and covered with buffer solution.
3. The DNA samples along with tracer dye are placed in the wells of gel.

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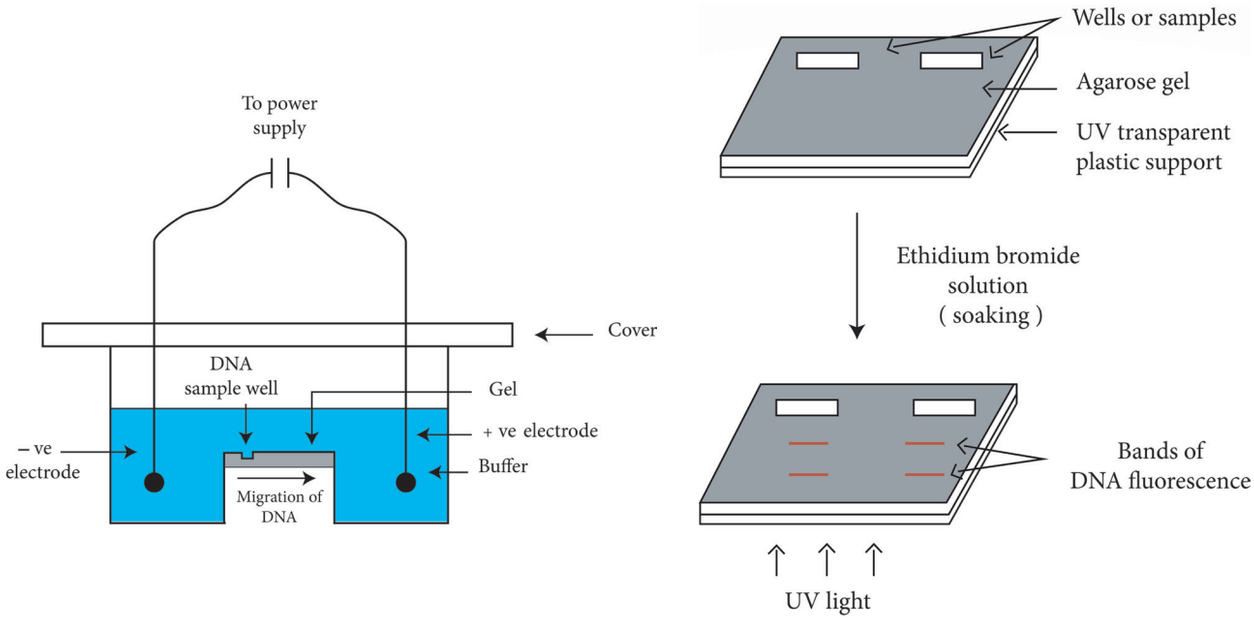


Figure 12.30: **(a)** agarose gel electrophoresis unit **(b)** DNA electrophoresis gel

4. Power supply is switched on and gel is run till the tracer dye reaches the end of the gel.

As the DNA is negatively charged, DNA fragments move through the gel towards the positive electrode. The rate of migration of DNA is dependent on the size and shape. In general, smaller linear fragments move faster than the larger ones. Hence, gel electrophoresis can be conveniently used for the separation of a mixture of DNA fragments, based on their size. The bands of the DNA can be detected by soaking the gel in ethidium bromide solution (Ethidium bromide can also be added to molten agarose prior to setting the gel). When activated by ultraviolet radiation, DNA base pairs in association with ethidium bromide, emit orange fluorescence. And in this way the DNA fragments separated in agarose electrophoresis can be identified (Figure 12.30b).

PAGE is composed of chains of acryl amide monomers crosslinked with methylene bisacryalmide units. The pore size of the gel is dependent on the total concentration of monomers and the cross links. PAGE is used for the separation of single stranded DNA molecules that differ in length by just one nucleotide. Agarose gels cannot be used for this purpose. This is because polyacrylamide gels have smaller pore sizes than agarose gels and allow precise separation of DNA molecule from 10–1500 bp.

HOTS

1. Explain how gel electrophoresis can be used to determine the size of a PCR product.

12.11.2 Polymerase Chain Reaction (PCR)

The PCR technique has already proven exceptionally valuable in many areas of molecular biology, medicine, and biotechnology. PCR technique has great practical importance and impact

TN_GOVT_XII_Micro_Biology_CH12.indd 271 07-12-2021 15:43:47

on biotechnology. Between 1983 and 1985 American biochemist Kary Mullis developed PCR technique that made it possible to synthesize large quantities of a DNA fragment without cloning it. Mullis received the 1993 Nobel Prize for Chemistry for his invention. PCR is a cell free amplification technique.

Figure 12.31 outlines how PCR technique works. To amplify (make large quantities) a particular DNA sequence by PCR a reaction mixture (often 100µl or less in volume) containing the following are required.

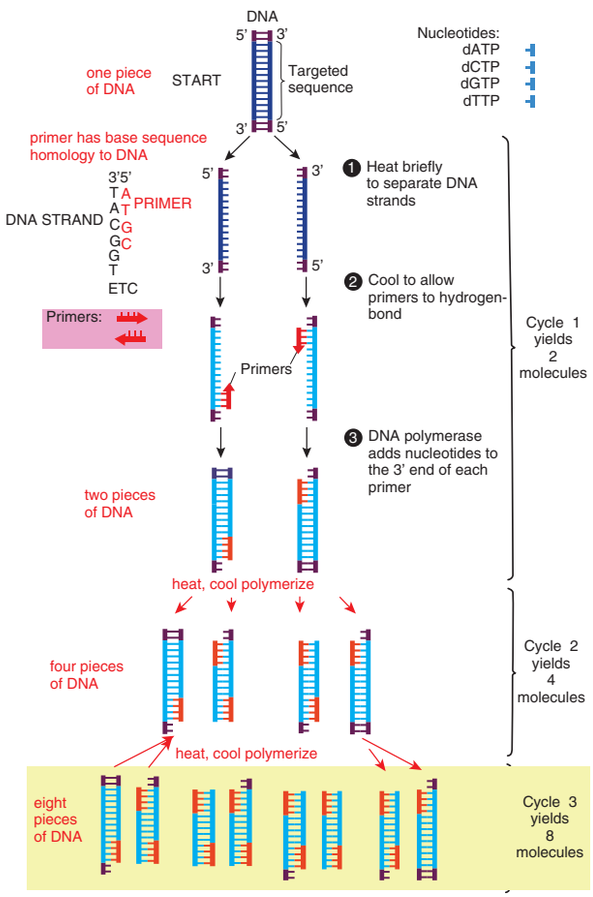


Figure 12.31: Steps in PCR

1. **Target DNA**
2. **Two primers**–These are synthetic oligonucleotides, usually about 20 nucleotides long. These are fragments with sequences identical to those flanking the targeted sequence.
3. **Thermostable DNA polymerase**– Two popular enzymes employed in the PCR technique are Taq polymerase from the thermophilic bacterium *Thermus aquaticus* and the vent polymerase from *Theromococcus litoralis*. These polymerases employed in PCR technique are able to function at high temperatures.
4. **Four deoxyribonucleoside triphosphates** (dNTPs)- dCTP, dATP, dGTP, dTTP

Infobits

Cloned genes and other DNA sequences often are analyzed to determine the arrangement and specific locations of restriction sites. The analytical process involves cleavage of the DNA with restriction enzymes, followed by separation of the resulting DNA fragments by agarose gel electrophoresis. The sizes of the DNA fragments are calculated, enabling restriction maps to be constructed. The many DNA fragments produced by cleaving genomic DNA show a wide range of sizes, resulting in a continuous smear of DNA fragments in the gel. In this case, specific gene fragments can be visualized only by transferring them to membrane filter by southern blotting, hybridizing a specific labelled probe with the DNA fragments, and detecting the hybrids. A similar procedure, Northern blotting is used to analyze the sizes and quantities of RNAs isolated from cell.

TN_GOVT_XII_Micro_Biology_CH12.indd 272 07-12-2021 15:43:47

DO YOU KNOW?

Taq polymerase lacks proof reading exonuclease (3′–5′) activity which might contribute to errors in the products of PCR. Some other thermostable DNA polymerases with proof reading activity have been identified. Example: Tma DNA polymerase from *Thermotoga maritana*.

Steps in PCR

1. **Denaturation:** The target DNA containing the sequence to be amplified is heat denatured to separate its complementary strands at temperature 94 °C–95 °C.
2. **Annealing:** The temperature is lowered to 37 °C–55 °C so that the primers can hydrogen bond or anneal to the DNA on both sides of the target sequence. Because the primes are present in excess the targeted DNA strands normally anneal to the primers rather than to each other.
3. **Extension:** Heat resistant DNA polymerase extends the primers and synthesizes copies of the target DNA sequence using the deoxyribonucleoside triphosphate's at 70 °C–75 °C.

The three – step cycle (Figure 12.32) is repeated to obtain copies of target DNA in large numbers. At the end of one cylcle, the targeted sequences on both strands have been copied. When the three – step cycle is repeated, the four strands from the first cycle are copied to produce eight fragments. The third cycle yields 16 products. Theoretically, 20 cycles will produce about one million copies of the target DNA sequence. Each cycle of PCR takes about 3 – 5 minutes.

