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
Contents

Chapter 1  Developments in Microbiology ............................................. 01
Chapter 2  Microscopy ............................................................................. 12
Chapter 3  Control of Microorganisms by Chemical Methods .......... 28
Chapter 4  Microbial Metabolism ............................................................ 45
Chapter 5  Food Microbiology ................................................................. 68
Chapter 6  Industrial Microbiology .......................................................... 85
Chapter 7  Medical Bacteriology ........................................................... 114
Chapter 8  Medical Parasitology ............................................................ 151
Chapter 9  Medical Mycology ................................................................. 188
Chapter 10 Medical Virology ................................................................. 206
Chapter 11 Immunology ..................................................................... 230
Chapter 12 Microbial Genetics .............................................................. 249
Practical ................................................................................................. 291

E-Book  [QR Code]
Assessment  [QR Code]
Digi-Link  [QR Code]

Lets use the QR code in the text books ! How ?
- Download the QR code scanner from the Google PlayStore/ Apple App Store into your smartphone
- Open the QR code scanner application
- Once the scanner button in the application is clicked, camera opens and then bring it closer to the QR code in the text book.
- Once the camera detects the QR code, a url appears in the screen.Click the url and goto the content page.
Chapter Outline
Presents a complete overview of the chapter

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

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

Evaluation
Assess students to pause, think and check their understanding

Career corner
List of professions particular to that chapter
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 a question, a 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 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.

1.2 Emerging Microbes

Microbes and the diseases they cause have emerged as a major threat to human populations. Some of them like malaria have been major pathogens since they emerged in the depths of antiquity.

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.

Ebola

Ebola virus disease (EVD), also known as Ebola hemorrhagic fever (EHF) or simply Ebola, is a viral hemorrhagic fever of humans and other primates caused by Ebola virus.

Ebola is a rare but deadly virus that causes fever, body aches, and diarrhea, and sometimes bleeding inside and outside the body. The virus spreads through the body; it damages the immune system and organs. Ultimately, it causes levels of blood-clotting cells to drop. This leads to severe, uncontrollable bleeding. The disease was known as Ebola hemorrhagic fever but is now referred to as Ebola virus. It kills up to 90% of people who are infected.

Ebola is a contagious as more common viruses like colds, influenza, or measles. It spreads to people by contact with the skin or bodily fluids of an infected animal, like a monkey, chimp, or fruit bat. Then it moves from person to person the same way early on, Ebola can feel like the flu or other illnesses. Symptoms show up 2 to 21 days after infection and usually include: High fever, Headache, Joint and muscle aches, Sore throat, Weakness, Stomach pain, Lack of appetite. It causes bleeding inside the body, as well as from the eyes, ears, and nose. Some people will vomit or cough up blood, have bloody diarrhea, and get a rash (Figure 1.1).

Zika

Zika virus (ZIKV) is a member of the virus family Flaviviridae. It is spread by daytime-active Aedes mosquitoes, such as A. aegypti and A. albopictus. Its name comes from the Zika Forest of Uganda, where the virus was first
isolated in 1947. Zika virus is related to the dengue, yellow fever, Japanese encephalitis, and West Nile viruses.

Zika virus do not develop symptoms. Symptoms are generally mild including fever, rash, conjunctivitis, muscle and joint pain, malaise, and headache, and usually last for 2–7 days.

Zika virus is primarily transmitted by the bite of an infected mosquito from the *Aedes* genus, mainly *Aedes aegypti*, in tropical and subtropical regions. *Aedes* mosquitoes usually bite during the day, peaking during early morning and late afternoon/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. An increased risk of neurologic complications is associated with Zika virus infection in adults and children, including Guillain-Barré syndrome, neuropathy and myelitis (Figure 1.2).

### 1.3 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 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.”

1.3.1 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 1.3).

![Figure 1.3: Monoclonal antibody](image)

1.3.2 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
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.

1.4 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.

**Figure 1.4:** Stem cells
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 nucleic acids (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.

**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 1.5).

**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 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.

### 1.5 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 1.6).

1.6.2 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 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. EHF stands for ______________
   a. Ebola hemorrhagic fever
   b. Ebola heart fever
   c. Ebola human fever
   d. none of these

5. 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.
10. Write about Vaccines and its importance.
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
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 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

![Components of phase contrast microscope](image.png)

**Figure 2.1:** Components of phase contrast microscope
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.
The difference in the phases between the retarded (deviated) and un-retarded (non-deviated) light rays is about \( \frac{1}{4} \) of original wave length (i.e., \( \lambda/4 \)). 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.

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.

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 in 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.

2.1.4 Applications

- Phase contrast microscope enables the visualization of unstained living cells.
- It makes highly transparent objects more visible.
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 fluorescence 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.
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), FITC (emits green), and Texas Red (emits red).

2.2.1 Principle

Light source such as Xenon or Mercury Arch Lamp which provides light in a wide range of wavelength, from ultraviolet to the infrared is directed through on 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).

Infobits

Some substances have the property of luminescence. They emit light of one colour when exposed to light of a different colour. If light emission occurs within one millionth of a second of light exposure, the luminescence is phosphorescence. The colour of the emitted light has a longer wavelength than the colour of the exciting light. For example, fluorescein isothiocyanate (FITC) is excited by blue light and emits green light; rhodamine isothiocyanate is excited by green light and emits red light.

Figure 2.6: Components of fluorescence microscope
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 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.

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.
2.2.4 Application

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

---

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

**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 excitation 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.

---

**Infobits**

Figure 2.7: (a) Fluorescence microscope (b) Fluorescent stained tubercule bacilli
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. Table 2.1 differentiate electron microscope from light microscope.

**Table 2.1:** Difference between light and electron microscope

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

**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 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 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.

**Figure 2.8:** Interaction between electron beams with specimen
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.

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. The following 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.
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 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 their relative amounts

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

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

**DO YOU KNOW?**

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.
## Table 2.2: Difference between SEM and TEM

<table>
<thead>
<tr>
<th>S.no</th>
<th>Properties</th>
<th>SEM</th>
<th>TEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Types of electrons</td>
<td>It is based on scattered electrons that are emitted from the surface of a specimen</td>
<td>It is based on transmitted electrons</td>
</tr>
<tr>
<td>2</td>
<td>Sample preparation</td>
<td>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</td>
<td>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</td>
</tr>
<tr>
<td>3</td>
<td>Resolution</td>
<td>The resolution is up to 20nm</td>
<td>TEM has much higher resolution than SEM. It can resolve objects as close as 1nm</td>
</tr>
<tr>
<td>4</td>
<td>Magnification</td>
<td>The magnifying power of SEM is up to 100,000X</td>
<td>The magnifying power of TEM is up to 5,000,000X</td>
</tr>
<tr>
<td>5</td>
<td>Image formation</td>
<td>SEM provides a 3 dimensional image. Secondary or back scattered electrons are captured, detected and displayed on computer screen</td>
<td>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</td>
</tr>
<tr>
<td>6</td>
<td>Application</td>
<td>SEM is used to study the topography and atomic composition of specimens</td>
<td>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</td>
</tr>
</tbody>
</table>
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 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, STEM requires very thin samples and looks primary at beam electrons 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 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**

1. Who invented PCM/  
   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

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 fluorescent 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 fluorescent microscopy
4. What are the different types of filters used in fluorescent microscopy
5. Define flurochromes.
6. Write the functions of Barrier filter
7. Write about 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 about the Principle, Components and Mechanism of EM.
Control of Microorganisms by Chemical Methods

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

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.
- Knows the resistance mechanisms developed by pathogens against antibiotic or chemotherapy drugs.

3.6 Antibiotics
3.7 Antimicrobial Susceptibility Testing
3.8 Drugs Resistance Mechanisms

Control of microorganisms is essential in order to prevent the transmission of diseases, infection, spoilage and to or remove 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.
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 are produced by microorganisms kill or inhibit the growth of other microbes.

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

<table>
<thead>
<tr>
<th>Disinfection</th>
<th>Antiseptics</th>
<th>Antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorine, Copper</td>
<td>Phenol, Tincture of Iodine</td>
<td>Penicillin, Streptomycin</td>
</tr>
</tbody>
</table>

Basic terms used in chemical control of microorganism are mentioned in Table 3.1 and Table 3.2 Describes the difference between chemical disinfectant against bacteria.

Table 3.1: Basic terms used in Chemical sterilization

<table>
<thead>
<tr>
<th><strong>Term</strong></th>
<th><strong>Meaning</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Disinfection</td>
<td>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.</td>
</tr>
<tr>
<td>Antisepsis</td>
<td>Prevention of the growth or activity of microorganisms by inhibition or killing; applies to the use of chemicals on living tissue</td>
</tr>
<tr>
<td>-cide</td>
<td>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.</td>
</tr>
<tr>
<td>-static</td>
<td>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.</td>
</tr>
</tbody>
</table>

Table 3.2: Action and Examples of Chemical sterilization

<table>
<thead>
<tr>
<th><strong>Term</strong></th>
<th><strong>Action</strong></th>
<th><strong>Examples</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Algicide</td>
<td>Agent that kills algae</td>
<td>Copper sulfate</td>
</tr>
<tr>
<td>Bactericide</td>
<td>Agent that kills bacteria</td>
<td>Chlorohexidine, ethanol</td>
</tr>
<tr>
<td>Biocide</td>
<td>Agent that kills living organisms</td>
<td>Hypochlorite (bleach)</td>
</tr>
<tr>
<td>Fungicide</td>
<td>Agent that kills fungi</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Germicide</td>
<td>Chemical agent that specifically kills pathogenic microorganisms</td>
<td>Formaldehyde, silver, mercury</td>
</tr>
<tr>
<td>Sporicide</td>
<td>Agent that kills bacterial endospores</td>
<td>Glutaraldehyde</td>
</tr>
<tr>
<td>Virucide</td>
<td>Inactivates viruses so that they lose the ability to replicate</td>
<td>Cationic detergents (quaternary ammonium salts of acetates, chlorides)</td>
</tr>
</tbody>
</table>
3.2 Factors Influencing the Antimicrobial Activity of Chemical Agents

The following factors will affect 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.

g. Mode of action of antimicrobial agent

The nature of the material bearing the microorganism.

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.

Table 3.2: Difference between Bactercidal and Bacteriostatic

<table>
<thead>
<tr>
<th>Bactercidal</th>
<th>Bacteriostatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bactercidal refers to antibiotics that kill bacteria</td>
<td>Bacteriostatic refers to antibiotics that prevent the growth of bacteria</td>
</tr>
<tr>
<td>Action is irreversible</td>
<td>Action is reversible</td>
</tr>
<tr>
<td>Inhibit the cell wall formation of bacteria</td>
<td>Inhibit DNA replication and protein synthesis of bacteria</td>
</tr>
<tr>
<td>Do not work with the immune system of the host</td>
<td>Work with the immune system of the host to prevent the growth and reproduction of bacteria</td>
</tr>
<tr>
<td>MBC refers to the concentration of the drug required to kill 99.99% of the bacterial population</td>
<td>MIC is the minimum drug concentration which inhibits the bacterial growth</td>
</tr>
<tr>
<td>Examples include betalactam antibiotics, cephalosporins, and vancomycin</td>
<td>Examples include tetracyclines, spectinomycin, chloramphenicol, sulfonamides, etc.</td>
</tr>
</tbody>
</table>
Chemical disinfectants are grouped by the types of microbes and infectious agents they are effective against.

High-level germicides kill vegetative cells, fungi, viruses and endospores and can ultimately lead to sterilization.

Intermediate-level germicides cannot kill all viruses and are less effective against endospores.

Low-level germicides kill vegetative cells and some enveloped viruses, but are ineffective against endospores.

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. However, it can cause brain damage and is now used in hospital nurseries to control and outbreak of gram positive (Staphylococcal) infections. 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 soaking 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.
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.

Heavy metals combine with sulphydryl (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

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 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
They 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.
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 chlorhexidine 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 quaternaries as disinfectants and sanitizing agents.

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

6. Aldehydes

Aldehydes are highly effective, broad spectrum disinfectant. The most which typically achieve its antimicrobial 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 for moldehyde is used as a surface disinfectant and a fumigant and has been used to decontaminate in animate objects. A concentration 2% of glutaraldehyde is an effective disinfectant.

It is less irritating than formaldehyde and is used to disinfect hospital and laboratory equipments. Glutaraldehyde usually disinfects objects within 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.

### 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 server 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, whereas 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 required 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 specified microorganism referred to as the test organism.

**Chemical Methods of Microbial Control**

i. Chemical agents are used on living tissue (as antiseptics) and on inanimate objects (as disinfectants).

ii. Few chemical agent achieve sterility.

**Evaluating a disinfectant**

1. The phenol coefficient is the comparison of one chemical’s disinfecting action with that of phenol, applied for the same length of time on the same organisms under identical conditions.
2. In the use-dilution test, a series of tubes contain increasing concentrations of disinfectant; the more the chemical can be diluted and still be effective, the higher its rating.

3. In the filter paper method, a disk of filter paper is soaked with a chemical and placed on an inoculated agar plate; a clear zone of inhibition indicates effectiveness.

**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.

**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 by the concentration of phenol 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),

<table>
<thead>
<tr>
<th>Chemical Agent and Dilution</th>
<th>Presence of Growth in Subcultures (minutes)</th>
<th>5</th>
<th>10</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:80</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1:90'</td>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1:100</td>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Test Chemical</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:400</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1:450'</td>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1:500</td>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Phenol dilution of 1:90 showed at 5 minutes but no growth at 10 minutes. Test Chemical dilution of 1:450 should grow at 5 minutes but not at 10 minutes. Phenol coefficient of test chemical = 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 trials were done in 1940.
Antibiotics are produced by species of Streptomyces, Bacillus, Penicillium and Cephalosporium.