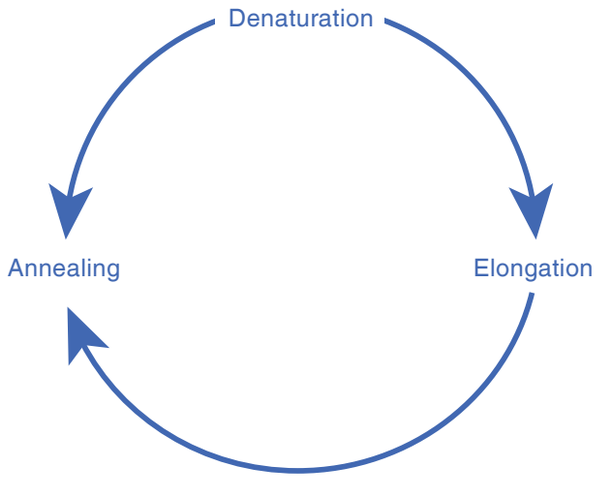


Figure 12.32: Three steps PCR cycle

The PCR technique has now been automated and is carried out by a specially designed machine (Figure 12.33) PCR machines are now fully automated and microprocessor controlled. They can process up to 96 samples at a time. PCR machines can carry out 25 cycles and amplify DNA 10^5 times in as little as 57 minutes.

The PCR has many applications in research and in commercial arena, including generating specific DNA segments for cloning or sequencing, amplifying DNA to detect specific genetic defects, and amplifying DNA for fingerprinting in crime scene investigation.

PCR technology is improving continually. Various forms of PCR are available. RNA too can be efficiently used in PCR procedures. Cellular RNAs and RNA viruses may be studied even when the RNA is present in very small amounts (as few as 100 copies can be transcribed and amplified). Quantitative PCR is quite valuable in virology and gene impression studies. PCR is

TN_GOVT_XII_Micro_Biology_CH12.indd 273 07-12-2021 15:43:47

modified as per the specific demands of the situation. Thus there are many variations in the original PCR Examples nested PCR, inverse PCR, reverse transcription PCR, time quantitative PCR, RAPD, RFLP, AFLP.

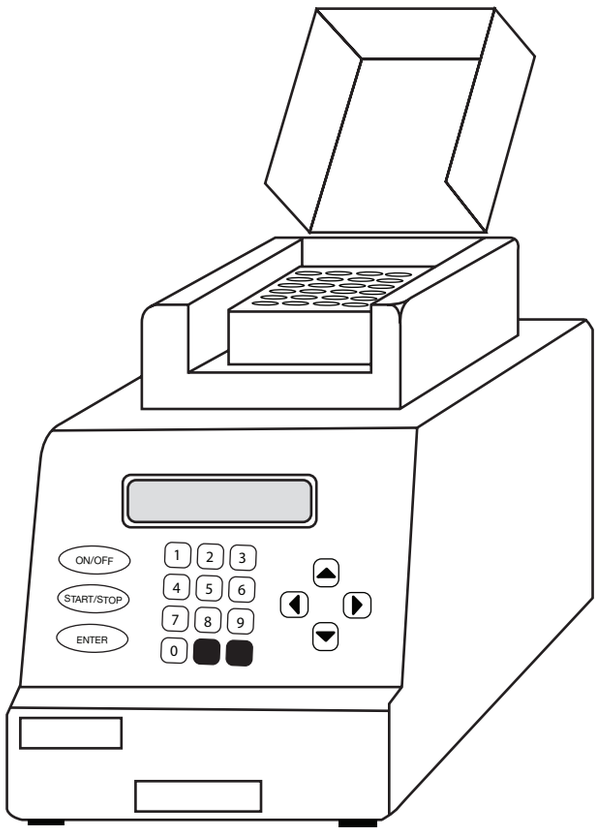


Figure 12.33: PCR machine

HOTS

Both PCR and Cloning allow for the production of many copies of a DNA sequence. What are the advantages of using PCR instead of cloning to amplify a DNA template?

What advantages are there to using a DNA polymerase for PCR that has proofreading activity?

Infobits

In 1970s American molecular biologists Allan M. Maxam and Walter Gilbert and English biochemist Frederick Sanger developed some of the first techniques for DNA sequencing. Gilbert and Sanger shared the 1980 Nobel Prize for Chemistry for their work. Dideoxy procedure is one of the procedure used to sequence DNA.

Summary

The fundamental unit of information in living systems is the gene. Genome is the set of all genes and genetic signals of a cell. Gene is expressed through a sequence of events. The central dogma of molecular biology, comprises the three major processes replication, transcription and translation. The genetic message encoded in messenger RNA (mRNA) is translated on the ribosomes into a polypeptide with a particular sequence of amino acids. An RNA base sequence (a set of 3 bases) corresponding to a particular amino acid is called a codon. The genetic code is the set of all codons. The genetic code is a triplet code, and all 64 possible codons carry information of some sort. The code is highly redundant. Polypeptides are synthesized from the amino terminus toward the carboxyl terminus, by adding amino acids one by one to the carboxyl end. An important feature of intiation of polypeptide synthesis in both prokaryotes and eukaryotes is the use of a specific initiating tRNA molecule.

Mutation is the process by which the sequence of base pairs in a DNA molecule is altered. Mutations can be divided into

TN_GOVT_XII_Micro_Biology_CH12.indd 274 07-12-2021 15:43:47

base pair substitution mutation and base pair insertion or deletions.

Frame shift mutation usually results in a nonfunctional protein. An addition or deletion of one base pair, for example, shifts the mRNA's downstream reading frame by one base, so that incorrect amino acids are added to the polypeptide chain after the mutation site. Point mutations are single base changes, that do not affect the reading frame, that is, the mutation only makes a single change in a single codon,

Mutations can also be defined according to their effects on amino acid sequences in proteins. They are missense mutation, silent mutation, nonsense mutation. Forward mutations change the genotype from wild type to mutant and reverse mutations (or reversions or back mutations) change the genotype from mutant to wild type or to partially wild type.

The term mutant refers to an organism in which either the base sequence of DNA or the phenotype has been changed. The

Infobits

DNA Fingerprinting Or DNA Profiling:

DNA fingerprinting is the present day genetic detective in the practice of modern medical forensics. The underlying principles of DNA fingerprinting are briefly described.

The structure of each person's genome is unique. The only exception being monozygotic identical twins (twins developed from a single fertilized ovum). The unique nature of genome structure provides a good opportunity for the specific identification of an individual. The DNA fingerprint is an analysis of the nitrogenous base sequence in the DNA of an individual.

The original DNA fingerprinting technique was developed by Alec Jaffreys in 1985. Although the DNA fingerprinting is commonly used, a more general term DNA profiling is preferred. This is due to the fact that a wide range of tests can be carried out by DNA sequencing with improved technology.

Applications of DNA fingerprinting:

The amount of DNA required for DNA fingerprint is remarkably small. The minute quantities of DNA from blood strains, body fluids, hair fiber or skin fragments are enough. Polymerase chain reaction is used to amplify this DNA for use in fingerprinting. DNA profiling has wide range of applications – most of them related to medical forensics. Some important ones are listed below.

- Identification of criminals, rapists, thieves etc.
- Settlement of paternity disputes.
- Use in immigration test cases and disputes.

In general, the fingerprinting technique is carried out by collecting the DNA from a suspect (or a person in a paternity of immigration dispute) and matching it with that of a reference sample (from the victim of a crime, or a close relative in a civil case).

TN_GOVT_XII_Micro_Biology_CH12.indd 275 07-12-2021 15:43:47

process of formation of mutant organism is called mutagenesis. Ames test is an indicator of whether the chemical is a mutagen. The Ames test assays the ability of chemicals to revert mutant strains of the bacterium Salmonella typhimurium to wild type. The most commonly employed gene transfer methods are transformation, conjugation, transduction, electroporation, lipofection and direct transfer of DNA. Transformation is genetic alteration of a cell resulting from the direct uptake, incorporation and expression of exogenous genetic material (exogenous DNA) from its surroundings. Competence refers to the state of being able to take up exogenous DNA from the environment. During conjugation, two live bacteria (a donor and a recipient) come together, join by cytoplasmic bridges (e.g. pilus) and transfer single stranded DNA (from donor to recipient). Transduction is the transfer of bacterial genes from one bacteria to other by viruses, e.g. Bacteriophage (Bacterial viruses). **Recombination** is the process in which one or more nucleic acids molecules are rearranged or combined to produce a new nucleotide sequence. Cloning in the molecular biology sense (as opposed to cloning whole organisms) is the making of many copies of a segment of DNA, such as a gene. Cloning makes it possible to generate large amounts of pure DNA, such as genes, which can then be manipulated in various ways

Vectors are the DNA molecules, which carry a foreign DNA fragment to be cloned. They are cloning vehicles, examples of which are Plasmids, Bacteriophages, cosmids, phagemids and artificial chromosomes. Bacterial plasmids are extra chromosomal elements that replicate autonomously in cells. They are viruses that replicate within the bacteria. Cosmids are the vectors possessing the characteristics of both plasmid and bacteriophage. Phagemids are the combination of plasmid and phage, and can function as either plasmid or phage. The plasmid vectors that are specifically designed to replicate in two or more different host organisms (say in E. *coli* and yeast) are referred to as shuttle vectors. An expression vector is a cloning vector containing the regulatory sequences (promoter sequence) necessary to allow the transcription and translation of a cloned gene or genes. Restriction enzymes are the bacterial enzymes that recognize a specific base sequence in a DNA molecule (from any source) and make two cuts one in each strand generating 3′ – OH and 5′ – P termini.