**Infobits**

1904 Ehrlich found that the dye try pan 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 microbially synthesized.

**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](image)

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

![Classification based on Antimicrobial spectrum](image)

Antimicrobial spectrum or antibiotic spectrum refers to the range 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
- 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
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.

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. Polymixins are such drugs of clinical importance.

E.g. Polymixin B and Polymixin 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, 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.
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. (Figure) 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
growth is determined by the turbidity in each tube. Tubes containing moderate to high concentrations of the antimicrobial agent would normally expected to have no growth.

**Minimal Inhibitory Concentration (MIC) Test**

The potency of an effective antimicrobial agent is expressed in terms of **minimal inhibitory concentration** (MIC). It is the smallest amount 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.

**Etest**

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.

![Etest](image)

**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 inhibit its growth. 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.

The tube dilution method is considered accurate for determining susceptibility of a pathogen to precise quantities 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 antibiotics.

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 drugs 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.
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 agent used to disinfect inanimate objects but generally toxic to use on human tissues. An antibiotic is a metabolic product produced by one microorganism 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.

However, bacteria acquire drugs resistance using resistance mechanisms such as reduced permeability to antibiotic, efflux (pumping) antibiotic out of the cell, drugs inactivation through chemical modification, target modification and development of a resistant biochemical pathway (Figure 3.5).

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

Figure 3.5: Drugs resistance mechanisms
6. 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

7. Which of the following agents are used as a preservative in ophthalmic solution?
   a. Alcohol
   b. Quaternary ammonium salts
   c. Phenol
   d. Aldehydes

8. Which of the following chemical lack penetrating power?
   a. Phenol
   b. Iodine
   c. Ethylene oxide
   d. Beta-propiolactone

9. 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

10. 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. List out 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?
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.

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

Chapter Outline

4.1 Metabolism
4.2 Energy of Chemical Reaction

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
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 microorganisms 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 synthesis 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](image-url)
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, meaning that energy is directed inward. When a bond is broken, energy is released. A chemical reaction that release energy is an exergonic reaction, meaning that energy is directed outward.

During chemical reaction energy is either released or absorbed and the quantum of energy liberated or taken up is that 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 chemical 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**

<table>
<thead>
<tr>
<th>Name of the Nucleotide</th>
<th>Biosynthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uridine triphosphate (UTP)</td>
<td>Polysaccharide</td>
</tr>
<tr>
<td>Cytidine triphosphate (CTP)</td>
<td>Lipid</td>
</tr>
<tr>
<td>Guanidine triphosphate (GTP)</td>
<td>Protein</td>
</tr>
</tbody>
</table>

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.

\[
\text{ATP} + \text{H}_2\text{O} \rightarrow \text{ADP} + \text{pi} \quad (\Delta G^\circ = -7.3 \text{ K Cal/mol})
\]

\[
\text{ATP} + \text{H}_2\text{O} \rightarrow \text{AMP} + \text{ppi} \quad (\Delta G^\circ = -10.9 \text{ K Cal/mol})
\]

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).
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.

\[
\begin{align*}
F_2 + 2e^- &\rightarrow 2F^- \\
H_2 + 2e^- &\rightarrow 2H^+ + 2e^- \\
NAD^+ + 2H^+ + 2e^- &\rightarrow NADH + H^+
\end{align*}
\]

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 molecules of oxygen. The process of oxidative phosphorylation occurs during electron transport chain (Figure 4.3).
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 NO3, SO₄²⁻ other than Oxygen.

4.3.3 Photo Phosphorylation

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 bacteriochlorophylls 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.
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**

\[
\text{Glucose} \xrightarrow{\text{Glycolysis}} \text{Pyruvate} \xrightarrow{\text{Respiration}} \text{CO}_2 + \text{H}_2\text{O}
\]

**Anaerobic condition**

\[
\text{Glucose} \xrightarrow{\text{Glycolysis}} \text{Pyruvate} \xrightarrow{\text{Fermentation}} \text{Fermented products}
\]

\[
\text{C}_6\text{H}_12\text{O}_6 + 2 \text{NAD} + 2 \text{ADP} + 2 \text{P} \rightarrow 2 \text{CH}_3\text{COCOOH} + 2 \text{ATP} + 2 \text{NADH} + 2 \text{H}^+
\]

(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 are produced 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?
Figure 4.4: Glycolysis Pathway
52

4.5 Tricarboxylic Acid Cycle

TCA cycle was first elucidated by in 1937 “Sir Hans Adolf Krebs, a German biochemist. 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 (CO₂) 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 FADH₂ 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.

\[
\text{Pyruvate} + \text{CoA-SH} + \text{NAD}^+ \rightarrow \text{Acetyl CoA} + \text{CO}_2 + \text{NADH}
\]

Pyruvate dehydrogenase complex

Pyruvate cannot enter the Krebs cycle directly. In a preparatory step, it must loses one molecule of CO₂ 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. This multi enzyme complex is responsible for the conversion of pyruvate to acetyl-coA. Therefore (PDHC) contribute to linking the Glycolysis metabolic 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.

Pyruvate dehydrogenase complex is a complex of three enzymes that convert pyruvate into acetyl-coA by a process called pyruvate decarboxylation (Figure 4.5). Acetyl-CoA may then be used in the citric acid cycle to carryout cellular respiration, and this complex links the Glycolysis metabolic pathway to the TCA cycle.

The Krebs cycle generates a pool of chemical energy (ATP, NADH, and FADH₂) from the oxidation of Pyruvic acid and it loses one carbon atom as CO₂ 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 (oxaloacetate) 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).
4.6 Electron Transport Chain

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\text{2}, 1 ATP (substrate level phosphorylation), 3 NADH and 1 FADH\text{2}. Then NADH and FADH\text{2} can be oxidized by electron transport chain to provide more ATPs.

**Figure 4.5:** Krebs cycle
inner mitochondrial membrane that transfer electrons from electron donors NADH and FADH\textsubscript{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\textsubscript{2} and H\textsuperscript{+} to form water (Figure 4.6).

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\textsubscript{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\textsuperscript{2+}) and oxidized form (Fe\textsuperscript{3+}). Cytochromes involved in ETC include cyt (b), cyt c\textsubscript{1}, cyt c, cyt a, cyt a\textsubscript{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\textsuperscript{−} to FMN, which then picks up an additional H\textsuperscript{+} from the surrounding aqueous medium. As

![Figure 4.6: Electron Transport Chain and Chemiosmotic mechanism of ATP](image-url)
a result of the first transfer, NADH is oxidized to NAD⁺, and FMN is reduced to FMNH₂.

In the second step, FMNH₂ passes 2 H⁺ to the other side of the mitochondrial membrane and passes 2 e⁻ to coenzyme Q. As a result, FMNH₂ 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 b₁, cyt c₁, cyt c, cyt a, cyt a₃. 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 O₂ which picks up protons from the surrounding medium to form H₂O.

FADH₂ derived from the Krebs cycle is another source of electrons. Thus at the end of ETC, NADH pumps three protons (synthesizes 3ATPs) whereas FADH₂ 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 O₂ in the matrix fluid to form H₂O. Thus O₂ 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 × 3 = 30 + 2 FADH₂ = 2 × 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.

\[
\begin{align*}
\text{C}_6\text{H}_{12}\text{O}_6 + 6\text{CO}_2 + 38\text{ADP} + 38\text{P}_i & \rightarrow 6\text{CO}_2 + 6\text{H}_2\text{O} + 38\text{ATP}
\end{align*}
\]
Figure 4.7: Overview of aerobic respiration

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

<table>
<thead>
<tr>
<th>Glycolysis</th>
<th>Preparatory step</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Oxidation of glucose to Pyruvic acid.</td>
<td>2 ATP (substrate level phosphorylation)</td>
</tr>
<tr>
<td>2. Production of 2 NADH</td>
<td>6 ATP (Oxidative phosphorylation in ETC)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Preparatory step</th>
<th>Krebs cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Formation of acetyl CoA produces 2NADH</td>
<td>2 ATP (Substrate level phosphorylation)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Krebs cycle</th>
<th>Preparatory step</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Oxidation of succinyl CoA to succinic acid</td>
<td>18 ATP (Oxidative phosphorylation in ETC)</td>
</tr>
<tr>
<td>2. Production of 6 NADH</td>
<td>4 ATP (Oxidative phosphorylation in ETC)</td>
</tr>
<tr>
<td>3. Production of 2 FADH</td>
<td></td>
</tr>
</tbody>
</table>

| Total 38 ATP |  |

1 NADH = 3 ATPs and 1FADH₂ = 2 ATP
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.

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 catalase 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.

**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 borkumensis, 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.

**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.

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

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 produced by the bacteria which convert the lactose into lactic acid.

**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).

**Homolactic acid fermentation**

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

Glucose + 2ADP + 2P \[\rightarrow\] 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...
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.
4.10.1 Characteristics of Enzymes

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 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).

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) quarterly 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

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

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

\[
\text{Apoenzyme (Protein portion) + Cofactor (Non protein) \rightarrow Holoenzyme (Active enzyme)}
\]

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 or 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
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

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

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.
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 microorganisms have been found in industrial wastes, vegetable oil processing factories, dairy 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 enzyme catalyzing activity (Flowchart 4.1). This adverse affect of inhibitors on the rate of enzymatically catalyzed reactions are called inhibition.

Inhibition

- Reversible
- Irreversible
- Allosteric
- Competitive
- Uncompetitive
- Mixed
- Non-competitive

**Flowchart 4.1:** Types of Inhibition

An irreversible inhibitor inactivates an enzyme by bonding 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.
of reactions. This process is used to regulate the synthesis of amino acids (Flowchart 4.2). Example: Threonine deaminase is the first enzyme the conversion 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\textsubscript{2} function as the final electron acceptor. In anaerobic respiration, the final electron acceptor is an inorganic molecule NO\textsubscript{2}-, SO\textsubscript{4}2- other than O\textsubscript{2}.

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

- Glycolysis occurring in cytoplasm
- Krebs cycle occurring in mitochondrial 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, O\textsubscript{2} 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
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 glycolysis 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 \( \rightarrow \) Cytoplasm pyruvate
      \( \rightarrow \) Mitochondria Ethanol + CO\(_2\)
   b. Glucose \( \rightarrow \) Cytoplasm pyruvate
      \( \rightarrow \) Cytoplasm Lactic acid
   c. Glucose \( \rightarrow \) Cytoplasm pyruvate + Energy \( \rightarrow \) Mitochondria CO\(_2\) + H\(_2\)O
   d. Glucose \( \rightarrow \) Cytoplasm pyruvate
      \( \rightarrow \) 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.
   \[ 2 \text{ ADP} \rightarrow 2 \text{ ATP} \]
   
   Reason (R) : Phosphoenol pyruvic acid \( \rightarrow \) 2 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
   Apoenzyme + Cofactor = Holoenzyme
   Haloenzyme + Coenzyme = Apoenzyme
   Apoenzyme + Holoenzyme = Coenzyme
   Coenzyme + Cofactor = Holoenzyme
9. Match the correct order given below
   a. Catalase - 1. Detect Blood glucose level
   b. Glucose oxidase - 2. Break down of \( \text{H}_2\text{O}_2 \)
   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.
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.
17. These are three mechanism for the phosphorylation of ADP to ATP. Write the name of the mechanism in the following reaction given below.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>An electron liberated from chlorophyll by light, is passed down an ETC</td>
</tr>
<tr>
<td>2</td>
<td>Cytochrome c passes two electrons to cyt a</td>
</tr>
<tr>
<td>3</td>
<td>Phosphoenol pyruvic acid</td>
</tr>
<tr>
<td></td>
<td>pyruvic acid</td>
</tr>
</tbody>
</table>

18. Name the stages of aerobic respiration?
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. Apart from the pathogenic microorganisms, some microorganisms are used in the preparation and preservation of food products.
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 plants 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
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 poising capacity, (the resistance to change in potential, of the food.)
   iii. The oxygen tension of the atmosphere about the food and
   iv. The access which the atmosphere has to the food.

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.

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).

![Figure 5.1: Food spoilage](image)

**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. When flies carrying germs sit on our food, they pass on the germs to our 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.

### 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

### Flowchart 5.1: Types of Food – Poisoning

- **Food Poisoning**
  - **Food intoxication**
    - Bacterial Food Poisoning (Bacterial food intoxication)
    - Fungal Food poisoning (mycointoxications)
    - Algal Food poisoning (phycointoxications)
  - **Food infection**
    - Botulism
      - Example: Clostridium botulinum
    - Mushroom poisoning
      - Example: Amanita
    - Fish poisoning
      - Example: Gymnodymium
    - Staphylococcal – poisoning
      - Example: Staphylococcus aureus
    - Viral disease:
      - Example: Polio, Hepatitis A& E, Gastro enteritis viruses
    - Bacterial diseases:
      - Example: Shigellosis (Bacillary dysentry)
      - Escherichia Cholera
      - Brucellosis
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.

### 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.

#### 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).

<table>
<thead>
<tr>
<th>S.No</th>
<th>Disease</th>
<th>Organism</th>
<th>Incubation period and characteristics</th>
<th>Major Foods Involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Salmonellosis</td>
<td>Salmonella typhimurium, S. enteritidis</td>
<td>8–48 hr Enterotoxin and cytotoxins</td>
<td>Meat, poultry, Fish, eggs, dairy product.</td>
</tr>
<tr>
<td>4.</td>
<td><em>Escherichia coli</em> (Diarrhea and colitis)</td>
<td><em>Escherichia coli</em>, includes serotype 0157:H7</td>
<td>24–72 hrs Entero toxigenic Positive and negative strains : hemorrhagic colitis</td>
<td>Cooked ground beef, raw milk</td>
</tr>
<tr>
<td>5.</td>
<td>Shigellosis</td>
<td>Shigella sonnei, S. flexneri</td>
<td>24–72 hrs</td>
<td>Egg products, puddings</td>
</tr>
<tr>
<td>6.</td>
<td>Vibrio parahamolyticus</td>
<td>Vibrio parahaemolyticus</td>
<td>16–48 hr</td>
<td>Seafood, shellfish</td>
</tr>
</tbody>
</table>
1. Microorganism of plant 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.
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](image)

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, air is removed from food and put in airtight cans so that germs do not grow on them. 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.
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.

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.

Methods of Food Preservation

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

<table>
<thead>
<tr>
<th>S.No</th>
<th>Approach</th>
<th>Examples of process</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Removal of microorganisms</td>
<td>Avoidance of microbial contamination, physical filtration, centrifugation.</td>
</tr>
<tr>
<td>2.</td>
<td>Low temperature</td>
<td>Refrigeration, Freezing</td>
</tr>
<tr>
<td>3.</td>
<td>High temperature</td>
<td>Partial or complete heat inactivation of microorganisms (pasteurization and canning)</td>
</tr>
<tr>
<td>4.</td>
<td>Reduced water availability</td>
<td>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.</td>
</tr>
<tr>
<td>5.</td>
<td>Chemical – based preservation</td>
<td>Addition of specific inhibitory compounds (Example: organic acids, nitrates, sulfur dioxide)</td>
</tr>
<tr>
<td>6.</td>
<td>Radiation</td>
<td>Use of ionizing (gamma rays) and non-ionizing (UV) radiation</td>
</tr>
<tr>
<td>7.</td>
<td>Microbial product – based inhibition</td>
<td>The addition of substances such as bacteriocins to foods to control food-borne pathogens</td>
</tr>
</tbody>
</table>

“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.
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.
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).

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),

![Flowchart 5.2: Various products obtained from raw milk](image)

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 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
Teats have been reported to contribute up to $10^5 \text{ cfu ml}^{-1}$ 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

### Table 5.3: Complex mixture

<table>
<thead>
<tr>
<th>S.No</th>
<th>Composition</th>
<th>Approximate percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Liquid (Water)</td>
<td>87%</td>
</tr>
<tr>
<td>2.</td>
<td>Solids</td>
<td>13%</td>
</tr>
<tr>
<td>3.</td>
<td>Fat</td>
<td>4%</td>
</tr>
<tr>
<td>4.</td>
<td>Protein</td>
<td>3.3%</td>
</tr>
<tr>
<td>5.</td>
<td>Lactose (Milk Sugar)</td>
<td>5%</td>
</tr>
<tr>
<td>6.</td>
<td>Ash content (Vitamins and minerals)</td>
<td>0.7%</td>
</tr>
</tbody>
</table>

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 $10^2$–$10^3 \text{ cfu ml}^{-1}$

- The udder exterior and its immediate environment can be contaminated with organisms from the cow’s general environment. Heavily contaminated

### Table 5.4: Microbiological Standard and Grading of Milk

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

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.

![Figure 5.4: Methylene Blue dye Reduction Test](image)

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:

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.

Table 5.5: Microbiological Quality of Milk

<table>
<thead>
<tr>
<th>S.No</th>
<th>Grade</th>
<th>Methylene blue reductase test in hrs</th>
<th>Total plate count/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Very good</td>
<td>5 and above</td>
<td>Not exceeding 0.2 million</td>
</tr>
<tr>
<td>2.</td>
<td>Good</td>
<td>3–4</td>
<td>Between 0.2 to 1.0 million</td>
</tr>
<tr>
<td>3.</td>
<td>Fair</td>
<td>1–2</td>
<td>Between 1–5 million</td>
</tr>
<tr>
<td>4.</td>
<td>Very poor</td>
<td>0.5</td>
<td>over 5 million</td>
</tr>
</tbody>
</table>
They are two groups of cheese, fresh cheese and ripened cheese. The fresh cheese are made up of mild coagulated by acid or high heat example, cottage cheese, while 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.

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).

<table>
<thead>
<tr>
<th>Major categories and Examples of Fermented Milk Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Category</td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>i. Lactic Fermentations</td>
</tr>
<tr>
<td>Mesophilic</td>
</tr>
<tr>
<td>Thermophilic</td>
</tr>
<tr>
<td>Therapeutic</td>
</tr>
<tr>
<td>ii. Yeast-lactic fermentations</td>
</tr>
<tr>
<td>iii. Mold – lactic fermentations</td>
</tr>
</tbody>
</table>

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).

**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...
Types of Cheese

Cheese can be divided among different categories or types, according to their firmness. There are various systems 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 with the help of their cellular enzymes on lactose, fat and proteins that creates the ripened cheese flavour. The gas forming culture of *Propionibacterium shermanii* is essential 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

Table 5.6: Types of Cheese

<table>
<thead>
<tr>
<th>Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soft cheese</td>
<td>Soft, Smooth and creamy texture. Soft cheese is not pressed or cooked during the manufacturing process. Example: Camembert</td>
</tr>
<tr>
<td>Semi-soft cheese</td>
<td>A little more firm and compact than soft cheese, the semi-soft category contains the largest variety of cheese. Example: Havarti</td>
</tr>
<tr>
<td>Firm cheese</td>
<td>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</td>
</tr>
</tbody>
</table>
### 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”.

---

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 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 and milk appear form. The milk is then heated at 80–90ºC 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 40–43ºC 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 (10⁶–10⁷ 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). 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 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

---

**Figure 5.7: Yoghurt**

**Figure 5.8: Curd**

**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...
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 organisms in food some of the factors are intrinsic and some others are extrinsic factors. Spoilage of food can be defined as any visible or invisible change which can make food or product from food unacceptable for human consumption. Spoilage of food not only causes health hazard to the consumer but also cause large economic losses. 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 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 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

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.
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.
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, CO₂ (gas bubbles) Figure 6.1 are formed during boiling of liquid.
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 of 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 produce during Logarithmic phase. Secondary metabolites do not play a role in development, growth and reproduction of microorganisms. They are produce 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
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 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

<table>
<thead>
<tr>
<th>Product</th>
<th>Microorganisms</th>
<th>Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin B12</td>
<td><em>Streptomycyes</em></td>
<td>Vitamin supplements</td>
</tr>
<tr>
<td>Lactic acid</td>
<td><em>Lactobacillus delbrueckii</em></td>
<td>Chemical reagents</td>
</tr>
<tr>
<td>Citric acid</td>
<td><em>Aspergillus niger</em></td>
<td>Food preservative</td>
</tr>
<tr>
<td>Acetic acid</td>
<td><em>Acetobacter</em></td>
<td>Vinegar, solvent</td>
</tr>
<tr>
<td>Ethanol</td>
<td><em>Saccharomyces</em></td>
<td>Chemical reagents drinks</td>
</tr>
<tr>
<td>Penicillin</td>
<td><em>Pencillium chrysogenum</em></td>
<td>Antibiotic</td>
</tr>
</tbody>
</table>
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

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.

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.
5. The strains should be able to protect themselves from contamination.

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.

**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. 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 are used in primary screening. For screening of antibiotic producing organisms crowded plate technique is described here,

**Crowded plate technique**

1. It is used for the screening of antibiotic producing organisms.
2. Soil is serially diluted
3. The serially diluted sample is spread on the nutrient agar plates
4. The plates are incubated and the agar plate having 300 to 400 colonies are observed for antibiotic producing activity
5. The ability of a colony to exhibit antibiotic activity is indicated by the presence of a zone of growth inhibition surrounding the colony
6. The technique is improved by using test organism
7. The antibiotic produced by the organisms in the soil may inhibit the growth of test organism
8. The formation of inhibitory zones around certain colonies indicates their antibiotic sensitivity
9. 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

---

**Figure 6.3:** Crowded plate technique
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.

<table>
<thead>
<tr>
<th>HOTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Why microorganisms are exploited more than plant and animal cells for production of commercial products?</td>
</tr>
</tbody>
</table>

1. As primary screening allows the deflection 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 that formed is very less. So, through secondary screening, further sorting out is performed. In this method, only microorganisms with real commercial value are selecting and those it lacks 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 micro organism 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 of the product 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 of even destroying 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's 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 microorganisms 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 Streptomyces growth. The plates are further incubated to allow the growth of the test organism. The growth of the test organism has been inhibited by antibiotic in the vicinity of the Streptomyces is then measured in millimeters. These Streptomyces that have produced antibiotics with observable microbial inhibition spectrum are retained for further testing (Figure 6.4).

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.
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 removed by either mechanical or enzymatic means. Protoplast has nucleus other protoplasmic contents which are surrounded by cytoplasmic membrane.

1. Chromosome
2. Plasma membrane
3. Cell wall
4. Bacterial cell walls are enzymatically digested, producing protoplasts.
5. In solution, protoplasts are treated with polyethylene glycol.
6. Protoplasts fuse
7. Segments of the two chromosomes recombine
8. Recombinant cell grows new cell wall
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 microorganisms 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 dried under vacuum, filled in ampoules (glass vessels) then frozen. 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.
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. (Figure 6.6) 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
Table 6.2: Components and their uses:

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

Figure 6.6: Components of Fermentor
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 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
Table 6.3: Some common substrates used in the industrial fermentation process:

<table>
<thead>
<tr>
<th>Carbon source</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Molasses</td>
<td>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</td>
</tr>
<tr>
<td>Malt extract</td>
<td>It is an aqueous extract of malted barley.</td>
</tr>
<tr>
<td>starch, dextrin cellulose</td>
<td>They can be metabolized by microorganisms. They are used for the industrial production of alcohol.</td>
</tr>
<tr>
<td>Whey</td>
<td>It is a byproduct of dairy industry used in the production of alcohol, SCP, vitamin B12, lactate acid, gibberllic acid</td>
</tr>
<tr>
<td>Methanol ethanol</td>
<td>Methanol is the cheapest substrate. It is utilized only by few bacteria. Methanol is used for SCP. Ethanol is used for acetic acid production</td>
</tr>
<tr>
<td>Hydro molasses</td>
<td>It is a byproduct in glucose production from corn</td>
</tr>
<tr>
<td>Sulphate waste liquor</td>
<td>It is a spent sulfite liquor from 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</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nitrogen sources</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Inorganic: Ammonium salts and ammonia</td>
<td>It is a cheap source of nitrogen</td>
</tr>
<tr>
<td>Urea (Organic)</td>
<td>It is a good and cheap source of organic source</td>
</tr>
<tr>
<td>Corn steep liquor (Organic)</td>
<td>It is formed during starch production from corn. It is rich in several amino acids</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>It is rich in amino acids, peptides vitamins</td>
</tr>
<tr>
<td>Soy meal</td>
<td>It is a left over residue on preparing soybean oil from soybean seeds. It is used in antibiotic production</td>
</tr>
<tr>
<td>Peptones</td>
<td>The proteins hydrolysates are called as peptones. The source of peptones includes meat, cotton seeds and sunflower seeds</td>
</tr>
</tbody>
</table>

pure form. Examples: Phenyl acetic acid or phenylacetamide added as side chain precursors in penicillin production.

6.5.3 Large Scale Production

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 6.7).

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 microorganisms 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 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 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 6.8)

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) whereas 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 sterilized 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**

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

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

6.6 Penicillin Production

Penicillin is a broad spectrum antibiotic. Penicillin is first obtained from the mould, *Penicillium notatum* (Figure 6.9).

*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.

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 which had bacterial inflations due to their wounds.

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.
Flowchart 6.2: Downstream processing methods
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) Amphiicillin. Nowadays, semi synthetic pencillins makeup the bulk of the penicillin market.

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)

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

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.
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 NH₃.

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
- 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)

**Figure 6.10:** Production of Penicillin
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 and dried and used for commercial purpose.

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 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

<table>
<thead>
<tr>
<th>Wine Type</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red wine</td>
<td>It has red pigments</td>
</tr>
<tr>
<td>White wine</td>
<td>It does not contain red pigments</td>
</tr>
<tr>
<td>Rose wine</td>
<td>It has less red pigments</td>
</tr>
<tr>
<td>Dry wine</td>
<td>It has less red pigments</td>
</tr>
<tr>
<td>Sweet wine</td>
<td>It has more alcohol content</td>
</tr>
<tr>
<td>Fortified wine</td>
<td>It is fortified with other alcoholic beverage</td>
</tr>
<tr>
<td>Sparkling wine</td>
<td>It has considerable amount of CO₂</td>
</tr>
<tr>
<td>Still wine</td>
<td>It does not contain carbon dioxide</td>
</tr>
<tr>
<td>Distilled wine</td>
<td>Brandy (alcohol content 21%)</td>
</tr>
<tr>
<td>Table wine</td>
<td>It has low alcohol and sugar content</td>
</tr>
</tbody>
</table>
alcohol tolerant and produce undesirable compounds, affecting the quality of the wine.

The cultivated wine yeast, *Saccharomyces ellipsoideus*, is used for commercial production. Figure 6.11 shows steps involved in wine production

**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 carlsbergensis*). 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).

**Steps involved in Wine production**

Grapes are stemmed, cleaned and crushed

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

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”.

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 6.12

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
### Some Commercial Products of Yeast

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Product</th>
<th>Micro Organism</th>
<th>Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Baker’s yeast, beer, wine, ale, bread</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>Baking industry brewing industry</td>
</tr>
<tr>
<td>2.</td>
<td>Soy sauce</td>
<td><em>Saccharomyces rouxii</em></td>
<td>Food Condiment</td>
</tr>
<tr>
<td>3.</td>
<td>Sour French bread</td>
<td><em>Candida milleri</em></td>
<td>Baking</td>
</tr>
<tr>
<td>4.</td>
<td>Commercial alcohol (ethanol)</td>
<td><em>Saccharomyces cerevisiae</em>&lt;br&gt;<em>Kluyveromyces fragilis</em></td>
<td>Fuel, Solvent</td>
</tr>
<tr>
<td>5.</td>
<td>Riboflavin</td>
<td><em>Eremotherium ashbyi</em></td>
<td>Vitamin supplement</td>
</tr>
<tr>
<td>6.</td>
<td>Microbial protein</td>
<td><em>Candida utilis</em>&lt;br&gt;<em>Saccharomyces lipolytica</em></td>
<td>Microbial protein from petroleum products. Animal food supplement (single cell protein) from paper-pulp waste</td>
</tr>
</tbody>
</table>

---

**Figure 6.12:** Large scale production of SCP

Pruteen was the 1st commercial SCP used as animal feed additive with 72% of protein. Pruteen was produced from bacteria named *Methylophilus methylotrophus* 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 CFTRI, SCP is produced from algae cultured on sewage.