There are several techniques used in recombinant DNA technology or gene manipulation. The most frequently used methods are agarose gel electrophoresis, isolation and purification of nucleic acids, nucleic acid blotting techniques, DNA sequencing, chemical synthesis of DNA, gene transfer methods, polymerase chain reaction, construction of gene library, radiolabeling of nucleic acids etc. Gel electrophoresis is a routinely used analytical technique for the separation and purification of specific DNA fragments. PCR is a cell free amplification technique. The three – step cycle is repeated to obtain copies of target DNA in large numbers.

The DNA markers are highly useful for genetic mapping of genomes. RFLPS (Restriction Fragment Length Polymorphisms), VNTRs (mini satellites or Variable Number Tandem Repeats),

TN_GOVT_XII_Micro_Biology_CH12.indd 276 07-12-2021 15:43:47

STRs (Microsatellites or Simple Random Repeats), SNPs (Single Nucleotide Polymorphisms) are types of DNA sequences (stretch of DNA) which can be used as markers. These markers are used in disease diagnosis and DNA fingerprinting.

Evaluation

Multiple choice questions

1. Which of the following properties is essential for the function of a tRNA molecule?



   a. Recognition of a codon
   b. Recognition of an anticodon
   c. Ability to distinguish one amino acid from another
   d. Recognition of DNA molecule

2. Which chain termination codon could be formed by a single base change from UCG, UGG and UAU?
   a. UAA b. UAG
   c. UGA d. AUG

3. Which of the following base-pair changes are transitions?
   a. AT → TA b. AT → GC
   c. Both a and b d. GC → AT

4. UV light usually causes mutations by a mechanism involving
   a. One-strand breakage in DNA
   b. Deletion of DNA segments
   c. Induction of thymine dimers and their persistence
   d. Inversion of DNA segments

5. The form of genetic information used directly in protein synthesis is
   a. DNA b. mRNA
   c. rRNA d. tRNA

6. _____ display one anticodon each
   a. eukaryotic mRNAs
   b. transfer RNAs
   c. ribosomal RNAs
   d. mRNAs

7. _____ contains exons and introns.
   a. Eukaryotic mRNAs b. rRNA
   c. tRNAs d. primers

8. The symbol lac^+ refer to
   a. genotype b. phenotype
   c. both a & b d. none

9. _____ sequence terminates protein synthesis
   a. UAA b. UAG
   c. UGA d. All the above

10. The principal start codon corresponds to which amino acid?
   a. Valine
   b. arginine
   c. Methionine
   d. Isoleucine

11. Number of nucleoprotein subunit in a prokaryotic ribosome
   a. 2 b. 4
   c. 5 d. 6

12. A deletion occurs that eliminates a single amino acid in a protein. How many base pairs were deleted?
   a. 1 b. 2
   c. 3 d. 4

13. During conjugation plasmids undergo
   a. Theta replication

TN_GOVT_XII_Micro_Biology_CH12.indd 277 07-12-2021 15:43:47

b. rolling circle replication

c. sigma replication

d. gamma replication

14. If a plasmid is mobilizable but nonconjugative, what function does it lack?

a. Antibiotic resistance

b. Fertility

c. Colicinogenic

d. Restriction sequences

15. The uptake of naked DNA from the surrounding is known as

a. Transduction

b. Conjugation

c. Transformation

d. Lysis

16. The F plasmid makes a cell

a. Donor b. Recipient

c. Resistant d. None

17. Which of the following is more efficient in transferring chromosomal DNA

a. F^+cell b. F^-cell

c. Hfr cell d. R^+cell

18. Which of the following statement is true

a. Protein is the only gene product

b. A functional gene product is protein or might also be one of several classes of RNA molecules

c. Carbohydrate is the only gene product

d. Lipids are the only gene product

19. DNA is transcribed into

a. mRNA b. tRNA

c. sRNA d. hnRNA

20. Which of the following is found as part of all prokaryotic promoters

a. Pribnow box

b. Shine dalgarno sequence

c. AUG sequence

d. UAG sequence

Answer the following

1. What is the direction of synthesis of RNA?
2. Define coding strand.
3. What parts of a mRNA molecule not translated? Ans. Leader & Introns
4. How many codons could be contained in a four-letter code? Ans 4^4=256
5. What is the principal start codon and to what amino acid does it correspond?
6. Restriction endonucleases are naturally found in bacteria. What purpose do they serve?
7. There are many varieties of cloning vectors that are used to propagate cloned DNA. One type of cloning vector used in E.coli is a plasmid vector. What features does a plasmid vector have that makes it useful for constructing and cloning recombinant DNA molecules?
8. What is shuttle vector and why is it used?
9. What information and materials are needed to amplify a segment of DNA using PCR?
10. In most PCR reactions, a DNA polymerase that can withstand short periods of very high (near boiling) temperatures is used. why?

TN_GOVT_XII_Micro_Biology_CH12.indd 278 07-12-2021 15:43:47

11. The sequence of nucleotides in an mRNA is 5′-AUG-ACCCAUU-CAUUGGUCUCGUUAG-3′. Assuming that ribosomes could translate this mRNA, how many amino acids long would you expect the resulting polypeptide chain to be?
12. The N-terminus of a protein has the sequence Met-His-Arg-Lys-Val-His-Cys-Gly. A molecular Biologist wants to synthesize a DNA chain that can encode this portion of the protein. How many DNA sequences can encode this polypeptide?
13. Explain the process by which an infected bacterium releases progeny phage.
14. Define coding strand.
15. Distinguish a missense and a nonsense mutation.
16. By what mechanism does 5-bromouracil induce mutations?
17. Define the term conjugative.
18. How does an Hfr cell differ from F^+ cell?
19. How are F′ plasmids produced?
20. Define a lysogen.
21. Restriction enzymes generate two types of termini. What are they?
22. Explain cosmids and the advantages resulting from the use of a cosmid?
23. Explain the use of bacteriophage in cloning DNA fragment.
24. What are expression vectors?
25. Diagramatically describe the plasmid cloning vector PUC19.
26. How is the natural phenomenon of conjugation used to transfer foreign gene?
27. List the stages involved in Recombinant DNA technology.
28. Discuss RAPD and RFLP.

TN_GOVT_XII_Micro_Biology_CH12.indd 279 07-12-2021 15:43:47

MICROBIOLOGY

PRACTICAL MANUAL

XII_Micro_Biology_Practical_Manual.indd 280 2/13/2020 5:13:50 PM

Std XII Microbiology Practical Manual

S.No	Major Practicals .	Page. No
1	Gram's staining of curd/idly batter/yeast	284
2	Identification of the fungus(*Aspergillus/Mucor/Rhizopus*) by wet mount using LPCB.	285
3	Blood grouping	287
4	Blood staining	288
5	Test for catalase	290
6	Widal test (slide test)	292
7	Demonstration of rhizobium from root nodules and its isolation	294
S.No	**Spotters**	**Page. No**
	II A) Specimen	
1	Root nodules of leguminous plant	297
2	Tikka leaf spot of groundnut plant	297
3	Mushroom	298
4	Sand fly	298
5	Ascaris	299
	II B) Slide	
6	Cyst of *Entamoeba histolytica*	299
7	*Penicillium* species	300
8	Microfilariae	300
9	Egg of *Ascaris lumbricoides*	301
10	Heterocysts of Nostoc	301
11	Acid fast bacilli	302
	II C) Spotter	
12	Antibiotic sensitivity plate set up by Kirby Bauer technique	302
13	Sugar fermentation tube showing acid and gas production	303
14	Agarose gel electrophoresis apparatus.	303
15	Spoiled food	304

XII_Micro_Biology_Practical_Manual.indd 281 2/13/2020 5:13:50 PM

Higher Secondary – Second Year Practical Examination

Microbiology	
Marking Scheme	
Allotment of Marks	
Internal Assessment	05 marks
External Assesment	15 marks
Total	**20 marks**
Internal Assessment (Practicals) Marks Break Up	
1. Record Note Book	03 marks
2. Skill of performing Experiments	02 marks
Total	**05 marks**
External Assessment Mark Break Up	
1. Major Practical	09 marks
2. Spotters	06 marks
Total	**15 marks**
I. Major Practical (Any one out of 5 questions) 9×1 = 9 marks	
• Aim	01 mark
• Principle	02 marks
• Procedure	03 marks
• Diagram	01 marks
• Observation	01 marks
• Results	01 marks
Total	**09 marks**
II. Spotters (Any three – one from each category) 2×3 = 6 marks	
• Identification	½ marks
• Two salient points	1 mark
• Diagram	½ mark
Total	**02 marks × 3 spotters = 6marks**

XII_Micro_Biology_Practical_Manual.indd 282 2/13/2020 5:13:50 PM

Key for Practical Examination

I. Major Practical (Any one) 9×1 = 9 marks

1. Determine the gram nature of microorganism present in the given sample (curd/idly batter/yeast)
2. Identify whether the given fungus is *Aspergillus* or *Mucor* or *Rhizopus* based on its microscopic characteristics.
3. Determine the blood group of the given blood sample.
4. Carry out blood staining using field's stain and observe the erythrocytes and leucocytes.
5. Identify whether the given culture is catalase positive.