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.
Steps involved in SCP production

Provision of carbon source with added nitrogen, CO₂, ammonia, trace minerals for growth

Prevention of contamination by using sterilized medium and fermentation equipments

Selected microorganism is inoculated in a pure form

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.
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.

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

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

During the cultivation of SCP, care must be taken to prevent and control the contamination by other microorganisms, which produce mycoxins or cyanotoxins. This is controlled by using the fungus *Scytalidium acidophilum* which grows at a low PH. It allows the hydrolysis 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 *Hansenula polymorpha*
and *Candida lipolytica* are generally 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 between 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

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 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 an 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 in 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.

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
1. Carrier–binding
2. Cross–linking
3. Entrapping
4. 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. The diagrammatic representation of entrapping methodology is shown in Figure 6.13.

Advantages of immobilization

1. Immobilized growing cells serve as self proliferating and self regenerating bio catalyst

Figure 6.13: Immobilization (Entrapping)
2. They are stable
3. They are used either repeatedly in a series of batch wise reactions or continuously in flow systems.

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.

Down stream processing, includes the separation and purification of products. The industrially important products like penicillin, wine and citric acid, single cell protein, are produced in large scale by using microorganisms.

Immobilization is need for the physical or chemical fixation of plant, animal cells, organelles, enzymes or other proteins into a solid matrix or retained by a membrane. Immobilized cells can be used for repeated doses.

Evaluation

Multiple choice questions

1. Theterm fermentation originates from a Latin verb _________
   a. Wear
   b. Fervere
   c. Severe
   d. Cheer

2. _______________ metabolites are produced in small quantities during industrial production.
   a. Secondary metabolites
   b. Primary metabolites
   c. Tertiary metabolites
   d. Neutral metabolites

3. _______________ enrichment technique for isolation of secondary metabolite producing Actinomycetes.
   a. Soil-Sodium Carbonate
   b. Soil-Calcium Carbonate
   c. Soil-Strontium Carbonate
   d. Soil-Potassium Carbonate

4. 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
5. Match the following:
   A. Lactic acid 1. Penicillium chrysogenum
   B. Citric acid 2. Lactobacillus delbruekii
   C. Penicillin 3. Saccharomyces
   D. Ethanol 4. Aspergillus niger
   a. 4 2 1 3
   b. 2 4 1 3
   c. 1 3 4 2
   d. 2 4 3 1

6. _____________ is an example for primary screening.
   a. Photography
   b. Cinematography
   c. Auxanography
   d. Telegraphy
7. 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
8. 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. 4 1 2 3
   b. 3 2 1 4
   c. 2 4 1 3
   d. 2 3 4 1
9. _______ are produced at the end of the growth phase or stationary phase.
   a. Tertiary
   b. Secondary
   c. Primary
   d. All the above
10. Assertion (A): Red wine is prepared from red grapes.
    Reason (R): Anthocyanin is responsible for pigmentation.
    a. The statement A is not supported by Statement R
    b. Statement A alone is correct
    c. Statement R alone is correct
    d. The statement A is supported by Statement R

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.
9. Define secondary screening with example.
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 microorganisms.
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.
31. Define Immobilization.
32. What are the materials used for Immobilization?

**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
Medical Bacteriology

Chapter 7

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

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, nuclease 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.
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
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

<table>
<thead>
<tr>
<th>Infections</th>
<th>Clinical Specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suppurative lesions</td>
<td>Pus</td>
</tr>
<tr>
<td>Respiratory infections</td>
<td>Sputum</td>
</tr>
<tr>
<td>Septicemia</td>
<td>Blood</td>
</tr>
<tr>
<td>Meningitis</td>
<td>CSF</td>
</tr>
<tr>
<td>Food poisoning</td>
<td>Faeces, food or vomitus</td>
</tr>
</tbody>
</table>

Specimens should be transported immediately to the laboratory and processed.

**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
They are non-motile, non-sporing. Some strains are capsulated (Figure 7.4).

**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.

### 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)

#### 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*.

---

**DO YOU KNOW?**

<table>
<thead>
<tr>
<th>Year</th>
<th>Scientist</th>
<th>Contributions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1874</td>
<td>The odor Billroth</td>
<td><em>Streptococci</em> were discovered</td>
</tr>
<tr>
<td>1884</td>
<td>Friedrich Julius Rosenbach</td>
<td>The cocci were isolated from human lesions and gave the name <em>Streptococcus pyogenes</em></td>
</tr>
</tbody>
</table>
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.

### 7.4.3 Antigenic Structure

**Capsule:** It inhibits phagocytosis

**Cell wall:** The outer layer of cell wall consists of protein and lipoteichoic acid

---

**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:

- **Alpha haemolytic**
- **Beta haemolytic**
- **Gamma haemolytic**
- **Incomplete haemolytic**
- **Complete haemolytic**
- **No haemolytic**

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.

---

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

---

**Infobits**

**Capsule:** It inhibits phagocytosis

**Cell wall:** The outer layer of cell wall consists of protein and lipoteichoic acid

---

TN_GOVT_XII_Micro_Biology_CH07.indd 120
27-02-2019 14:48:42
### Table 7.3: Enzymes of *Streptococcus pyogenes* and its virulence nature

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Virulence nature</th>
</tr>
</thead>
<tbody>
<tr>
<td>streptokinase (fibrinolysin)</td>
<td>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.</td>
</tr>
<tr>
<td>Deoxyribonucleases</td>
<td>It liquefy the highly viscous DNA that accumulate in thick pus and responsible for thin serous character of streptococcal exudates.</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>It breaks down hyaluronic acid of the tissues and favors spread of streptococcal lesion along intercellular spaces.</td>
</tr>
<tr>
<td>Other enzymes</td>
<td>NADase, lipase, amylase, esterase, phosphates and other enzymes</td>
</tr>
</tbody>
</table>

**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:

- **Direct Contact**
- **Fomites**
- **Airborne droplets**
- **Carriers**

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

#### Flowchart 7.1: Classification of *Streptococcal* diseases

- **Suppurative diseases**
  - Respiratory tract infection
  - Skin Infections
  - Streptococcal toxic shock syndrome
  - Deep infections
  - Genital infections
- **Non-suppurative complication**
  - Acute Rheumatic fever
  - Acute glomerulonephritis

**Suppurative Infections**

1. **Respiratory tract infection**
   - **Streptococcal sore throat**: Sore throat (acute tonsillitis and pharyngitis) is the
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.

DO YOU KNOW? 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
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.

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 O titration. ASO titles 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 reinfection 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 sufferers do not need to give up chocolate, they need to reduce their lipid consumption.
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. meningitidis* 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% CO₂ 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).

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.
and young adults. Meningococci are strict human pathogens. Human nasopharynx is the reservoir of *N. meningitidis*. The pathogenesis is discussed 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% CO₂ for 18–24 hours. After incubation period, meningococcus is identified by gram staining, colony morphology and biochemical reactions. *N. meningitides* is catalase and oxidase positive (Figure 7.8).

![Oxidase test](image)

**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 penetrates intoneary 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 multlsystem 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*
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 7.9: (a) Gram staining of *Corynebacterium diphtheriae* (b) Albert’s staining showing metachromatic granules](image)

![Figure 7.10: Gram staining of *Corynebacterium tetani*](image)
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

<table>
<thead>
<tr>
<th>Media</th>
<th>Colony Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loeffler’s Serum slope</td>
<td>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</td>
</tr>
<tr>
<td>Tellurite Blood Agar</td>
<td>Grey or black colonies. Based on colony morphology on tellurite medium, three main biotypes – Gravis, Intermedius and Mitis.</td>
</tr>
</tbody>
</table>

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 2–10 years.

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
Culture: The swabs 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).

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 preparation and 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.
Table 7.6: Immunization for diphtheria

<table>
<thead>
<tr>
<th>Immunization</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Active</td>
<td>Passive</td>
</tr>
<tr>
<td>DPT (Triplevaccine)</td>
<td>500–1000 units of Antidiphtheritic serum (ADS) is administered subcutaneously.</td>
</tr>
<tr>
<td>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.</td>
<td></td>
</tr>
</tbody>
</table>

Table 7.7: Clostridium sp. causing pathogenic diseases.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Clostridium tetani</em></td>
<td>Tetanus</td>
</tr>
<tr>
<td><em>Clostridium perfringens</em></td>
<td>Gas gangrene</td>
</tr>
<tr>
<td><em>Clostridium botulinum</em></td>
<td>Food poisoning</td>
</tr>
</tbody>
</table>

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*

<table>
<thead>
<tr>
<th>Media</th>
<th>Colony Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood agar</td>
<td>They produce α – hemolysis which subsequently develop into β – hemolysis (due to tetanolysis) it produces swarming growth.</td>
</tr>
<tr>
<td>Cooked meat broth (CMB)</td>
<td>Growth occurs as turbidity with gas formation. The meat is not digested but becomes black on prolonged incubation</td>
</tr>
</tbody>
</table>

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:** Tetanospasmin is a neurotoxin, which blocks the release of...
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.
Table 7.9: Immunization for tetanus

<table>
<thead>
<tr>
<th>Active immunization</th>
<th>Passive immunization</th>
<th>Combined prophylaxis</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Tetanus toxoid (TT)</td>
<td>Antitetanus serum (ATS)</td>
<td>Tetanus toxoid in one arm and ATS or HTIG in another arm.</td>
</tr>
<tr>
<td>b. DPT</td>
<td>Human Antitetanus immunoglobulins (HIIG)</td>
<td></td>
</tr>
</tbody>
</table>

Tetanus develops by the contamination of wound with *Clostridium 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 affects most of the voluntary 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.

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.
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*

<table>
<thead>
<tr>
<th>Media</th>
<th>Colony Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient Agar</td>
<td>Colonies are circular, convex smooth and translucent</td>
</tr>
<tr>
<td>MacConkey Agar</td>
<td>Colourless colonies</td>
</tr>
<tr>
<td>SS – Agar</td>
<td>Colourless colonies</td>
</tr>
</tbody>
</table>

### 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
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).

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.
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*

<table>
<thead>
<tr>
<th>Media</th>
<th>Colony Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient Agar</td>
<td>Colonies are large, circular, smooth, translucent</td>
</tr>
<tr>
<td>MacConkey Agar</td>
<td>Colourless colonies (non – lactose fermenters)</td>
</tr>
<tr>
<td>SS – Agar</td>
<td>Colourless colonies with black centered.</td>
</tr>
</tbody>
</table>

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.
7.9.4 Clinical Manifestations

- The illness is usually gradual, with headache, malaise (feeling of discomfort), anorexia (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.

<table>
<thead>
<tr>
<th>Duration of disease</th>
<th>Specimen examination</th>
<th>% Positivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st Week</td>
<td>Blood culture</td>
<td>90</td>
</tr>
<tr>
<td>2nd Week</td>
<td>Blood culture</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>Faeces culture</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Widal test</td>
<td>Low titer</td>
</tr>
<tr>
<td>3rd Week</td>
<td>Widal test</td>
<td>80–100</td>
</tr>
<tr>
<td></td>
<td>Blood culture</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Faeces culture</td>
<td>80</td>
</tr>
</tbody>
</table>

Flowchart 7.6: Pathogenesis of *Salmonella typhi*

**Route of entry – faecal oral route (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 neutrophils 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 discharged 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.

**Figure 7.15:** Colony morphology of *Salmonella typhi* on SS agar
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 isolated from 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.

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Doses</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAB – Vaccine</td>
<td>2 doses of 0.5 ml at an interval of 4–6 weeks</td>
</tr>
<tr>
<td>Typhoral</td>
<td>3 doses on alternate days. It gives 65–96% protection for 3–5 years and is safe</td>
</tr>
<tr>
<td>typhim – Vi</td>
<td>A single dose of 25µg</td>
</tr>
</tbody>
</table>

**Table 7.14:** Isolation method of typhoid bacilli from various clinical specimens.

<table>
<thead>
<tr>
<th>Specimen culture</th>
<th>Isolation methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood culture</td>
<td>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.</td>
</tr>
<tr>
<td>Clot Culture (An alternative method to blood culture)</td>
<td>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.</td>
</tr>
<tr>
<td>Faeces culture</td>
<td>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.</td>
</tr>
<tr>
<td>Urine culture</td>
<td>Urine samples are centrifuged, and the deposit is inoculated into enrichment media and then on selective media.</td>
</tr>
</tbody>
</table>
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.

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 Vibrios 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

<table>
<thead>
<tr>
<th>Media</th>
<th>Colony morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient agar</td>
<td>The colonies are moist, translucent round disks (1–2mm in diameter) with a bluish tinge in transmitted light.</td>
</tr>
<tr>
<td>MacConkey agar</td>
<td>The colonies are colorless at first but become reddish on prolonged incubation due to late fermentation of lactose.</td>
</tr>
<tr>
<td>Thiosulphate citrate bile sucrose agar (pH 8.6)</td>
<td>It is used as a selective medium for isolation of <em>Vibrios</em>. It produces large yellow convex colonies due to sucrose fermentation.</td>
</tr>
</tbody>
</table>
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.

**Do YOU KNOW?**

Natural infection of *Vibrio cholerae* occurs only in human beings.
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 – enterotoxin mode 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 are odourless and contains flecks of mucus, hence it is known as Rice watery stool

**Flowchart 7.7: Pathogenesis of Vibrio cholerae**

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 disinfecte
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 Rehydration therapy:** 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, lipid-rich waxy cell walls. This high lipid content (Mycolic acids) imparts the characteristic of acid fastness or resistance to decolorization by 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*
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.

- Tubercl 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 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 macrophages 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.
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 – Neelson staining.

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.

7.11.5 **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 – Neelsen technique. It is examined under oil immersion objective lens. The acid fast bacilli appear as bright red bacilli against a blue background.

7.11.6 **Treatment**

The antitubercular drugs include two types of agents which are;
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 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.

### 7.12.1 Culture

- Pathogenic Treponema cannot be grown in artificial culture media.
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.

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.
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 tabes dorsalis 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 Reagin (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 Reagin test
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 interrogans 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.

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.
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.
- Aseptic meningitis is common in canicola infection.

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 thereafter up to 6 weeks. Centrifuged deposit of urine may be observed by Dark filed microscopy.

**Culture:** Blood (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. Cultures are 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
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.

**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.
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 aureus
   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. Clostridium perfringens

5. Drumstick appearance of spores is a characteristic feature of
   a. Clostridium difficile
   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
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/
10. Define chancre.
11. Write the morphology of Clostridium tetani
12. Write short notes on Canicola fever.
13. List out the antituberculosis drugs.
14. Write the colony morphology of salmonella typhi shigella dysenteriae on ss-agar.
15. Discuss the pathogenesis of typhoid fever.
16. Describe the pathogenesis of pulmonary tuberculosis.
17. Write briefly about Lab/diagnosis of tuberculosis.
18. Explain the pathogenesis of V. Cholerae
19. Write the mode of action of tetanospasmin
20. Describe the pathogenesis of lock-jaw disease.
21. What is staphylococcal toxic shock syndrome (STSS).
22. Describe the mode of entry of Meningococci from nusopharynx to brain.
23. Why antibiotics are avoided in dysentery except in severe cases.
24. Define zoonotic bacterial disease and give two examples.
25. Describe the pathogenesis of primary syphilis.
26. Enlist the recent serological methods used to diagnose tuberculosis.
27. Write the colony morphology of Lowenstein – Jensen.
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 dealing 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?
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
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 where 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 where asexual reproduction 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*.

**Paraténic 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.

### 8.1.4 Classification of Medically 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
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 on 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
Flowchart 8.2: Classification of medically important parasites

Protozoa (Protozoology)
Kingdom – Protista (Unicellular)

Amoebae
(Typically amoeboid and use pseudopodia or protoplasmic flow to move)
Entamoeba histolytica

Flagellates
(have one or more whiplike flagella)
Intestinal and genitourinary normal flagellates
• Giardia
• Trichomonas
• Blood and tissue flagellates
• Leishmania
• Trypanosoma

Sporozoans
(undergo a complex life cycle with alternating sexual and asexual reproductive phases)
Plasmodium
Toxoplasma

Ciliates
(Complex protozoa bearing cilia)
Balantidium coli

Nematodes (round worms)
They are elongated and tapered at both ends, round in cross section and unsegmented.
• Wuchereria bancrofti
• Ascaris sp

Platyhelminths (flatworms)

Cestodes (tapeworms)
have a ribbon like chain of segments (proglottids)
Taenia solium
T. saginata

Trematodes (Flukes)
Typically flattened and leaf shaped with 2 muscular sucker
They lack cuticle.
They are hermaphrodites
Fasciola (Liver fluke)
Schistosoma (blood fluke)
Parasites having direct life cycle

**Protozoa**
- *Giardia lamblia*
- *Trichomonas vaginalis*
- *Balantidium coli*

**Helminths**
- *Ascaris lumbricoides*
- *Trichuris trichiura*
- *Ancylostoma duodenale*

Parasites having indirect life cycle

<table>
<thead>
<tr>
<th>S.No</th>
<th>Protozoa</th>
<th>Definite host</th>
<th>Intermediate host</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Plasmodium</em> spp.</td>
<td>Female <em>Anopheles</em> mosquito</td>
<td>Man</td>
</tr>
<tr>
<td>2.</td>
<td><em>Toxoplasma</em> gondii</td>
<td>Cat</td>
<td>Man</td>
</tr>
<tr>
<td>3.</td>
<td><em>Cestodes</em> Taenia solium</td>
<td>Man</td>
<td>Pig</td>
</tr>
<tr>
<td>4.</td>
<td><em>Trematodes</em> Fasciola hepatica</td>
<td>Man</td>
<td>Snail</td>
</tr>
<tr>
<td>5.</td>
<td><em>Nematodes</em> Wuchereria bancrofti</td>
<td>Man</td>
<td>Mosquito</td>
</tr>
</tbody>
</table>

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.

**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.
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 trophozoite.

The mature quadrinucleate cyst, passed in the stool, do not undergoes 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.
The cyst that are swallowed along with food and water enters into the alimentary canal. The cyst wall is resistant to action of gastric juice. The cyst 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.

Figure 8.1: Trophozoite, precyst and cyst of *Entamoeba histolytica*

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*
• 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.
Infection of *E. histolytica*

**Intestinal amoebiasis**
Infection is limited entirely to the large intestine, the initial site of location 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*
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.

**Table 8.1**: Difference between the stools of amoebic and bacillary dysentery

<table>
<thead>
<tr>
<th></th>
<th>Amoebic dysentery</th>
<th>Bacillary Dysentery</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macroscopic observation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>6–8</td>
<td>Over 10 motions a day</td>
</tr>
<tr>
<td>Amount</td>
<td>Relatively large</td>
<td>Small</td>
</tr>
<tr>
<td>Odour</td>
<td>Offensive</td>
<td>Odorless</td>
</tr>
<tr>
<td>Colour</td>
<td>Dark red</td>
<td>Bright red</td>
</tr>
<tr>
<td>Nature</td>
<td>Blood and mucus mixed with faeces</td>
<td>Blood and mucus no faeces</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Microscopic observation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RBC</td>
<td>In clumps, reddish – yellow in colour</td>
<td>RBC in rouleaux, bright red in colour</td>
</tr>
<tr>
<td>Pus cells</td>
<td>Scanty</td>
<td>Numerous</td>
</tr>
<tr>
<td>Parasite</td>
<td>Trophozoites of <em>E. histolytica</em></td>
<td>Nil</td>
</tr>
<tr>
<td>Charcot – Leyden crystals</td>
<td>Present</td>
<td>Nil</td>
</tr>
</tbody>
</table>

**Eurointestinal 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.

2. **Pulmonary Amoebiasis**: It is very rare, but this may occur when direct hemogogenous spread from the colon. The patient presents with severe chest pain.

**Figure 8.4**: Amoebic liver abscess
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 cerebellum 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.

### 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 trophozoites 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.
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*)

**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.

8.3.1 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)
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.
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
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
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.

<table>
<thead>
<tr>
<th>Host</th>
<th>Forms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human and other mammals</td>
<td>Amastigote</td>
</tr>
<tr>
<td>(Example: Dogs)</td>
<td></td>
</tr>
<tr>
<td>Sandfly of Genus Phlebotomus</td>
<td>Promastigote</td>
</tr>
</tbody>
</table>

**Promastigote:** This form is found in the mid-gut of sandfly 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-azar 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 staphylococcal or streptococcal infection, mycobacterial ulcer, leprosy, fungal infection, cancer, sarcoidosis and tropical ulcer.

**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 deposits promastigotes on the 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.

**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.
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)
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 20 years, creating a persistent human reservoir of infection.

**Laboratory diagnosis**

**Specimens:** Aspiration from spleen, bone marrow, lymph node, liver biopsy and peripheral blood.

**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.
Flowchart 8.5: Laboratory diagnosis of kala-azar

Direct evidence
- Demonstration of LD bodies
- Culture (NNN)
- Animal inoculation in hamster or mice

Indirect evidence
- Serodiagnosis
  - DNA probe
  - PCR
- Molecular diagnosis
  - Non-specific serum test
  - Aldehyde test
  - Chopra’s antimony test
- Skin test
  - Leishmanin skin test
- Blood picture
  - Anemia
  - Progressive leucopenia

Detection of antigen
- ELISA

Detection of antibody
- CFT

Lab picture
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 malaria parasites.

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 malaria 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.

Three basic types of malaria

1. Benign tertian (P. vivax and P. ovale) 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 cardiac 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.
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).

**Intermediate host:** 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).

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).
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 schizogony

- 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 exoerythrocytic 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. Erythrocytic stage

- 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 a 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 reticuloendothelial 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.
• A mature schizont contains 8–32 merozoites and haemoglobin. 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.
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 parasitized 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
when the level of Sodium in the blood is too low) occur in both uncomplicated and severe malaria.

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 haemoglobin aemia (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 complications 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 infected P. falciparum infection within 1 to 3 days.

**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.
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.

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.

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...
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.

**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.

---

**Table 8.2: Comparison of course of infection – *P. falciparum* and *P. vivax* in man**

<table>
<thead>
<tr>
<th>Stage</th>
<th><em>P. falciparum</em></th>
<th><em>P. vivax</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-erythrocytic schizonts</td>
<td>Stage lasts for 6 days. Each Schizont produces about 40,000 merozoites approximately</td>
<td>Lasts for 8 days. Each Schizont produces about 12,000 approximately</td>
</tr>
<tr>
<td>Erythrocytic schizonts</td>
<td>Each cycle lasts for 36–48 hours. First temperature peak occurs by 12th day of infection. Primary attack lasts for 10–14 days.</td>
<td>Each cycle lasts for 48 hours. First fever peak occur by 16th day of infection. Primary attack lasts for 3–4 weeks.</td>
</tr>
<tr>
<td>Gametogony</td>
<td>Gametocytes in peripheral blood may be seen on 21st day of infection.</td>
<td>Gametocytes in peripheral blood may be seen on 16th day of infection.</td>
</tr>
<tr>
<td>Exo-erythrocytic schizonts</td>
<td>Absent. Relapses do not occur</td>
<td>Present. Can continue for up to 3 years. Relapses often occur.</td>
</tr>
</tbody>
</table>

---

**Figure 8.14: Blood smear**

A. Thin smear  
B. Thick smear
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 defection of serum antibodies.

Rapid Antigen defection 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
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.

![Female Male](image)

**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.
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](image-url)
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 $80\mu m \times 105\mu m$ 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 mouls 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

![Ascaris life cycle](image)

*Figure 8.17: Lifecycle of Ascaris lumbricoides*
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,
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.

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 live on its host for its nourishment and survival. The relationship between host and the parasite can be symbiotic,
commensal 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 carrying 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.
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 syndrome.
   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 disease
   b. Gardiasis
   c. Malaria
   d. Ascariasis

10. The common name for A. Lambricoides 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?
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 paratonic 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.

<table>
<thead>
<tr>
<th>Human Nematode Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parasite</td>
</tr>
<tr>
<td>Ascaris lumbricoides</td>
</tr>
</tbody>
</table>

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.
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 to prevent and treat the fungal infection.
- Study the taxonomy, structure and classification of medically important fungi.
- Study about the mycoses and its pathogenesis, clinical feature, and its treatment and prophylaxis.
- Study about the collection, processing of the sample and its molecular diagnosis.

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 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.

Based on their wide spectrum of adaptability, fungi causing human mycoses can be categorized into:

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.

### 9.1.1 Mycoses

Diseases caused by the medically important fungi are called Mycoses. Mycoses are classified according to the specific site of involvement.

a. **Superficial Mycoses**: The infection is limited to the outermost 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. **Subcutaneous 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 airborne 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.

### 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 internal membrane bound organelles. The cell wall is composed of polysaccharides and chitin. A fungus
varies in size and shape. They are broadly divided into two main groups.

a. **Yeast**: 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 are reproduced 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 **Yeast**: 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*
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
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, homo polymer of N-acetyl glucosamine. Chitin synthase is responsible for the bio synthesis of chitin.
c. Plasma lemma: Cytoplasmic membrane or plasma lemma encloses complex cytosol. It is composed of glycoprotein, lipids and ergosterol.

d. Cytosol: Cytosol comprises of mitochondria, microtubules ribosome’s, 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 (micro conidia) and macrospores (macro conidia). Spores that are present inside sporangium are

**Figure 9.2:** Microscopic view of yeast Moulds

**Figure 9.3:** Dimorphic 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
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.

### 9.2.1 Dermatophytoses

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 skin and hair]

iii. **Epidermophyton** [cause infection skin and nail]

The fungal species affecting humans are known as **anthropophilic**. Those inhabiting 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?

### 9.2.2 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.

### 9.2.3 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:
1. **Tinea Capitis:** This is an infection of the **shaft of scalp hairs.** It can be inflammatory (e.g., 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 margined 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 the **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
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 dermatophytes

---

**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.

---

**9.2.4 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). 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, they are identified.

**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.
Table 9.1: Microscopic and macroscopic characteristics of Dermatophytes.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Dermatophytes</th>
<th>Macro conidia</th>
<th>Micro conidia</th>
<th>Macroscopic Morphology – SDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td><em>Trichophyton</em></td>
<td>Rare, thin-walled, smooth</td>
<td>Abundant</td>
<td><img src="image1.png" alt="Image" /></td>
</tr>
<tr>
<td>2.</td>
<td><em>Microsporum</em></td>
<td>Numerous, thick-walled, rough</td>
<td>Rare</td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>3.</td>
<td><em>Epidermophyton</em></td>
<td>Numerous, smooth-walled</td>
<td>Absent</td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure 9.6: LPCB wet mount of major Dermatophytes
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.

9.3 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.

### 9.3.1 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 caused by fungi and actinomycetoma caused by higher bacteria of class actinomycets.

#### 9.3.2 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](image-url)
9.3.3 Classification of Mycetoma

The Mycetoma is classified on the basis of the causative agent aerobic actinomycetes causes actinomycetoma whereas hyaline and phaeoid fungi cause eumycetoma.

9.3.4 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.