II. Spotters

A. Specimen	2 marks
B. Slide	2 marks
C. Spotter	2 marks

XII_Micro_Biology_Practical_Manual.indd 283 2/13/2020 5:13:50 PM

1. Gram's staining of curd/idly batter/yeast

Aim: To determine the gram nature of microorganism present in the given sample(curd/ idly batter/yeast) by Gram's staining technique.

Theory and Principle:

Based on gram staining reaction bacteria can be divided into two large groups called gram positive and gram negative. Gram positive bacteria have thicker peptidoglycan compared to the gram negative bacteria. The lipid content of the cell wall of gram negative bacteria is higher than that of gram positive bacteria. Both gram positive and gram negative bacteria take up the primary stain crystal violet and stain red. When decolorized the porosity and permeability of the gram negative bacteria increases due to which the crystal violet iodine complex is given out by the gram negative bacteria. Further the gram negative bacteria takes up the counterstain safranin and stains red. Hence when bacteria are observed under microscope after gram staining the gram positive cells appear violet and gram negative cells appear red.

Requirements:

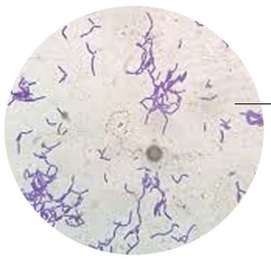
- Clean grease free slide
- Nichrome loop
- Given culture
- Crystal violet
- Grams iodine
- Decolorizer(Acetone Alcohol)
- Safranin
- D/W

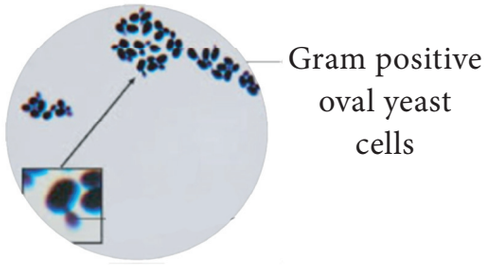
Procedure:

1. Take a loopful of the given culture and place on the slide.
2. Prepare a smear and heat fix it.
3. Cover the smear with Crystal Violet for one minute.
4. Wash gently
5. Add Grams iodine for one minute
6. Decolorise with acetone alcohol
7. Wash the slide immediately
8. Cover the smear with safranin for a minute
9. Wash and Air dry.
10. Observe the slide under high power and oil immersion objectives.
11. Record your observations.

XII_Micro_Biology_Practical_Manual.indd 284 2/13/2020 5:13:51 PM

Diagram: (Any one Diagram)





Observation Table: (any one shape and stain)

Sr. no	Morphology	Arrangement	Colour of Cytoplasm	Colour of Background	Inference
1.	Rod (bacilli)	Singles, chains	Violet	colourless	Gram positive
2.	Oval yeast cells	Singles, budded	Violet	colourless	Gram positive

Results: Gram staining of the given culture revealed gram positive violet colored rod-shaped bacteria in chains.

2. Identification of the fungus (Aspergillus/Mucor/Rhizopus) by wet mount using LPCB.

Aim: To identify whether the given fungus is *Aspergillus* or *Mucor* or *Rhizopus* based on microscopic characteristics by wet mount method using lactophenol cotton blue stain.

Theory and Principle :

Filamentous fungi are reliably identified by their characteristics microscopic morphology such as shape, size and arrangement of spores and hyphae. Fungi are eukaryotic and range from unicellular yeast to multicellular molds. They reproduce by producing spores.

Common fungi are Aspergillus, Mucor and Rhizopus. They are filamentous and collectively form mycelium. The morphology of the hyphae and spores can be identified using a simple wet mount technique using lactophenol cotton blue stain.

The organism suspended in the stain are killed due to the presence of phenol. Lactic acid preserves fungal structures and cotton blue stains the fungal cell wall.

Fungi	Characteristics of Hyphae	Spores borne in
Aspergillus sp.	Septate	Conidiophore bear conidia
Mucor and Rhizopus sp.	Aseptate	Sporangiosphore bear sporangium containing sporangiospore.

XII_Micro_Biology_Practical_Manual.indd 285 2/13/2020 5:13:52 PM

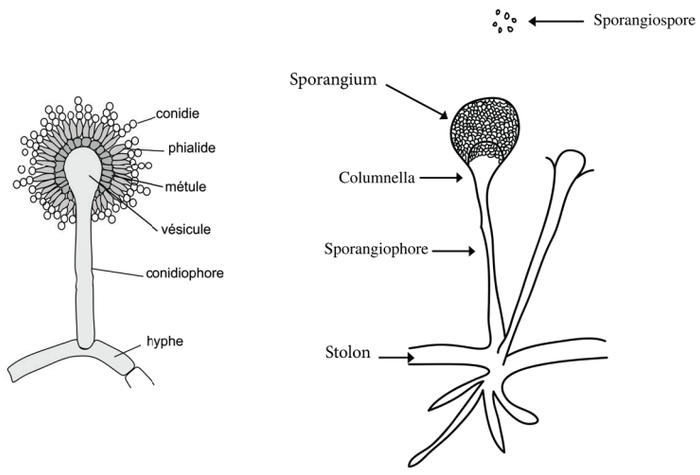
Requirements :

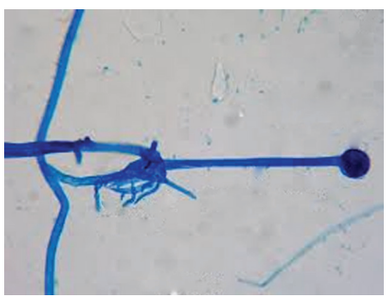
- Clean grease free slide
- Coverslip
- Forcep
- Teasing needle
- Distilled water
- Lactophenol Cotton Blue

Procedure:

1. Take a clean slide.
2. Place a drop of water on the slide.
3. With the help of forceps transfer the fungal mycelium.
4. Tease it with needle to separate the filaments (hyphae).
5. Add a drop of lactophenolcotton blue.
6. Gently place a coverslip avoiding air bubble formation.
7. Observe under low power and high power objective lens.
8. Read the observations and interpret.

Diagram:





XII_Micro_Biology_Practical_Manual.indd 286 2/13/2020 5:13:56 PM

Observation;

Filamentous hyphae bearing sporangia were observed.

Results:

Wet mount using lactophenol cotton blue was carried to identify the fungus sample. Hyphae with sporangium bearing sporangiospores were observed. It is likely to be of mucor species.

3. Blood Grouping

Aim: To determine the blood group of the blood sample by the slide agglutination test.

Theory and Principle:

Blood grouping is an essential requirement before blood is transfused from one person to another.It is also useful in settling paternity disputes and medicolegal problems.

Red blood cells contain blood group antigens. Antibodies to the blood group antigens are present in the blood plasma.The antigens are generally determined and are responsible for blood types. When RBCs of a person are mixed with corresponding antiserum, agglutination occurs due to antigen-antibody reactions.

Materials Required

- Blood sample (anticoagulated)
- Sterile cotton
- Sterile lancet
- Clean dry grease free slides or white tile
- Toothpicks
- Marker pen
- Commercially available Anti A sera, Anti B sera and Anti D sera

Procedure

1. Prick the finger under aseptic conditions
2. Place a drop of blood on the slide on each side marked as A, B and D.
3. Add a drop of antiserum A , B and D on A, B and D side respectively.
4. Mix with toothpick using separate toothpicks for each mixture.
5. Wait for 2 mins and observe for clumping reaction if any confirm it by observing under microscope.
6. Interpret the results and report.

XII_Micro_Biology_Practical_Manual.indd 287 2/13/2020 5:13:56 PM

Interpretation

If agglutination on A side the blood group is A

If agglutination seen on B side the blood group is B

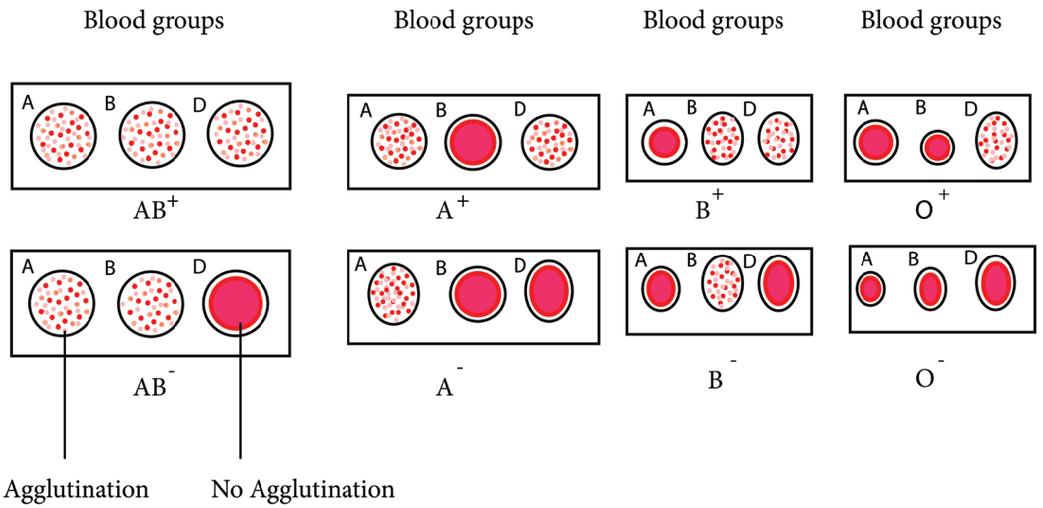
If Agglutination on both A and B side the blood group is AB

If No agglutination on A and B side the blood group is O

If agglutination is seen on D side the blood group is Rh(D) positive

If No agglutination on D side the blood group is Rh(D) negative.