9.3.5 Laboratory Diagnosis

i. Samples
The clinical sample in Mycetoma is usually grains, pus exudates or biopsy were collected.

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 -Jonson 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.
9.4 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 caused **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.

9.4.1 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**.

9.4.2 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.

9.4.3 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.

9.4.4 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
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.

9.5 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.

9.5.1 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 Candidias 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 Candida colonizes on the oral
201 cavity (oral thrush) infection on 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* colonize on the cutaneous layer causes cutaneous Candidiasis leads to maculopapules vesicles with erythematous rash. This is common among infants and known as *Diaper rash*.

ii. Intertrigo – This is an inflammatory 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 *Candidiasis*, *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.

The 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
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.

**Chlamydosposes 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 chlamydosposes 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.

**9.5.2 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.
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.
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 Dermatophytes, 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?
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.
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?
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.
After studying this chapter the students will be able to,

- Study the importance of viruses causing disease in humans and from Animals. Viral infection are very common so its our main concern to protect us from the viral infection.
- Study the classification, structure and cultivation of Viruses.
- Study about the virus its pathogenesis, clinical feature and its treatment and prophylaxis.
- Study about the collection, processing of the sample and its molecular diagnosis.

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.
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 (cubical) and helical. Virions may be envelo ped 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
as RNA dependent DNA polymerase or transcriptase which can transcribe RNA into DNA.

**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.

**DO YOU KNOW?**

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 multiplication cycle can be divided into six steps and they are as follows, 1. Adsorption or attachment, 2. Penetration, 3. Uncoating, 4. Biosynthesis, 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.

Biosynthesis consists of the following steps:

i. Transcription of messenger RNA (mRNA) from the viral nucleic acid.

ii. Translation of the mRNA into ‘early proteins’

iii. Replication of Viral nucleic acid.

iv. Synthesis of late or structural proteins, which are the components of daughter virion capsids.
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 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

Viroids 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.
Prions are small proteinaceous infectious agents without genetic material. These are responsible for a number of degenerative brain diseases (Example: Creutzfeldt) and hereditary dementia.

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: varcola or vaccinia.

b. Allantonic Cavity

Inoculation on the allantonic cavity provides a rich yield of influenza and some paramyxoviruses.

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 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.

Detection of virus Growth in Cell Cultures

Virus growth in cell cultures can be detected by the following methods.

1. Cytopathic effect
Many viruses cause morphological changes in cultured cells in which they grow. These changes are known as cytopathic effects (CPE) and the viruses causing CPE are called as ‘cytopathogenic viruses’.

2. Metabolic inhibition
In normal cell cultures, the medium turns acidic due to cellular metabolism. This can be made out by the colour of the indicator (Phenol red) incorporated in the medium.

3. Haemadsorption
When haemagglutinating viruses grow in cell cultures, their presence can be identified by the addition of guinea pig
The erythrocytes will adsorb on to the surface of viral cells multiplying in the culture. This is known as ‘haemadsorption’.

4. Interference
The growth of non cytopathogenic virus in cell culture can be tested with the cytopathogenic virus. The growth of the first will inhibit infection by the second virus by ‘interference’.

5. Transformation
Tumour forming viruses induce cell transformation and loss of contact inhibition, so that growth appears in a piled up fashion producing ‘microtumours’.

6. Immuno fluorescence
Cells from virus infected cultures are stained by fluorescent conjugated antiserum and examined under the UV microscope for the presence of virus antigen.

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 between the envelope and capsid. The enveloped virion measures about 200nm and the naked virion about 100 nm in diameter.

Classification
The family Herpesviridae is divided into three subfamilies based on biological, physical and genetic properties (Table 10.1).

Table 10.1: Classification of Human Herpes Viruses

<table>
<thead>
<tr>
<th>Species</th>
<th>Common name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human herpes virus type 1</td>
<td>Human Simplex virus type1</td>
</tr>
<tr>
<td>Human herpes virus type 2</td>
<td>Human Simplex virus type2</td>
</tr>
<tr>
<td>Human herpes virus type 3</td>
<td>Varicella Zoster virus</td>
</tr>
<tr>
<td>Human herpes virus type 4</td>
<td>Epstein - Barr Virus</td>
</tr>
<tr>
<td>Human herpes virus type 5</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>Human herpes virus type 6</td>
<td>Human B cell lymphotropic virus*</td>
</tr>
<tr>
<td>Human herpes virus type 7</td>
<td>R K Virus *</td>
</tr>
<tr>
<td>Human herpes virus type 8</td>
<td>-</td>
</tr>
</tbody>
</table>
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 or HHV 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 humans, the sources of infection are saliva, skin lesions or respiratory secretions. In type 2, transmission occurs by close contact and may be venereal 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) was observed.

Virus isolation
Inoculation in mice and on chick embryo CAM is insensitive. Primary human 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 childhood infections. The disease may, occur at any age.

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, cytomagalovirus 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’.

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 named it has ‘EB Virus’ affecting cells of B lymphocyte. 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, 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 lymph tropic virus, renamed as HHV - 6. HHV- 7 was isolated in 1990 from peripheral CD4 cells of a healthy
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 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 appears peaks in 3-4 months and persists much longer for life. ELISA kits for detection of IgM and IgG antibodies are available.
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 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 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 circulation. 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 excreations such as saliva, breast
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.

HBcAg 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 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.

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 infection has largely been eliminated by donor screening 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, box 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 1-2 months 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 (2-6 months). Anti HBs is the protective antibody.

HBcAg is not demonstrable in circulation because it’s enclosed within the HBsAg coat but its antibody, anti
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.

**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).

**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

![Figure 10.6: Structure of Rabies Virus](image)
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 disseminated 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 year. 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 phase begins with hyperactivity. Attempts to drink on such painful spasms of the
pharynx and larynx producing 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 3–5 days.

**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 at death.

**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**

- Prompte cauterization of the wounds helps to destroy the virus.
- Antirabic serum may be applied topically.
- Antitetanus measures and antibiotics to prevent sepsis.

**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 no residual live virus.

2. **Beta propiolactone (BPL) Vaccine:** Beta propiolactone is used as the inactivating agent instead of Phenol.

3. **Infant Brain Vaccine:** The encephalitogenic 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**

HIV is known as Human Immuno Deficiency Virus, the etiological agent of AIDS, belongs to the lentivirus subgroup of the family Retroviridae.
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 T4 cells decrease in numbers. Infected T4 cells do not release normal amounts of interleukin-2, gamma interferon and other lymphokines, this is a 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 infection which may last up to several years. The infection progresses in course of time through various stages, CD4 lymphocytopenia, minor opportunistic infections, ARC AIDS-related complex, ultimately terminating to AIDS.

3. Persistent generalized lymphadenopathy (PGL)
It’s defined as the presence of enlarged lymph nodes at least 1 cm, in diameter in two or more non contiguous extrainguina, sites persist for at least three months.
4. AIDS related complex (ARC)
This group includes patients with considerable immuno deficiency, 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. Pneumonia 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 encephalopathy 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/mm3.
ii. Platelet count will show thrombocytopenia.
iii. Raised IgG and IgA levels.

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 to appear in blood.

2. Polymerase Chain reaction
It the most sensitive and specific test. Two forms of PCR have been used, DNA PCR and RNA PCR.

3. Antibody detection
Demonstration of antibodies is the simpest and widely employed technique.
2–8 weeks to months for antibodies to appear after infection, during part of 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. Immunorestorative measure
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 **encephalitides**, the liver in **yellow fever** and the capillary endothelium in **hemorrhagic fever**.

Clinical syndromes are fever with or without rash encephalitis, hemorrhagic fever with or without rash encephalitis, hemorrhagic fever and systemic disease, 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.

**ELISA**

Virus isolated from insect vectors and from reservoir animal.

**Toga Viruses**

Toga viruses are **spherical enveloped viruses** with a diameter of 50-70nm. Single stranded RNA genome. The virus replicates in the **cytoplasm** 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.

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, retrobulbar pain, 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 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.

![Figure 10.9: Aedes aegypti](image)
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,
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 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**

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 Arbovirus, 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 the prevention or treatment of Zika virus infection. Development of a Zika vaccine remains an active area of research.
   a. HIV virus
   b. EB Virus
   c. Rabies virus
   d. None of these
5. The process of elution is caused by ________ enzyme
   a. Neuramidase
   b. Isomerase
   c. Polymerase
   d. None of these
6. Beta propiolactone (BPL. Vaccine is given for ________
   a. HIV virus
   b. Influenza Virus
   c. Rabies virus
   d. None of these
7. ________ is an example for mosquito-borne and tick borne viruses
   a. Dengu virus
   b. Flavi virus
   c. Chikungunya virus
   d. None of these

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 infective.
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 on 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 hemorrhagic 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
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.

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.

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 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.

\[ \text{Ag} + \text{Ab} \rightarrow \text{Ag} – \text{Ab complex} \]

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...
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 immunofluorescence. 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
- Sandwich 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).

**Sandwich Method**

This is an immuno fluorescence method used to test the number of cells producing antibodies for a specific antigen. In this method, lymphocytes are fixed with ethanol. These fixed cells are treated with polysaccharide antigen of *Pneumococcus*. This antigen combines with those lymphocytes which have the capacity to produce antibody against pneumococcal antigen. Now fluorescent antibody is added. Antigen is sandwiched between antibodies.

**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 microtitre plates. (Figure 11.2 Micro titre plate).

If the antigen is to be detected the antibody is fixed in the microtitre 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 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-Phenyldiamine-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).

**Figure 11.2:** Micro titre plate  
**Figure 11.3:** Basic steps in ELISA  
**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).

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 microtitre 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 with 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 microtitre 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, 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 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 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
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

Figure 11.7: Type I 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$^{2+}$ 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.
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 in turn 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.

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 leucocytes 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
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

<table>
<thead>
<tr>
<th>Sn. No.</th>
<th>Immediate Hypersensitivity</th>
<th>Delayed Hypersensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>It appears and disappears rapidly</td>
<td>It appears slowly and last longer.</td>
</tr>
<tr>
<td>2.</td>
<td>It is induced by antigens or haptens by any route</td>
<td>Induced by infection, injection of antigen intra dermally or with adjuvants of by skin contact.</td>
</tr>
<tr>
<td>3.</td>
<td>The reaction is antibody mediated B-cell response</td>
<td>The reaction is T-cell mediated response.</td>
</tr>
<tr>
<td>4.</td>
<td>Passive transfer is possible with serum</td>
<td>Cannot be transferred with serum but can be transferred by lymphocytes</td>
</tr>
<tr>
<td>5.</td>
<td>Desensitization is easy, but does not last long</td>
<td>Desensitization is difficult but long lasting.</td>
</tr>
</tbody>
</table>
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

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.

**Figure 11.10: Type IV delayed hypersensitivity**
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 the graft as 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.
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.

Mechanism of the graft
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 graft should be used

11.5 Immunization/Vaccination
Father of Immunization 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.
Principles of Vaccination

- The primary goal in vaccination is to provide protective immunity by inducing a memory response to an infectious microorganism using a non-toxic antigen preparation. It is important to produce immunity of the appropriate kind: antibody / or cellular immunity.

- Antibodies produced as a result of immunization are effective primarily against extracellular organisms and their products e.g., toxins. Passively administered antibodies have the same effect as induced antibodies.

- Cell-mediated immunity (T cells, macrophages) induced by vaccination is important particularly in preventing intracellular bacterial and viral infections and fungal infections.

- The ultimate goal of any immunization program is the eradication of the disease.

- This requires that the infection is limited only to humans, with no animal or environmental reservoir, and the absence of any subclinical or carrier state in humans.

- Achieving elimination requires a high level of herd immunity to prevent person to person spread.

- This requires considerable infrastructure support to ensure that all at-risk populations are targeted for immunization.

- This has been achieved for small pox, although we are close to the elimination of polio.

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**: Uses 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 adn display them on their surfaces. The body's own cells become vaccine generating factories.
**Routes of Administration**
- Deep subcutaneous or intramuscular route
- Oral route
- Intradermal route
- Scarification
- Intranasal route

**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).

**Passive artificial immunization** - developed by injecting previously prepared

<table>
<thead>
<tr>
<th>Infection</th>
<th>Source of Antiserum</th>
<th>Indications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetanus</td>
<td>Immune human; horse</td>
<td>Post exposure (plus vaccine)</td>
</tr>
<tr>
<td>Diptheria</td>
<td>Horse</td>
<td>Post-exposure</td>
</tr>
<tr>
<td>Gas gangrene</td>
<td>Horse</td>
<td>Post-exposure</td>
</tr>
<tr>
<td>Botulism</td>
<td>Horse</td>
<td>Post-exposure</td>
</tr>
<tr>
<td>Varicella-Zoster</td>
<td>Immune human</td>
<td>Post-exposure in immunodeficiency</td>
</tr>
<tr>
<td>Rabies</td>
<td>Immune human</td>
<td>Post exposure (plus vaccine)</td>
</tr>
<tr>
<td>Hepatitis B</td>
<td>Immune human</td>
<td>Post-exposure prophylaxis</td>
</tr>
<tr>
<td>Hepatitis A</td>
<td>Pooled human Ig</td>
<td>Prophylaxis</td>
</tr>
<tr>
<td>Measles</td>
<td>Immune human</td>
<td>Prophylaxis</td>
</tr>
<tr>
<td>Snakebite</td>
<td>Horse</td>
<td>Post-bite</td>
</tr>
<tr>
<td>Some autoimmune disease</td>
<td>Pooled human Ig</td>
<td>Acute thrombocytopenia and neutropenia</td>
</tr>
</tbody>
</table>
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 intramuscularly or subcutaneously.

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).