Diagram: (any one depending on the results)



Observation: (will vary with the type of blood group an example is given below)

Agglutination is seen on A, B and D side

Result: The blood group of the blood sample was determined by slide agglutination test and was found to be AB Rh positive.

4. Blood Staining

AIM

To make a blood smear ,stain it using Field's stain and observe the erythrocytes and leucocytes.

Theory and Principle:

Blood smears are used to determine leukocyte differentials, to evaluate erythrocyte, platelet and leukocyte morphology, and, if necessary, to estimate platelet and leukocyte counts. It is also used for diagnosis of parasites like plasmodium in the blood.

Field's Stain is a romanowsky stain, used for rapid processing of blood specimens and is used to stain thick and thin films. It consists of two differential stain.**Field stain A**

XII_Micro_Biology_Practical_Manual.indd 288 2/13/2020 5:13:59 PM

which is methylene blue and Azure dissolved in a phosphate buffer solution.It is the basic component of the stain and **Field stain B** made up of Eosin Y in a buffer solution which is the acidic component of the stain.These basic and acidic dyes induce several colours when applied to cells.The fixator, methanol, does not allow any additional changes to the slide. The basic component of peripheral white blood cell(cytoplasm) is stained with acid dye and the acid component that is nucleic acid of the nucleus takes on the basic dye and is stained blue to violet. The neutral components of the cells are stained by both dyes(Field's stain A and B solution).

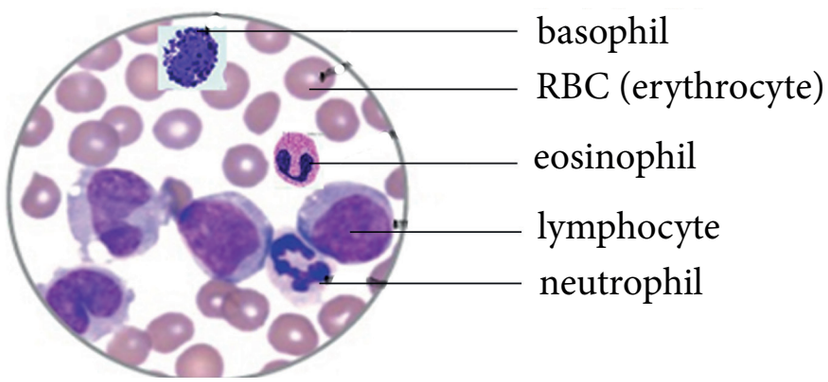
Requirements

- Cotton
- Spirit
- Blood sample
- Clean grease free slides
- Methanol fixative
- Field's stain A and Field's stain B.

Procedure

1. Finger Prick under aseptic condition.
2. Place a small drop of blood, on one side about 1-2 cm from one end of a slide.
3. Without delay place another slide at an angle of 45° to make contact with the drop.
4. Spread it over an area of about 2 cm^2(The film should be distributed so thinly that it appears transparent.
5. After air drying the thin blood film,immerse or fix the smear in methanol for 1 minutes.
6. Flood or dip the slide in Field's Stain A for 2-3 seconds.
7. Wash it with distilled water,
8. Flood or dip the slide in Field's Stain B for 2-3 seconds and wash with distilled water.
9. Now air dry the smear and observe under microscope.

Diagram



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Observation

TYPE OF CELL	COLOUR OF CYTOPLASM	COLOUR OF NUCLEUS	COLOUR OF GRANULES
RBC	pink	-	-
WBCs(leucocytes)			
Neutrophil	pink	blue	lilac
Eosinophil	pink	blue	orange
Basophil	pink	blue	Dark blue black
lymphocyte	blue	violet	-

Results

The blood smear was stained using field's stain and erythrocytes and leucocytes were observed under microscope.

5. Test for Catalase

Aim

To test whether the given culture is catalase positive by the catalase test

Theory And Principle

Catalase test demonstrates the presence of catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide (H_2O_2). It is used to differentiate those bacteria that produces an enzyme catalase, such as *staphylococci*, from non-catalase producing bacteria such as *streptococci*.

The enzyme catalase mediates the breakdown of hydrogen peroxide into oxygen and water. The presence of the enzyme in a bacterial isolate is evident when a small inoculum is introduced into hydrogen peroxide, and the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production. The culture should not be more than 24 hours old.

Requirements

- Slides
- Nichrome loop or toothpick
- 24hour old culture
- 3%hydrogen peroxide
- Dropper

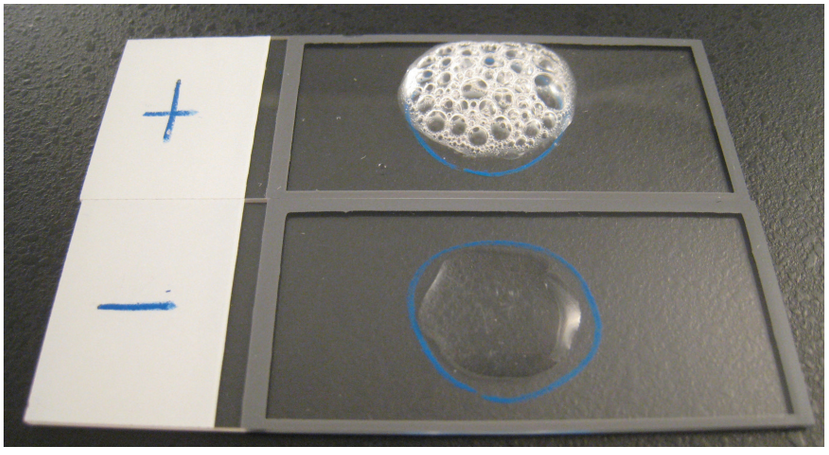
XII_Micro_Biology_Practical_Manual.indd 290 2/13/2020 5:14:00 PM

Procedure

Slide Method

1. Use a loop or sterile wooden stick to transfer a small amount of colony growth in the surface of a clean, dry glass slide.
2. Place a drop of 3% H_2O_2 in the glass slide.
3. Observe for the evolution of oxygen bubbles.

Diagram



$2H_2O_2 \xrightarrow[\text{catalase}]{} 2H_2O + O_2$ (gas bubbles)

Observation (any one to be reported depending on the culture)

Positive: Copious bubbles produced, active bubbling

Examples: *Staphylococci, E. coli, Enterobacter, Klebsiella, Shigella, Yersinia, Pseudomonas.*

Negative: No or very few bubbles produced.

Examples: *Streptococcus and Enterococcus* sps.

Result

The given culture was found to be catalase positive as determined by the catalase slide test.

XII_Micro_Biology_Practical_Manual.indd 291 2/13/2020 5:14:00 PM

6. Widal Test (Slide Test)

Aim

To carry out the widal test for the given blood sample and to determine the presence of antibodies against salmonella antigens.

Theory And Principle

Widal test is a serological test which is used for the diagnosis of enteric fever or typhoid fever. Typhoid or enteric fever is caused by a gram negative bacteria *Salmonella enterica* (*Salmonella* Typhi or *Salmonella* Paratyphi). *Salmonella* possess O antigen on their cell wall and H antigen on their flagella. On infection, these antigen stimulates the body to produce specific antibodies which are released in the blood. The Widal test is used to detect these specific antibodies in the serum sample of patients suffering from typhoid using antigen-antibody interactions. These specific antibodies can be detected in the patient's serum after 6 days of infection (fever).

Salmonella Typhi possesses O antigen on the cell wall and H antigen on flagella. *Salmonella* Paratyphi A and *S.* Paratyphi B also possess O antigen on their cell wall and but have AH and BH antigen on their flagella respectively.

Widal test is an agglutination test in which specific typhoid fever antibodies are detected by mixing the patient's serum with killed bacterial suspension of Salmonella carrying specific O, H, AH and BH antigens and observed for clumping ie. Antigen-antibody reaction. The main principle of Widal test is that if homologous antibody is present in patient's serum, it will react with respective antigen in the suspension and gives visible clumping on the test slide.

Requirements

Fresh serum

The complete kit containing five vials containing stained Salmonella antigen

- *S.* Typhi ⟶ O antigen
- *S.* Tyhhi ⟶ H antigen
- *S.* Paratyphi ⟶ AH antigen
- *S.* Paratyphi ⟶ BH antigen

Widal positive control

Widal test card or slide

v) Applicator stick

Procedure

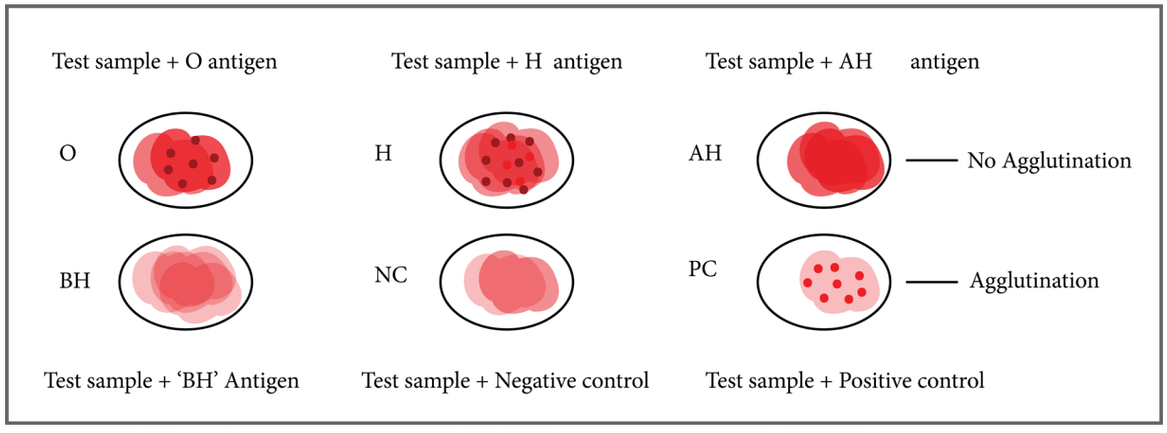
- Widal test can be done in two ways-one is rapid test on slide and another is tube test in which result may be obtained after one night of incubation.