Table 11.3: Antigen Preparations Used in Vaccines

<table>
<thead>
<tr>
<th>Type of antigen</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Viruses</td>
</tr>
<tr>
<td>Normal heterologous organism</td>
<td>Vaccinia (Cowpox)</td>
</tr>
<tr>
<td>Living attenuated organism</td>
<td>Measles</td>
</tr>
<tr>
<td></td>
<td>Mumps</td>
</tr>
<tr>
<td></td>
<td>Rubella</td>
</tr>
<tr>
<td></td>
<td>Polio (Sabin)</td>
</tr>
<tr>
<td></td>
<td>Yellow fever</td>
</tr>
<tr>
<td></td>
<td>Varicella-Zoster</td>
</tr>
<tr>
<td>Whole killed organism</td>
<td>Rabies</td>
</tr>
<tr>
<td></td>
<td>Poli (Salk)</td>
</tr>
<tr>
<td></td>
<td>Influenza</td>
</tr>
<tr>
<td>Inactivated toxin (toxoid)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Capsular polysaccharide</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Surface antigen</td>
<td></td>
</tr>
<tr>
<td>Hepatitis B</td>
<td></td>
</tr>
</tbody>
</table>
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.

Table 11.4: National immunization schedule

<table>
<thead>
<tr>
<th>Sn. No.</th>
<th>Vaccine</th>
<th>Due age</th>
<th>Max age</th>
<th>Route</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>BCG</td>
<td>At birth</td>
<td>till one year age</td>
<td>Intra dermal</td>
</tr>
<tr>
<td>2.</td>
<td>Hepatitis B-Birth dose</td>
<td>At birth</td>
<td>within 24 hours</td>
<td>Intra muscular</td>
</tr>
<tr>
<td>3.</td>
<td>OPV-O</td>
<td>At birth</td>
<td>within the first 15 days</td>
<td>Oral</td>
</tr>
<tr>
<td>4.</td>
<td>OPV 1, 2 &amp; 3</td>
<td>At 6 weeks, 10 weeks &amp; 14 weeks</td>
<td>till 5 years of age</td>
<td>Oral</td>
</tr>
<tr>
<td>5.</td>
<td>Pentavalent 1, 2 &amp; 3 (Diphtheria + Pertuss is + Tetanus + Hepatitis B + Hib)</td>
<td>At 6 weeks, 10 weeks &amp; 14 weeks</td>
<td>1 year of age</td>
<td>Intra muscular</td>
</tr>
<tr>
<td>6.</td>
<td>Inactivated polio vaccine</td>
<td>At 6 &amp; 14 weeks</td>
<td>1 year of age</td>
<td>Intra muscular</td>
</tr>
<tr>
<td>7.</td>
<td>Rotavirus (where applicable)</td>
<td>At 6 weeks, 10 weeks &amp; 14 weeks</td>
<td>1 year of age</td>
<td>Oral</td>
</tr>
<tr>
<td>8.</td>
<td>Pneumococcal conjugate vaccine (where applicable)</td>
<td>At 6 weeks &amp; 14 weeks</td>
<td>1 year of age</td>
<td>Intra muscular</td>
</tr>
<tr>
<td>9.</td>
<td>Measles/Rubella 1st dose</td>
<td>At 9 completed months – 12 months</td>
<td>5 years of age</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>10.</td>
<td>DPT Booster-1</td>
<td>16–24 months</td>
<td>7 years of age</td>
<td>Intra muscular</td>
</tr>
<tr>
<td>11.</td>
<td>Measles/Rubella 2nd dose</td>
<td>16–29 months</td>
<td>5 years of age</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>12.</td>
<td>OPV Booster</td>
<td>16–24 months</td>
<td>5 years</td>
<td>Oral</td>
</tr>
<tr>
<td>13.</td>
<td>DPT Booster – 2</td>
<td>5–6 years</td>
<td>7 years of age</td>
<td>Intra muscular</td>
</tr>
<tr>
<td>14.</td>
<td>TT</td>
<td>10 years &amp; 16 years</td>
<td>16 years</td>
<td>Intra muscular</td>
</tr>
</tbody>
</table>
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, 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.
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
10. Immunisation done in a community is called _____________ immunity.
    a. Combined
    b. General
    c. Local
    d. Herd

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
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 genet 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
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.
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 12.2:** Major events in transcription

**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
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. 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. 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).
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.

---

**Table 12.1: Genetic code**

<table>
<thead>
<tr>
<th>First letter</th>
<th>Second letter</th>
<th>Third letter</th>
</tr>
</thead>
<tbody>
<tr>
<td>U</td>
<td>UUU UUC UUA UUG</td>
<td>Phe UCU UCA UCG Ser UAU UAC UAG Stop UAG Stop Cys UGU UGC UGA Trp</td>
</tr>
<tr>
<td>C</td>
<td>CUC CUCC CUA CUG</td>
<td>Leu CCU CCC CCA CCG Pro CAU CAC CAA CAG Gln CGU CGC CGA CGG Arg</td>
</tr>
<tr>
<td>A</td>
<td>AUG AUA AUC</td>
<td>Met Ile ACC ACC ACA ACC Thr AUA AAG Asn AAC AUA AAG Lys AGU AGC AGA AGG Ser Arg</td>
</tr>
<tr>
<td>G</td>
<td>GGU GUC GUA GUG</td>
<td>Val GCU GCC GCA GCG Ala GAA GAG Glu GGU GCC GGA GGG Gly UCG UCA UGA UGA</td>
</tr>
</tbody>
</table>

**The 20 amino acids and their abbreviations**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>3-letter abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>Ala</td>
</tr>
<tr>
<td>Arginine</td>
<td>Arg</td>
</tr>
<tr>
<td>Asparagine</td>
<td>Asn</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>Asp</td>
</tr>
<tr>
<td>Aspartic acid &amp; Asparagine</td>
<td>Asx</td>
</tr>
<tr>
<td>Cysteine</td>
<td>Cys</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Gln</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>Glu</td>
</tr>
<tr>
<td>Glutaine or Glutamic acid</td>
<td>Glx</td>
</tr>
<tr>
<td>Glycine</td>
<td>Gly</td>
</tr>
<tr>
<td>Histidine</td>
<td>His</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>Ile</td>
</tr>
<tr>
<td>Leucine</td>
<td>Leu</td>
</tr>
<tr>
<td>Lysine</td>
<td>Lys</td>
</tr>
<tr>
<td>Methionine</td>
<td>Met</td>
</tr>
</tbody>
</table>
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

**Figure 12.4:** Major events in Translation
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 12.5: tRNA

tRNA is often designated tRNA^{Met}. Both tRNA^{Met} and tRNA^{Met} recognize the codon AUG, but only tRNA^{Met} 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.

Figure 12.6: Wobble hypothesis
Phenotype is an observable property of an 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 – pyrimidine 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.
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.

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 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 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 dimmers (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.

Figure 12.10: UV induced DNA damage

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. Like normal bases, base analogs exist in normal and rare tautomeric states. In each of the two states, the base analog pairs with a different normal base in DNA. Because base analogs are similar to the normal nitrogen bases, they may be incorporated into DNA in place of normal bases. E.g. 5 - bromouracil (5-BU). If 5-BU is incorporated in its more common normal state, it pairs with adenine. If it changes into its rare state during replication, it pairs with guanine instead. In the next round of replication, the 5-BU – G base pair is resolved into a C – G base pair instead of the T – A base pair. By this process a transition mutation is produced, from TA to CG (Figure 12.11).

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 (- NH₂) from the bases guanine, cytosine, and...
adenine. When cytosine is treated with nitrous acid it removes amino group from cytosine which changes to uracil resulting in transition mutation (Figure 12.12a).

2. Hydroxylamine ($\text{NH}_2\text{OH}$) 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. Mutations induced by hydroxylamine can only be CG to TA transitions (Figure 12.12b).

3. Alkylating agents like methymethane sulfonate (MMS) introduces alkyl groups onto the bases at a number of location. For example after treatment with MMS, some guanines are methylated at 6 – oxygen to produce O6 – methyl guanine. The methylated guanine pairs with thymine rather than cytosine giving G C to AT transitions (Figure 12.12c).

<table>
<thead>
<tr>
<th>Original base</th>
<th>Mutagen</th>
<th>Modified base</th>
<th>Predicted transition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosine</td>
<td>Nitrous acid ($\text{H}_2\text{NO}$)</td>
<td>Uracil</td>
<td></td>
</tr>
<tr>
<td>Cytosine</td>
<td>Hydroxylamine ($\text{NH}_2\text{OH}$)</td>
<td>Hydroxyl amino cystosine</td>
<td>Adenine</td>
</tr>
<tr>
<td>Guanine</td>
<td>Methylmethane sulfonate (MMS) (alkylating agent)</td>
<td>O-Methylguanine</td>
<td>Thymine</td>
</tr>
</tbody>
</table>

Figure 12.12: Action of three base modifying agents.
iii. **Intercalating agents**

Acridine, proflavin, ethidium bromide are examples of few examples of intercalating agents. These insert (intercalate) themselves between adjacent bases in one or both strands of the DNA double helix. Due to this an extra base (chosen at random, G in the Figure 12.13a) must be inserted into the new DNA strand opposite the intercalating agent. Intercalating agents can cause either additions or deletions.

**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,

---

**Figure 12.13: Mutations due to intercalating agents**
Once mutations are induced, 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.
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

**Infobits**

**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
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 take 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.

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.

**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.
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 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.

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.
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+ × 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 F plasmid'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
Figure 12.17: Mechanism of conjugation (a) $F^+ \times F^-$ (b) HFR cell Formation (c) HFR $\times F^-$ (d) $F'$ formation (e) $F' \times F^-$
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.

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).
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, excision 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.
**Generalized Transduction**

1. Phage infects bacterial cell
2. Destruction of bacterial DNA
3. Virus capsid synthesis and assembly
4. Lysis of bacterial cell, phage release, & infection of new cell
5. Crossover and stable gene transfer into recipient cell chromosome

**Specialized Transduction**

1. Phage DNA integrates into chromosome
2. Prophage de-integrates and picks up piece of bacterial chromosome
3. Lysis of bacterial cell, phage release, infection of new bacterial cell
4. Replication of viral DNA and destruction of bacterial DNA
5. Virus capsid synthesis and assembly

**Figure 12.21:** (a) Generalized Transduction (b) Specialized Transduction
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 the 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
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.

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 containing 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 1 to 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
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

**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. PI bacteriophage can carry fragments up to 95 kb

**Phage λ (Figure 12.26)** consists of a head and a tail (both proteins). The DNA, located in the head, is a linear molecule of about 50 kb. At each end of the DNA, there are single stranded extensions called

**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 plasmids are 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.
cohesive (cos) ends. On attachment with tail to E.coli, phage injects its DNA into the cell. Inside E.coli the phage linear DNA cyclizes and gets ligated through cos ends to form a circular DNA. The phage DNA has two fates – lytic cycle and lysogenic cycle (Figure 12.27).

Only about 50% of phage λ DNA is necessary for its multiplication and other functions. Thus, as much as 50% (i.e. up to 20 kb) of the phage DNA can be replaced by a donor DNA for use in cloning experiments. However, several restriction sites are present on phage which is not by itself a suitable vector. The λ based phage vectors are modifications of the natural phage with much reduced number of restriction sites.

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.

**Cosmids:** Cosmids are the vectors possessing the characteristics of both plasmid and bacteriophage. Cosmids can be constructed by adding a fragment of phage λ DNA including cos site, to plasmids. Once inside the host cell, cosmids behave just like plasmids and replicate. The advantage with cosmids is that they carry larger fragments of foreign DNA (35–45 kb) compared to plasmids.

**Figure 12.27:** Life cycle of phage λ

275
**Phagemids:** Phagemids are the combination of plasmid and phage and can function as either plasmid or phage. Since they possess 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.

1. Yeast artificial chromosome YAC is a synthetic DNA that can accept large fragments of foreign DNA between 0.2 to 2.0 Mb (particularly human DNA). In addition to origin of replication sequence and selectable markers they possess centromeric and telomeric regions, and therefore the recombinant DNA can be maintained like a yeast chromosome.

2. (BACs) Bacterial Artificial chromosomes: BACS can accept DNA inserts around 300 kb. A major part of the sequencing of human genome has been accomplished by using a library of BAC recombinant. BACS are vectors containing the origin replication of a natural plasmid called the F factor, a MCS, a selectable marker and often some other features.

**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 regulatory sequences (promoter sequence) necessary to allow the transcription and translation of a cloned gene or genes. Expression vectors are essentially derivatives of the plasmid cloning vectors used in the host. They are used to produce the protein encoded by a cloned gene in the transformed host. For example, the biotechnology industry produces pharmaceutically active proteins with the use of expression vectors and the appropriate host.

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.

12.10 Restriction Enzymes

In 1960s Swiss microbiologist Werner Arber and American microbiologists Hamilton 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 of restriction endonucleases (particularly type II) cut DNA at defined sites within recognition sequence. Type II restriction enzymes make
two single – strand 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 somatostatin, insulin, human growth hormone and interferon. It decreases the dependency on human tissues and solves problem of limited production.

b. Development of synthetic vaccines for instance, vaccines for malaria and rabies a recombinant hepatitis vaccine is already commercially available.

<table>
<thead>
<tr>
<th>Type</th>
<th>No of Enzyme and sub units</th>
<th>Cleavage site</th>
<th>Examples</th>
<th>Bacterial source</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>One with 3 sub units for recognition cleavage and methylation</td>
<td>1000 bp from recognition site</td>
<td>EcoK1 Cfr A1</td>
<td>Escherichia coli Citrobacter freundii</td>
</tr>
<tr>
<td>II</td>
<td>Two different enzymes to cleave or modify the recognition sequence</td>
<td>Same as recognition or close to recognition site</td>
<td>Eco R1 Alu I</td>
<td>Escherichia coli Arthrobacter luteus</td>
</tr>
<tr>
<td>III</td>
<td>One with 2 subunits</td>
<td>24-26 bp from recognition site</td>
<td>Hinf III Pst II</td>
<td>Haemophilus influenzae Providencia stuarti</td>
</tr>
</tbody>
</table>

Table 12.2: Types and features of restriction enzyme
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. 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.