XII_Micro_Biology_Practical_Manual.indd 292 2/13/2020 5:14:00 PM

Rapid slide test:

1. Clean the glass slide or test card supplied in the kit well and make it dry.
2. Label the circles (1, 2, 3, 4, 5 and 6) in the test card as O, H, AH, BH, Negative control and Positive control
3. Place a drop of undiluted test serum in each of the four labelled circle (1, 2, 3 and 4) ie O, H, AH and BH and place a drop of Negative control serum in circle 5 and Positive control in circle 6.
4. Place a drop of antigen O, H, AH and BH in circle 1, 2, 3, and 4 respectively and no antigen in circle 5 and O/H antigen in circle 6.
5. Mix the content of each circle with a separate wooden applicator stick and spread to fill the whole area of the individual circle.
6. Rock the test card for a minute and observe for agglutination.

Diagram



Observation

Agglutination was observed in O and H side within a minute which indicates the presence of antibodies in the serum sample against Salmonella typhi antigens.

Proceed for quantitative slide test or tube test for the quantitative estimation of the titre of the antibody.

Result

Qualtative widal test was carried out using rapid slide agglutination method. Antibodies against O and H antigens of Salmonella typhi were detected in the serum.

XII_Micro_Biology_Practical_Manual.indd 293 2/13/2020 5:14:01 PM

7. Demonstration of rhizobium from root nodules and its isolation

Aim:

To demonstrate the presence of rhizobium in root nodules by gram staining and isolate them on a nutrient medium.

Theory and Principle:

Leguminous plants like cowpea, red gram , black gram contain root nodules formed by rhizobium.Rhizobium in the soil enter into the roots of leguminous plant and form nodules and establish symbiotic association. Bacteria derive nutrients from the plants. The rhizobacteria fix nitrogen which is beneficial to the plant. Rhizobium is a symbiotic N_2 fixer found to occur as bacteroids in the root nodules of leguminous plants. They can be easily isolated and cultured in vitro.

Based on gram staining reaction bacteria can be divided into two large groups called gram positive and gram negative. Gram positive bacteria have thicker peptidoglycan compared to the gram negative bacteria. The lipid content of the cell wall of gram negative bacteria is higher than that of gram positive bacteria. Both gram positive and gram negative bacteria take up the primary stain crystal violet and stain red. When decolorized the porosity and permeability of the gram negative bacteria increases due to which the crystal violet iodine complex is given out by the gram negative bacteria. Further the gram negative bacteria takes up the counterstain safranin and stains red. Hence when bacteria are observed under microscope after gram staining the gram positive cells appear violet and gram negative cells appear red. **Rhizobia are Gram- negative rods which are motile with bi-polar, sub-polar and peritrichous flagella**.

Rhizobium grows well on Yeast Extract Mannitol Agar (YEMA). Congo red added to the medium differentiates rhizobia that stand out as white, translucent, glistening elevated, small colonies with entire margin, in contrast to the red stained colonies of Agrobacterium and other bacteria.

Requirements:

1. Root nodules (pink) of any leguminous plant
2. **Congo red, Yeast Extract, Mannitol Agar (pH 6.8 – 7.0):**

Mannitol	10.0 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g
Yeast extract	1.0 g
$CaCO_3$	3.0 g
Agar	25.0 g

XII_Micro_Biology_Practical_Manual.indd 294 2/13/2020 5:14:01 PM

Congo red (1% aqueous)

2.5 ml (1.0 g in 100 ml)

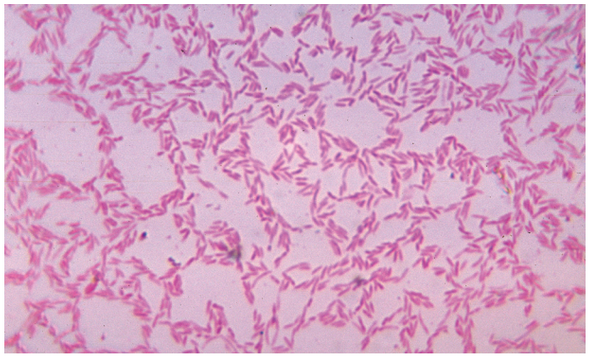
Distilled water 1000.0 ml

3. Inoculation loop
4. Bunsen burner/laminar clean air flow hood.
5. Slides and glass rod.
6. Petri plates with YEMACR medium.
7. Sterile distilled water.
8. 95% alcohol and 0.1% $HgCl_2$.

Procedure:

1. Wash the root system under a slow stream of running tap water, taking care to see that the nodules are intact.
2. Select pink nodules and remove them
3. Wash and keep the nodules in 95% ethanol for a minute, wash and transfer them to 0.1% $HgCl_2$.
4. Remove after five minutes and wash the nodules about four to five times with sterile distilled water.
5. Place the nodule on a sterile slide in a drop of sterile distilled water and crush it either with a sterile glass rod or a flat tipped forceps.
6. Remove a loopful of this cloudy suspension and streak inoculate on YEMACR plates and label.
7. Incubate in dark at 28°-30°C for 2-3 days and observe the colonies.
8. Make a smear of the remaining crushed material and gram stain and observe the gram negative bacilli. Even samples from the colonies can be gram stained.

Diagram:





XII_Micro_Biology_Practical_Manual.indd 295 2/13/2020 5:14:02 PM

Observation

Gram's stain

Organism	Morphology	Arrangement	Colour of cytoplasm	Colour of Background	Inference
Rhizobium from root nodule.	Rod (bacilli)	Singles	red	colourless	Gram negative

Colony characteristics of rhizobium on YEMA after incubation for 2-3 days at room temperature

Size – 2-4 mm

Shape- circular

Colour – White

Margin - entire

Elevation – convex, raised

Opacity - semitranslucent

Texture – creamy

Consistency – mucilaginous

Gram nature – gram negative

Motility – actively motile

Results: Gram staining of the root nodule exudate revealed the presence of gram negative rods.

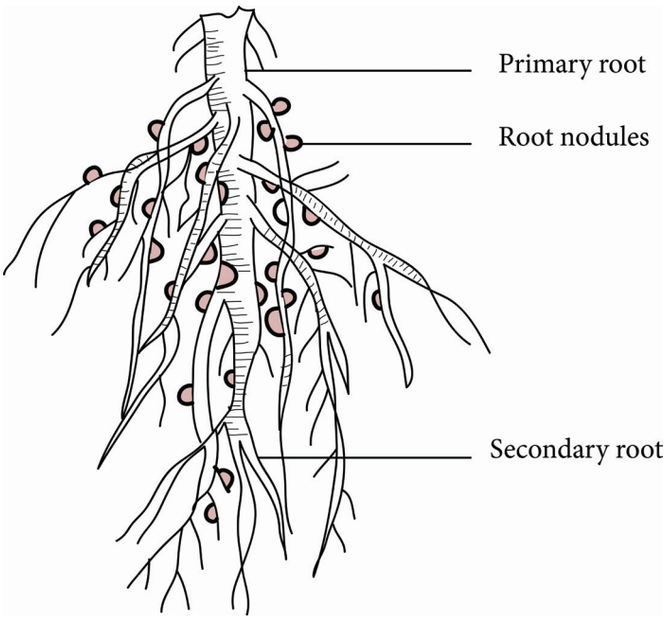
The colony characteristics of rhizobia were studied after isolation on YEMA medium. White, creamy, mucoid colonies were obtained.

XII_Micro_Biology_Practical_Manual.indd 296 2/13/2020 5:14:02 PM

Spotters

II A) Specimen

1. Root nodules of leguminous plant



- Leguminous plants like cowpea, red gram contain root nodules formed by rhizobium.
- Rhizobium in the soil enter into the roots of leguminous plant and form nodules and establish symbiotic association.
- Bacteria derive nutrients from the plants.
- The rhizobacteria fix nitrogen which is beneficial to the plant.

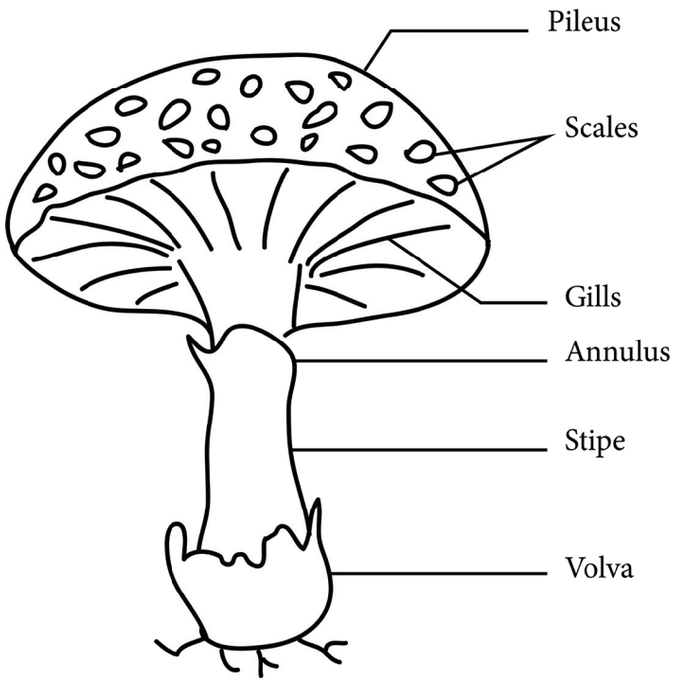
2. Tikka leaf spot of groundnut plant



XII_Micro_Biology_Practical_Manual.indd 297 2/13/2020 5:14:04 PM

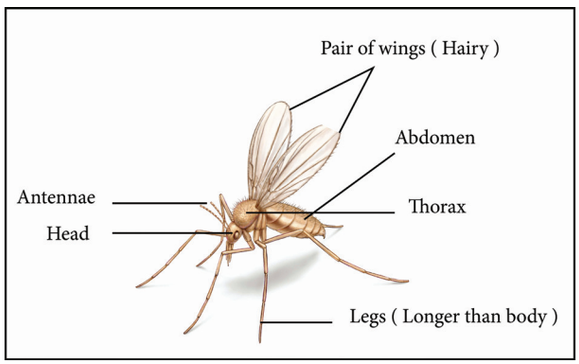
- Tikka leaf spot disease is a kind of fungal disease seen in groundnut leaf.
- This disease is caused by Cercaspora personata.
- Brown spots surrounded by a yellow halo appear on the upper surface of the leaf.
- The fungal spores can be demonstrated if the leaf is processed and observed under microscope.

3. Mushroom



- Mushroom is a saprophytic fungus.
- Primary mycelium grows from basidiospores.
- It has high protein content and edible mushrooms are used as food.
- Example: Agaricus species and Pleurotus species.

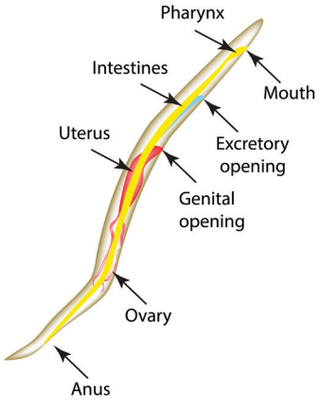
4. Sand fly



XII_Micro_Biology_Practical_Manual.indd 298 2/13/2020 5:14:07 PM

- Bite of an infected sandfly transmits leishmania donovani infection.
- Female sandfly during a blood meal ingest free as well as intracellular amastigotes in the blood.
- In the midgut these are transformed to flagellated promastigote.

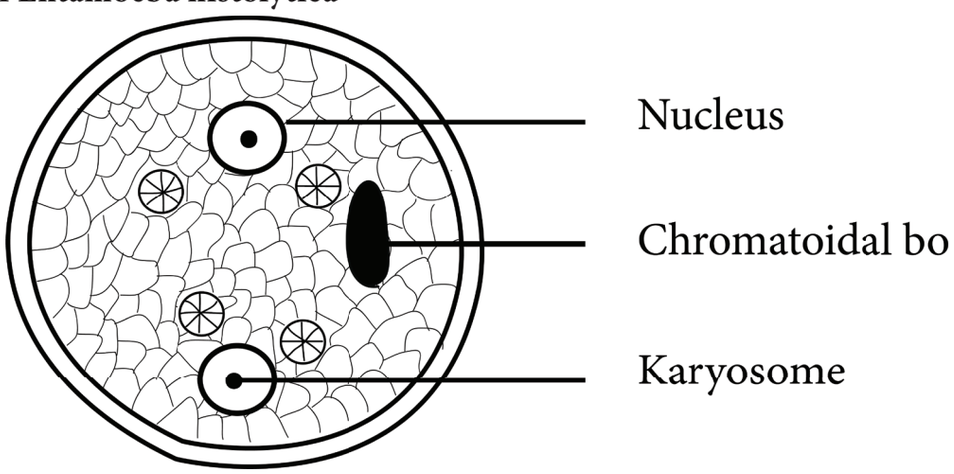
5. Ascaris



- The adult worm of ascaris lives in the small intestine of humans
- They are large cylindrical worms with tapering ends, the anterior end being thinner than the posterior end
- The adult male worm is smaller than female worms.

IIB) Slide

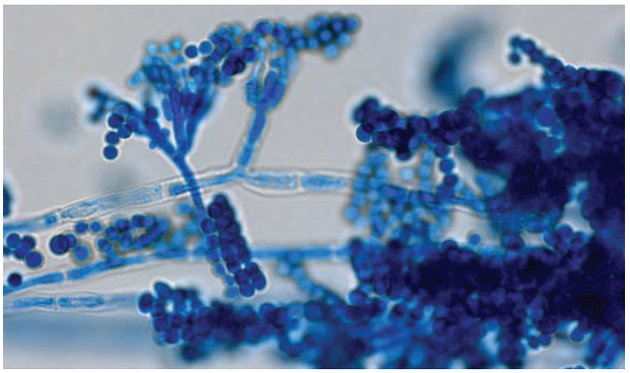
6. Cyst of Entamoeba histolytica



- Cyst is one of the three forms of entamoeba histolytica
- A mature cyst is a quadrinucleate spherical body.
- Mature cysts are passed in the stool of infected person
- Direct examination of wet mount of stool for cysts is diagnostic of intestinal amoebiasis

XII_Micro_Biology_Practical_Manual.indd 299 2/13/2020 5:14:08 PM

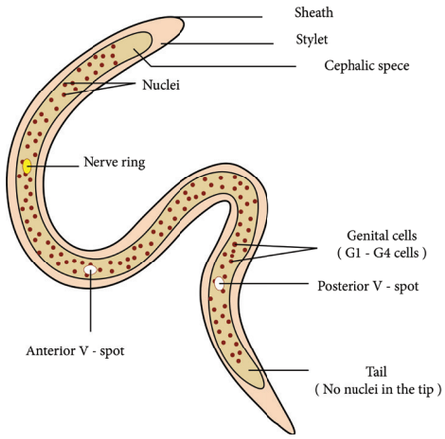
7. Penicillium species



- Colony of penicillium are initially white and fluffy and later produce pigmented spores and turn into shades of green or blue green
- Hyphae are hyaline and septate
- Condiophores are long, give rise to branching phialids
- Phialids branch and give the appearance of brush or penicillins
- They produce sterigmata bearing chain of conidia (spores) which are oval or spherical and measure 1-2micrometer.

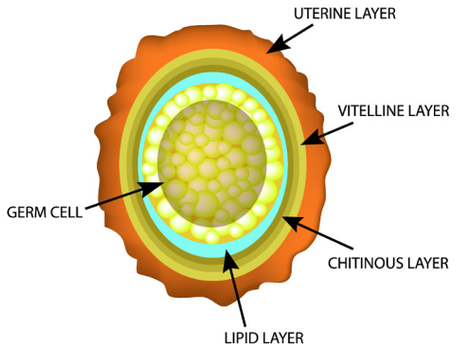
8. Microfilariae

- Filariasis is caused by nematodes (roundworms) like *Wuchereria bancrofti* that inhabit the lymphatics and subcutaneous tissues.
- The female worms release the first stage larvae called microfilariae, which are detected in the peripheral blood.
- Identification of microfilariae by microscopic examination is the most practical diagnostic procedure.
- The blood sample can be a thick smear, stained with Giemsa.
- The larva measures about 290microns in length and 6-7micron in breath.



XII_Micro_Biology_Practical_Manual.indd 300 2/13/2020 5:14:10 PM

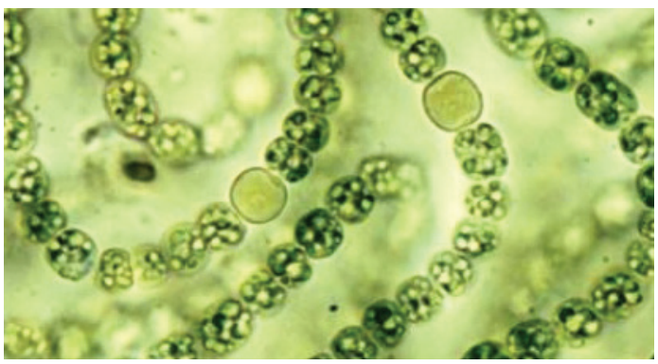
9. Egg of Ascaris lumbricoides



EGG OF ASCARID FERTILIZED

- These are passed in stool of the infected host.
- Brownish due to bile pigment.
- Fertilised eggs are rounded and have a thick shell (chitinous).
- Unfertilised eggs are elongated and larger than fertile eggs.
- When ingested through water or contaminated food by human it causes Ascariasis.
- Microscopic identification of eggs in the stool is the most common method for diagnosing intestinal ascariasis.

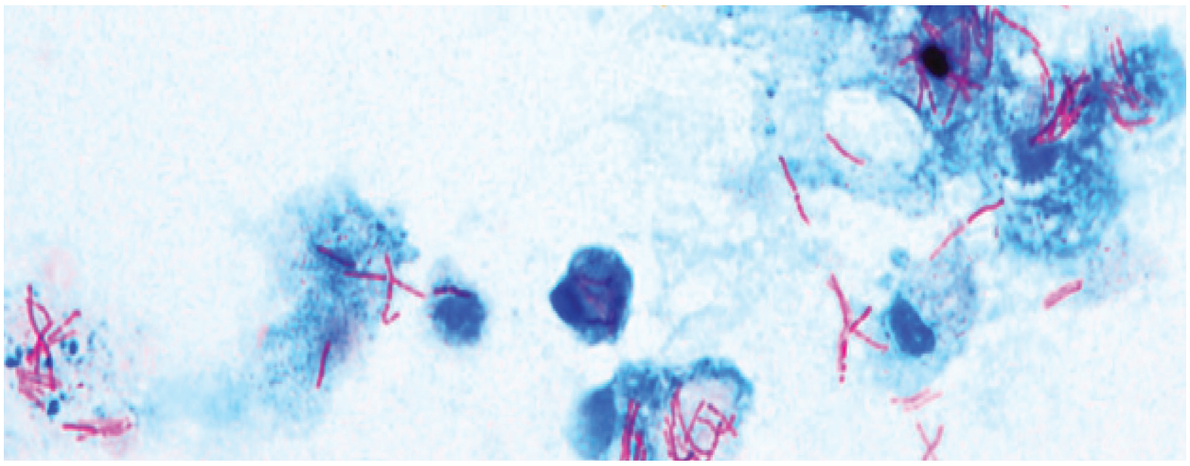
10. Heterocysts of Nostoc



- Heterocysts are specialized structures having thick cell wall formed in some filamentous blue green algae like Nostoc, Anabena.
- They may be terminal or found in between the vegetative cells attached to it by means of pores.
- They are sites of atmospheric nitrogen fixation.
- They serve as a store house of food material.

XII_Micro_Biology_Practical_Manual.indd 301 2/13/2020 5:14:10 PM

11. Acid fast bacilli

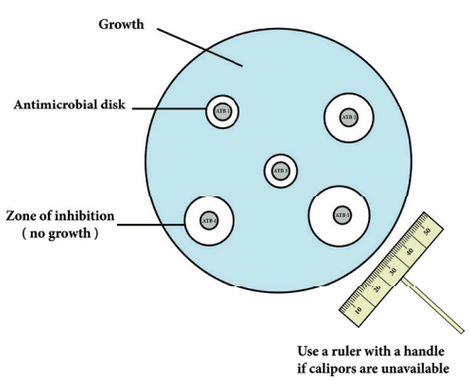


- Acid fast bacilli contains mycolic acid in their cell walls hence do not get stained easily, however once stained cannot be decolourised easily.
- Special method like Ziehl- Neelson's Carbol fuchsin is used to stain acid fast bacilli.
- The acid- fast bacilli are stained red in colour while the non acid fast cells appear blue when counterstained with methylene blue.
- Mycobacterium tuberculosis is and acid fast bacilli.

IIC) Spotter

12. Antibiotic sensitivity plate set up by Kirby Bauer technique

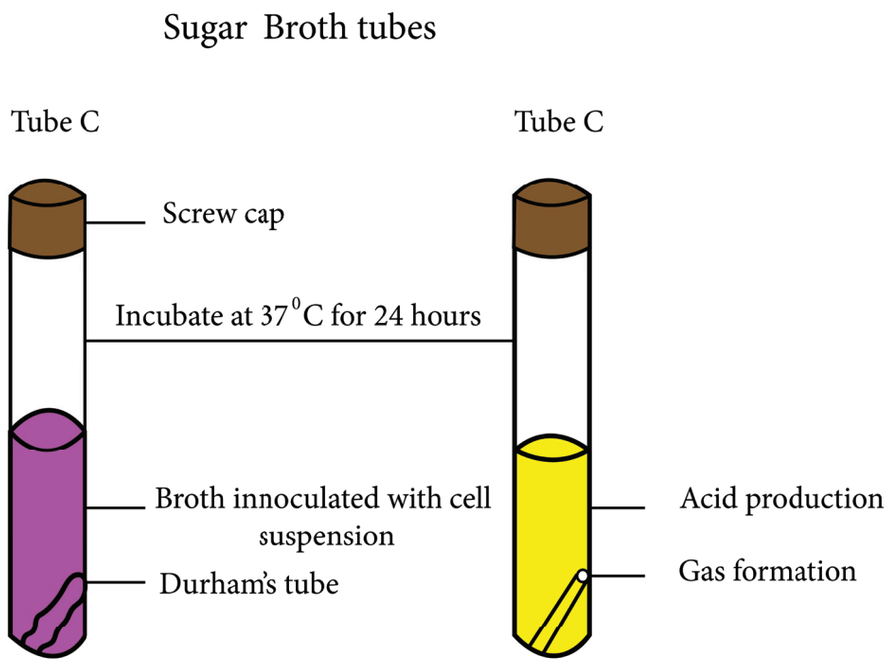
- Kirby Bauer technique is used to determine the susceptibility of the organism to various antimicrobial agents.
- Standard suspensions of rapidly growing test bacterium is inoculated on the surface of muller hinton agar plates.
- Antibiotic discs are pressed on the surface of the seeded plates.
- The zone of inhibition or the zone of growth determines the degree of susceptibility of the organism towards antibiotic.



XII_Micro_Biology_Practical_Manual.indd 302 2/13/2020 5:14:11 PM

13. Sugar fermentation tube showing acid and gas production

- Carbohydrate broth with bromocresol purple as indicator is used for testing the ability of pure bacterial culture to ferment a specific sugar like lactose, xylose, mannitol and other sugars.
- Acid production is indicated by colour change of the indicator from purple to yellow
- Gas production is indicated by an air bubble in the durham's tube.
- Escherichia coli ferments lactose producing acid and gas.

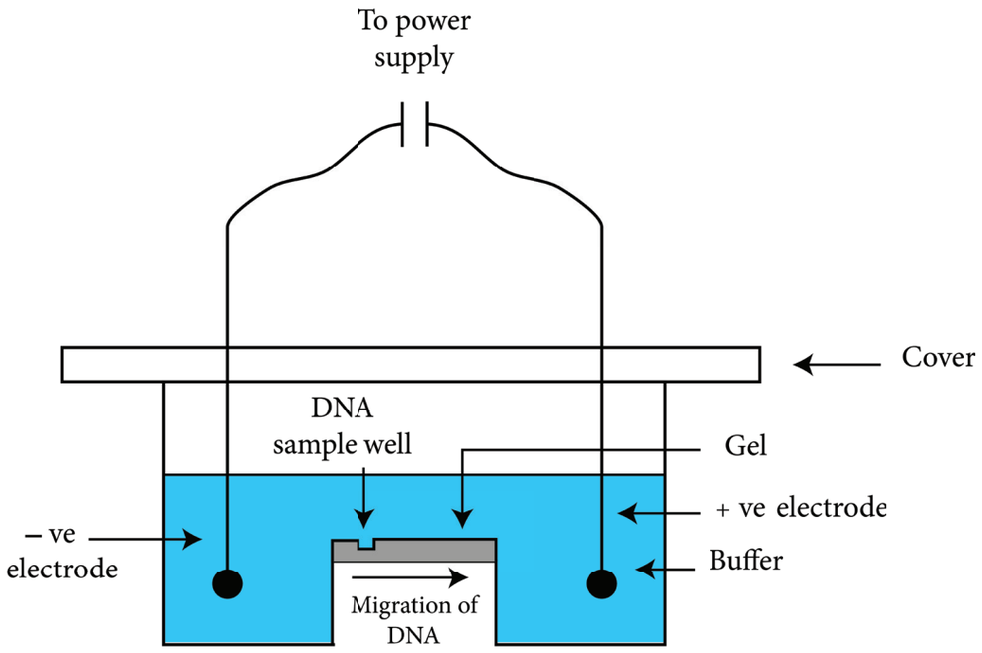


In the above image Test tube C was innoculated with cell suspension and incubated at 37 C for 24 hours and after innoculation the purple colour of broth was changed to yellow colour with gas bubble formation in durham's tube.

14. Agarose gel electrophoresis apparatus.

- Electrophoresis refers to the movement of charged molecules in an electric field.
- The negatively charged molecules move towards the positive electrode while the positively charged molecules migrate towards the negative electrode.
- Gel electrophoresis is a routinely used analytical technique for the separation and purification of specific DNA fragments.
- As the DNA is negatively charged, DNA fragments move through the gel towards the positive electrode. The rate of migration of DNA is dependent on the size and shape.

XII_Micro_Biology_Practical_Manual.indd 303 2/13/2020 5:14:16 PM



15. Spoiled food

- Spoilage is a process in which food deteriorates such that its quality of edibility is reduced.
- Food poisoning may result on eating contaminated or spoiled food.
- Foods spoil due to attacks from enzymes, oxidation and microorganisms.
- These include bacteria, mold, yeast, moisture, temperature and chemical reaction.





XII_Micro_Biology_Practical_Manual.indd 304 2/13/2020 5:14:22 PM

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This book has been printed on 80 G.S.M.
Elegant Maplitho paper.

Printed by offset at:

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