![Agarose gel electrophoresis unit](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 acrylamide monomers crosslinked with methylene bisacrylamide 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.

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 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.

![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.
Figure 12.31 outlines how PCR technique works. To amplify (make large quantities) a particular DNA sequence by PCR a reaction mixture (often 100ml or less in volume) containing the following are required.

4. **Four deoxyribonucleoside triphosphates** (dNTPs) - dCTP, dATP, dCTPP, dTTP

---

**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
cycle, 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.

![Diagram of PCR cycle](image)

**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.

PCRs 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 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.

![PCR machine](image)

**Figure 12.33:** PCR machine
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?

12.11.3 Molecular Markers – RFLP, RAPD

A molecular marker is a DNA sequence in the genome which can be located and identified. As a result of genetic alterations (mutations, insertions, deletions), the base composition at a particular location of the genome may be different in different cells. These differences, collectively called as polymorphisms can be mapped and identified. The DNA markers are highly useful for genetic mapping of genomes. RFLPS (Restriction Fragment Length Polymorphisms), VNTRs (mini satellites or Variable Number Tandem Repeats), 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.

Restriction fragment length polymorphism (RFLP) was the very first technology employed for the detection of polymorphism, based on the DNA sequence differences. RFLP is mainly based on the altered restriction enzyme sites, as a result of mutations and recombinations of genomic DNA. An outline of the RFLP analysis is given

Figure 12.34: (a) An outline of RFLP analysis as a molecular marker (b) A schematic representation of restriction fragment length polymorphism (RFLP) analysis as a molecular marker
in Figure 34 a. The procedure basically involves the isolation of genomic DNA, its digestion by restriction enzymes, separation by electrophoresis, and finally hybridization by incubating with cloned and labeled probes (Figure 12.34 b).

Based on the presence of restriction sites, DNA fragments of different lengths can be generated by using different restriction enzymes. In the Figure 12.34 b, two DNA molecules from two sources (A and B) are shown. In source A, a mutation has

Figure 12.35: An outline of RAPD analysis as a molecular marker

**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).
occurred leading to the loss of restriction site that can be digested by EcoRI.

The result is that when the DNA molecules are digested by the enzyme Hind III, there is no difference in the DNA fragments separated. However, with the enzyme EcoRI, source A DNA molecules is not digested while source B DNA molecule is digested. This results in a polymorphic pattern of separation.

**Random amplified polymorphic DNA (RAPD)** is a molecular marker based on PCR amplification. An outline of RAPD is depicted in Figure 12.35. The DNA isolated from the genome is denatured. The template molecules are annealed with primers, and amplified by PCR. Single short oligonucleotide primers (usually a 10-base primer) can be arbitrarily selected and used for the amplification of DNA segments of the genome (which may be distributed throughout the genome). The amplified products are separated on electrophoresis and identified.

Based on the nucleotide alterations in the genome, the polymorphisms of amplified DNA sequences differ which can be identified as bands on gel electrophoresis. Genomic DNA from two different sources often results in different amplification patterns i.e. RAPDs.

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 initiation 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
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 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, cosmid, 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), 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
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  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?

11. The sequence of nucleotides in an mRNA is 5′-AUG-ACCCAUU-CAUUGUCUCGUUAG-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.


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.
<table>
<thead>
<tr>
<th>Practicals</th>
<th>Page. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram’s staining of curd/idly batter/yeast</td>
<td></td>
</tr>
<tr>
<td>Identification of the fungus (aspergillus/mucor/Rhizopus) by wet mount using LPCB</td>
<td></td>
</tr>
<tr>
<td>Blood grouping</td>
<td></td>
</tr>
<tr>
<td>Blood staining</td>
<td></td>
</tr>
<tr>
<td>Test for catalase</td>
<td></td>
</tr>
<tr>
<td>Widal test (slide test)</td>
<td></td>
</tr>
<tr>
<td>Demonstration of rhizobium from root nodules and its isolation</td>
<td></td>
</tr>
</tbody>
</table>

**Spotters**

| II A) Specimen                                                             |           |
| Root nodules of leguminous plant                                          |           |
| Tikka leaf spot of groundnut plant                                        |           |
| Mushroom                                                                  |           |
| Sand fly                                                                   |           |
| Ascaris                                                                    |           |

| II B) Slide                                                                |           |
| Cyst of Entamoeba histolytica                                             |           |
| Penicillium species                                                       |           |
| Microfilariae                                                             |           |
| Egg of Ascaris lumbricoides                                               |           |
| Heterocysts of Nostoc                                                     |           |
| Acid fast bacilli                                                         |           |

| II C) Spotter                                                              |           |
| Antibiotic sensitivity plate set up by Kirby Bauer technique              |           |
| Sugar fermentation tube showing acid and gas                              |           |
| Agarose gel electrophoresis apparatus.                                   |           |
| Spoiled food                                                              |           |
## Higher Secondary – First Year Practical Examination

<table>
<thead>
<tr>
<th>Microbiology</th>
</tr>
</thead>
</table>

### Marking Scheme

#### Allotment of Marks

<table>
<thead>
<tr>
<th></th>
<th>Marks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internal Assessment</td>
<td>05</td>
</tr>
<tr>
<td>External Assessment</td>
<td>15</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20</strong></td>
</tr>
</tbody>
</table>

#### Internal Assessment (Practicals) Marks Break Up

<table>
<thead>
<tr>
<th>Item</th>
<th>Marks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Record Note Book</td>
<td>03</td>
</tr>
<tr>
<td>2. Skill of performing Experiments</td>
<td>02</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>05</strong></td>
</tr>
</tbody>
</table>

#### External Assessment Mark Break Up

<table>
<thead>
<tr>
<th>Item</th>
<th>Marks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Major Practical</td>
<td>09</td>
</tr>
<tr>
<td>2. Spotters</td>
<td>06</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>15</strong></td>
</tr>
</tbody>
</table>

### I. Major Practical (Any one out of 5 questions) $9 \times 1 = 9$ 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) $2 \times 3 = 6$ marks

- **Identification** | $\frac{1}{2}$ marks
- **Two salient points** | 1 mark
- **Diagram** | $\frac{1}{2}$ mark

**Total** | $02 \text{ marks} \times 3 \text{ spotters} = 6\text{ marks}$
Key for Practical Examination

I. Major Practical (Any one) 9×1 = 9 marks

1. Determine the gram nature of bateria 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

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Specimen</td>
<td>2 marks</td>
</tr>
<tr>
<td>B. Slide</td>
<td>2 marks</td>
</tr>
<tr>
<td>C. Spotter</td>
<td>2 marks</td>
</tr>
</tbody>
</table>
1. Gram’s staining of curd/idly batter/yeast

Aim: To determine the gram nature of bacteria 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.
Observation Table: (any one shape and stain)

<table>
<thead>
<tr>
<th>Sr. no</th>
<th>Morphology</th>
<th>Arrangement</th>
<th>Colour of Cytoplasm</th>
<th>Colour of Background</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Rod (bacilli)</td>
<td>Singles, chains</td>
<td>Violet</td>
<td>colourless</td>
<td>Gram positive</td>
</tr>
<tr>
<td>2.</td>
<td>Oval yeast cells</td>
<td>Singles, budded</td>
<td>Violet</td>
<td>colourless</td>
<td>Gram positive</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Characteristics of Hyphae</th>
<th>Spores borne in</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus sp.</td>
<td>Septate</td>
<td>Conidiophore bear conidia</td>
</tr>
<tr>
<td>Mucor and Rhizopus sp.</td>
<td>Aseptate</td>
<td>Sporangiosphere bear sporangium containing sporangiospore.</td>
</tr>
</tbody>
</table>
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:
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.
**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)**

![Blood groups diagram]

**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.

1. **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**
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 cm² (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 minute.
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**

![Image of stained cells](image-url)
OBSERVATION

<table>
<thead>
<tr>
<th>TYPE OF CELL</th>
<th>COLOUR OF CYTOPLASM</th>
<th>COLOUR OF NUCLEUS</th>
<th>COLOUR OF GRANULES</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC</td>
<td>pink</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>WBCs(leucocytes)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophil</td>
<td>pink</td>
<td>blue</td>
<td>lilac</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>pink</td>
<td>blue</td>
<td>orange</td>
</tr>
<tr>
<td>Basophil</td>
<td>pink</td>
<td>blue</td>
<td>Dark blue black</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>blue</td>
<td>violet</td>
<td>-</td>
</tr>
</tbody>
</table>

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 (H₂O₂). It is used to differentiate those bacteria that produces an enzyme catalasase, such as staphylococci, from non-catalase producing bacteria such as streptococci.

The enzyme catalasase 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

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% H₂O₂ in the glass slide.
3. Observe for the evolution of oxygen bubbles.

DIAGRAM

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.
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.
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) i.e. 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**

<table>
<thead>
<tr>
<th>Testsample + O antigen</th>
<th>Test sample + H antigen</th>
<th>Test sample + AH antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>O</td>
<td>H</td>
<td>AH</td>
</tr>
<tr>
<td>BH</td>
<td>NC</td>
<td>PC</td>
</tr>
</tbody>
</table>

**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**

Qualitative widal test was carried out using rapid slide agglutination method. Antibodies against O and H antigens of Salmonella typhi were detected in the serum.
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 N₂ 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 peritrichious 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):

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol</td>
<td>10.0</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.5</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>0.2</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.1</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1.0</td>
</tr>
<tr>
<td>CaCO₃</td>
<td>3.0</td>
</tr>
<tr>
<td>Agar</td>
<td>25.0</td>
</tr>
</tbody>
</table>
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% HgCl₂.

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% HgCl₂.
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:
Observation
Gram's stain

<table>
<thead>
<tr>
<th>Organism</th>
<th>Morphology</th>
<th>Arrangement</th>
<th>Colour of cytoplasm</th>
<th>Colour of Background</th>
<th>Inference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhizobium from root nodule.</td>
<td>Rod (bacilli)</td>
<td>Singles</td>
<td>red</td>
<td>colourless</td>
<td>Gram negative</td>
</tr>
</tbody>
</table>

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.
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
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**
- 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
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
- Conidiophores 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 1-2 micrometer.

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 290 microns in length and 6-7 microns in breadth.
9. Egg of Ascaris lumbricoides

- 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.
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 an 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.
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.

![Sugar Broth tubes](image)

In the above image Test tube C was inoculated with cell suspension and incubated at 37°C for 24 hours and after inoculation 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.
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.
Microbiology – Class XII
List of Authors and Reviewers

Authors
Gajalakshmi S
PGT., KBC GGHSS., Redhills, Thiruvallur District.

Ramachandran N
PGT., GHSS., Kovur, Kancheepuram District.

Manimegalai K
PGT., Govt. Model HSS., Saidapet, Chennai.

Pameela Fatmima H
PGT., JGGHSS., Thiruvottiyur, Thiruvallur District.

Parvin Zeenath Anwar
PGT., St. Mary's Girls, HSS., Perambur, Chennai.

Jaya Priya D
PG Asst., GHSS., Nandhivaram, Kancheepuram.

Academic Coordinator
Angeline Ruby G
Assistant Professor
SCERT, Chennai.

QR Code Management Team
S. Albert Valavan Babu
B.T. Asst., GHS., Perumal Kovil, Paramakudi, Ramanathapuram.

V. Padmavathi
B.T. Asst., GHS., Vetriyur, Ariyalur.

A. Devi Jesintha
B.T. Asst., GHS., N.M. Kovil, Vellore.

This book has been printed on 80 G.S.M.
Elegant Maplitho paper.
Printed by offset at:

Art and Design Team

Layout
Jaishree Anbalagan
Winmac Solutions, Chennai.

Wrapper Design
Kathir Arumugam

QC
Arun Kamaraj Palanisamy

Co-ordination
Ramesh Munisamy

Typist
Suresh Mani

Domain Expert
Dr. Elanchezhiyan Manickan
Professor & Chairman
Dr. ALM PG IBMS,
University of Madras, Chennai.

Reviewers
Mrs. C. Initha Lebanon Ebency
Principal and Co-ordinator of Academic Affairs,
St. Paul's College of Arts and Science for Women
and St. Paul's Matriculation Hr. Sec School,
Eden Garden, St. Paul's Nagar,
Thadagam Road, Coimbatore.

Mrs. B. Manjula Devi
HOD., Asst. Professor,
Dept of Microbiology,
Bhaktavatsalam Memorial College for Women,
Kovur, Chennai.

Ms. C. Banu Rekha
Asst. Professor,
Dr. MGR Janaki College,
Chennai.

Mrs. G. Sangeetha
HOD., Asst. Professor,
Dept of Microbiology,
Mahalashmi Women's College of Arts and Science,
Avadi, Chennai.

Authors
Gajalakshmi S
PGT., KBC GGHSS., Redhills, Thiruvallur District.

Ramachandran N
PGT., GHSS., Kovur, Kancheepuram District.

Manimegalai K
PGT., Govt. Model HSS., Saidapet, Chennai.

Pameela Fatmima H
PGT., JGGHSS., Thiruvottiyur, Thiruvallur District.

Parvin Zeenath Anwar
PGT., St. Mary's Girls, HSS., Perambur, Chennai.

Jaya Priya D
PG Asst., GHSS., Nandhivaram, Kancheepuram.

Academic Coordinator
Angeline Ruby G
Assistant Professor
SCERT, Chennai.

QR Code Management Team
S. Albert Valavan Babu
B.T. Asst., GHS., Perumal Kovil, Paramakudi, Ramanathapuram.

V. Padmavathi
B.T. Asst., GHS., Vetriyur, Ariyalur.

A. Devi Jesintha
B.T. Asst., GHS., N.M. Kovil, Vellore.

This book has been printed on 80 G.S.M.
Elegant Maplitho paper.
Printed by offset at: