মানুষের জ্ঞান ও ভাবকে বইয়ের মধ্যে সঞ্চিত করিবার যে একটা প্রচুর সুবিধা আছে, সে কথা কেহই অস্বীকার করিতে পারে না। কিন্তু সেই সুবিধার দ্বারা মনের স্বাভাবিক শক্তিকে একেবারে আচ্ছন্ন করিয়া ফেলিলে বুদ্ধিকে বাবু করিয়া তোলা হয়।

— রবীন্দ্রনাথ ঠাকুর

ভারতের একটা mission আছে, একটা গৌরবময় ভবিষ্যৎ আছে, সেই ভবিষ্যৎ ভারতের উত্তরাধিকারী আমরাই। নূতন ভারতের মুক্তির ইতিহাস আমরাই রচনা করছি এবং করব। এই বিশ্বাস আছে বলেই আমরা সব দুঃখ কষ্ট সহ্য করতে পারি, অন্ধকারময় বর্তমানকে অগ্রাহ্য করতে পারি, বাস্তবের নিষ্ঠুর সত্যগুলি আদর্শের কঠিন আঘাতে ধূলিসাৎ করতে পারি।

— সুভাষচন্দ্র বসু

Any system of education which ignores Indian conditions, requirements, history and sociology is too unscientific to commend itself to any rational support.

— Subhas Chandra Bose

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NETAJI SUBHAS OPEN UNIVERSITY

Choice Based Credit System (CBCS)

SELF LEARNING MATERIAL

HZO

ZOOLOGY

Cell Biology, Immunology & Parasitology (Practical)

CC-ZO-06

Under Graduate Degree Programme

PREFACE

In a bid to standardize higher education in the country, the University Grants Commission (UGC) has introduced Choice Based Credit System (CBCS) based on five types of courses viz. *core, generic, discipline specific elective, ability and skill enhancement* for graduate students of all programmes at Honours level. This brings in the semester pattern which finds efficacy in sync with credit system, credit transfer, comprehensive continuous assessments and a graded pattern of evaluation. The objective is to offer learners ample flexibility to choose from a wide gamut of courses, as also to provide them lateral mobility between various educational institutions in the country where they can carry their acquired credits. I am happy to note that the university has been recently accredited by National Assessment and Accreditation Council of India (NAAC) with grade "A".

UGC (Open and Distance Learning Programmes and Online Programmes) Regulations, 2020 have mandated compliance with CBCS for U.G. programmes for all the HEIs in this mode. Welcoming this paradigm shift in higher education, Netaji Subhas Open University (NSOU) has resolved to adopt CBCS from the academic session 2021-22 at the Under Graduate Degree Programme level. The present syllabus, framed in the spirit of syllabi recommended by UGC, lays due stress on all aspects envisaged in the curricular framework of the apex body on higher education. It will be imparted to learners over the six semesters of the Programme.

Self Learning Material (SLMs) are the mainstay of Student Support Services (SSS) of an Open University. From a logistic point of view, NSOU has embarked upon CBCS presently with SLMs in English / Bengali. Eventually, the English version SLMs will be translated into Bengali too, for the benefit of learners. As always, all of our teaching faculties contributed in this process. In addition to this we have also requisioned the services of best academics in each domain in preparation of the new SLMs. I am sure they will be of commendable academic support. We look forward to proactive feedback from all stakeholders who will participate in the teaching-learning based on these study materials. It has been a very challenging task well executed, and I congratulate all concerned in the preparation of these SLMs.

I wish the venture a grand success.

Professor (Dr.) Subha Sankar Sarkar
Vice-Chancellor

Netaji Subhas Open University

Under Graduate Degree Programme

Choice Based Credit System (CBCS)

Subject : Honours in Zoology (HZO)

Course : Cell Biology, Immunology & Parasitology (Practical)

Course Code: CC-ZO-06

First Print : August, 2022

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Netaji Subhas Open University

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Course Code: CC-ZO-06

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UG Zoology (HZO)

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Unit 1 □ Study of Polytene Chromosome from Chironomid Larvae

Structure

1.0 Objective

1. To visualize the chromosome under light microscope.
2. To study the physical structure of a chromosome within a nucleus at polytene phase.

1.1 Introduction

Chromosomes are thread-like structures located inside the nucleus of animal and plant cells. Each chromosome is made of protein and a single molecule of deoxyribonucleic acid (DNA). Passed from parents to offspring, DNA contains the specific instructions that make each type of living creature unique. The term chromosome comes from the Greek words for color (chroma) and body (soma). Scientists gave this name to chromosomes because they are cell structures, or bodies, that are strongly stained by some colorful dyes used in research.

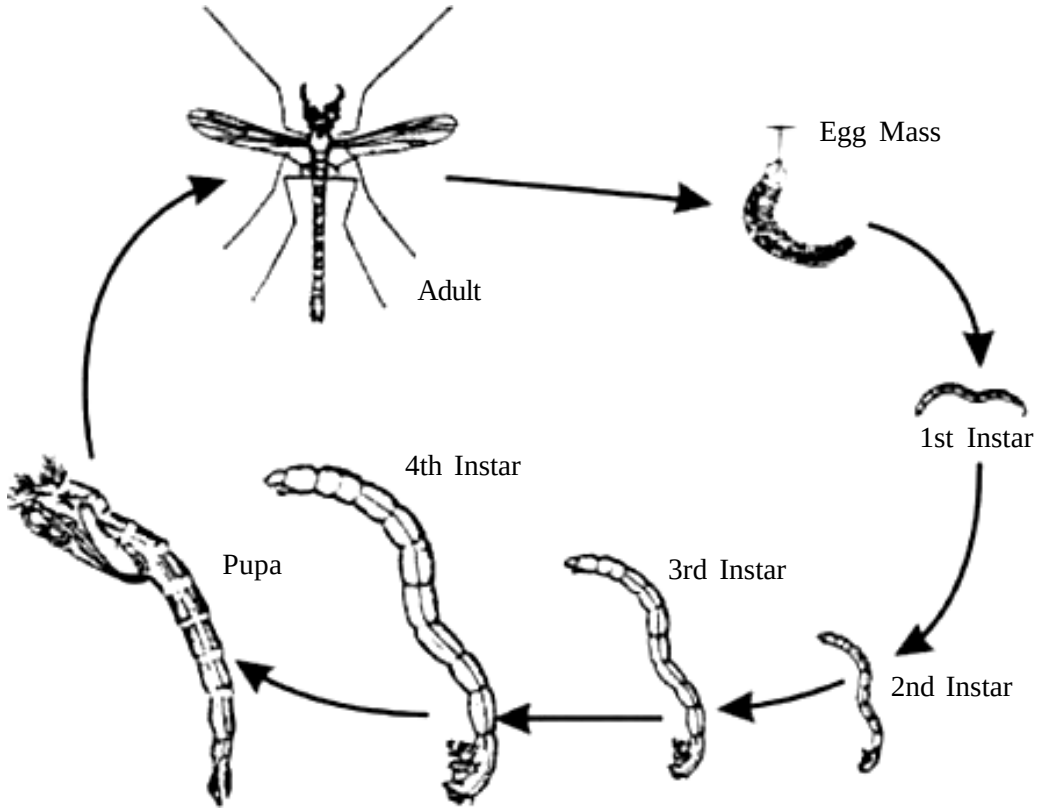


Fig. 1.1 : Chironomid Lifecycle (Source : intechopen.com)

1.2 Structure of Chromosome

1. In eukarytoic cells, chromosomes are composed of single molecule of DNA with many copies of five types of histones.
2. Histones are protein molecules and are rich in lysine and arginine residues, they are positively charged. Hence they bind tightly to the negatively-charged phosphates in the DNA sequence.
3. A small number of non-histone proteins are also present, these are mostly transcription factors. Transcription factors regulate which parts of DNA to be transcribed into RNA.
4. During most of the cell's life cycle, chromosomes are elongated and cannot be observed under the microscope.

5. During the S phase of the mitotic cell cycle the chromosomes are duplicated.
6. At the beginning of mitosis the chromosomes are duplicated and they begin to condense into short structures which can be stained and observed easily under the light microscope.
7. These duplicated condensed chromosomes are known as dyads.
8. The duplicated chromosomes are held together at the region of centromeres.
9. The centromeres in humans are made of about 1-10 million base pairs of DNA.
10. The DNA of the centromere are mostly repetitive short sequences of DNA, the sequences are repeated over and over in tandem arrays.
11. The attached, duplicated chromosomes are commonly called sister chromatids.
12. Kinetochores are the attachment point for spindle fibers which helps to pull apart the sister chromatids as the mitosis process proceeds to anaphase stage. The kinetochores are a complex of about 80 different proteins.
13. The shorter arm of the two arms of the chromosome extending from the centromere is called the p arm and the longer arm is known the q arm.

These chromosomes are primarily found in the nucleus of eukaryotic cells. But in certain animals like the Dipteran Insects during their developmental stages certain cells

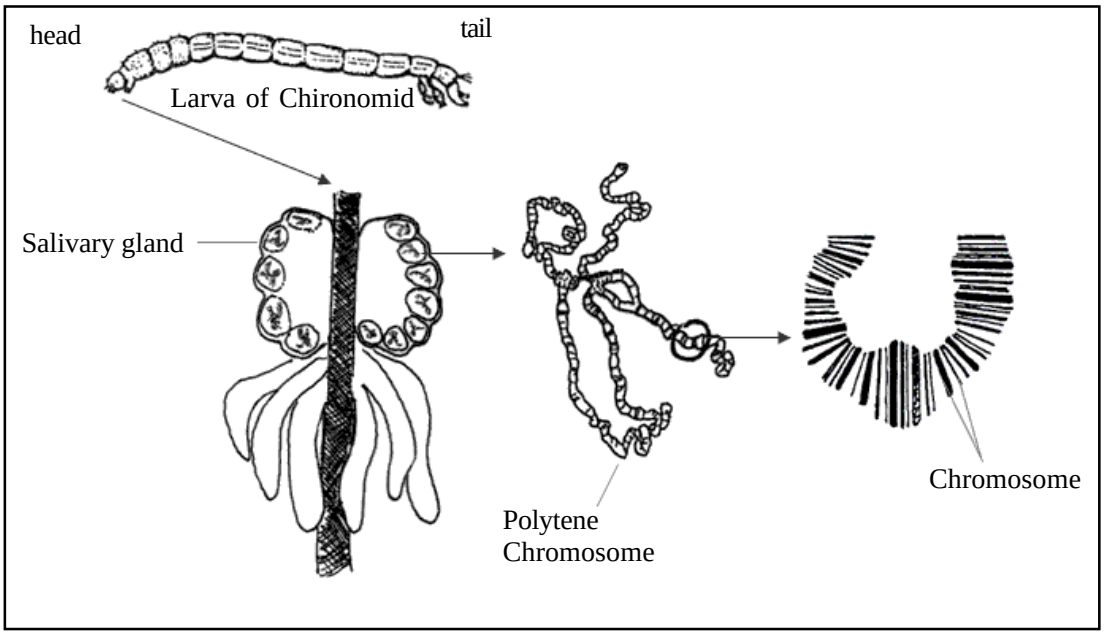


Fig. 1.2 : Chironomid Larvae Salivary gland and polytene chromosome (Source : cit.vfu.cz)

contain a large chromosome and these chromosomes are commonly known as giant chromosome. Giant chromosomes were first time observed by E.G. Balbiani in the year 1881 in nuclei of certain secretory cells (salivary glands) of Chironomas larvae (Diptera). However he could not conclude them to be chromosomes. They were conclusively reported for the first time in insect cells *(Drosophila)* by Theophilus Painter of the University of Texas in the year 1933. Since they were discovered in the salivary glands of insects they were termed as salivary gland chromosomes. The name polytene chromosome was proposed by Kollar due to the occurrence of many chromonemata (DNA) in them. Cells in the larval salivary gland of *Drosophila*, mosquito *and Chironema* contain chromosomes with high DNA content. However they may also occur in malphigian tubules, rectum, gut, foot pads, fat bodies, ovarian nurse cells etc. Polyteney of giant chromosomes happens by replication of the chromosomal DNA several times without nuclear division (endomitosis) and the resulting daughter chromatids do not separate but remain aligned side by side. During endomitosis the nuclear envelope does not rupture and no spindle formation takes place. The polytene chromosomes are visible during interphase and prophase of mitosis. They are about 100 times thicker contain 1000 to 2000 chromosomes, than the chromosomes found in most other cells of the organism. When stained and viewed under compound microscope at 40X magnification they display about 5000 bands. Polytene chromosomes are giant chromosomes common to many dipteran (two- winged) flies. They begin as normal chromosomes, but through repeated rounds of DNA replication without any cell division (called endomitosis), they become large, banded chromosomes. Polytene chromosomes are usually found in the larval stages, where it is believed these many-replicated chromosomes allow for much faster larval growth than if the cells remained diploid and contain normal chromosome. Simply because each cell now has many copies of each gene, it can transcribe at a much higher rate than with only two copies in diploid cells. In polytene chromosomes, condensed (bands), decondensed (interbands), genetically active (puffs), and silent (pericentric and intercalary heterochromatin as well as regions subject to position effect variegation) regions were found and their features were described in detail. Analysis of the general organization of replication and transcription at the cytological level has become possible using polytene chromosomes.

1.3 Materials and Methods

1.3.1 Materials

Following chemicals and glass goods are essential to prepare and study the polytene chromosomes :

l. Glacial Acetic Acid

2. Ethyl alcohol (Absolute)

3. Orcein stain

4. Glass slides

5. Cover glasses

6. Fine forceps

7. Fine needle

8. Watch glass

9. Sealing material (Nail polish or paraffin)

10. Dissecting binocular

11. Chironomus larvae or Drosophila larvae

1.3.2 Reagent Preparation

Fixative preparation : Fixative is kind of chemicals which fixes and preserves the cells or animals itself against decomposition and autolysis. In this experiment Aceto- alcohol will act as a fixative. It is prepared by mixing equal volume of glacial acetic acid and absolute ethyl alcohol at the ratio 1:3.

Stain preparation : 02 grams of Orcein powder is dissolved in 100 ml of glacial acetic acid (Remember the orcein powder is a very costly item and do not waste it.)

1.3.3 Protocols

1. The small head is brownish in colour with simple eyes and movable mouthparts. The tail region is characterized by the presence of tufty gills.
2. Hold the bloodworm firmly at a short distance behind the head with a pair of extra fine forceps under a dissecting binocular.

3. Then, with a downward and forward movement of another pair of forceps, pull the head end and separate it from the rest of the body. The salivary gland will generally drawn out completely with the head and can easily be recognized as two flat and whitish structures, attached to the anterior of the gut, with large cells and prominent nuclei.
4. After isolation of salivary glands add few drops aceto-alcohol(fixative) for 15-20 minutes.
5. Add a drop of aceto-orcein stain on the slide. Leave the gland in the aceto-orcein stain for about 30 minutes. If the gland begins to dry up, add another drop of stain.
6. Place a cover slip over the gland in the drop of stain. Lay a paper towel on the desk, turn the slide over, and lay it face down on the towel. Press firmly on the back of the slide with the tip of the finger, so as to blot thoroughly.
7. Place the slide on the desk with cover slip on top, hold the edge of the slide steadily with one hand, and press on the cover slip intermittently with the wooden handle of a dissecting needle.
8. Seal with proper sealing material(Nail-polish).
9. Observation of the giant chromosomes under the microscope.

1.4 Characters of Polytene chromosomes

1. The large size of the chromosome is due to the presence of many longitudinal strands called chromonemata; hence the name polytene (many stranded).
2. They are about 0.5 mm in length and 20 μm in diameter.
3. The chromosomal strands are formed after repeated division of the chromosome in the absence of cytoplasmic division. This type of division is called endo mitosis.
4. The polytene chromosome contains two types of bands, dark bands and inter bands.

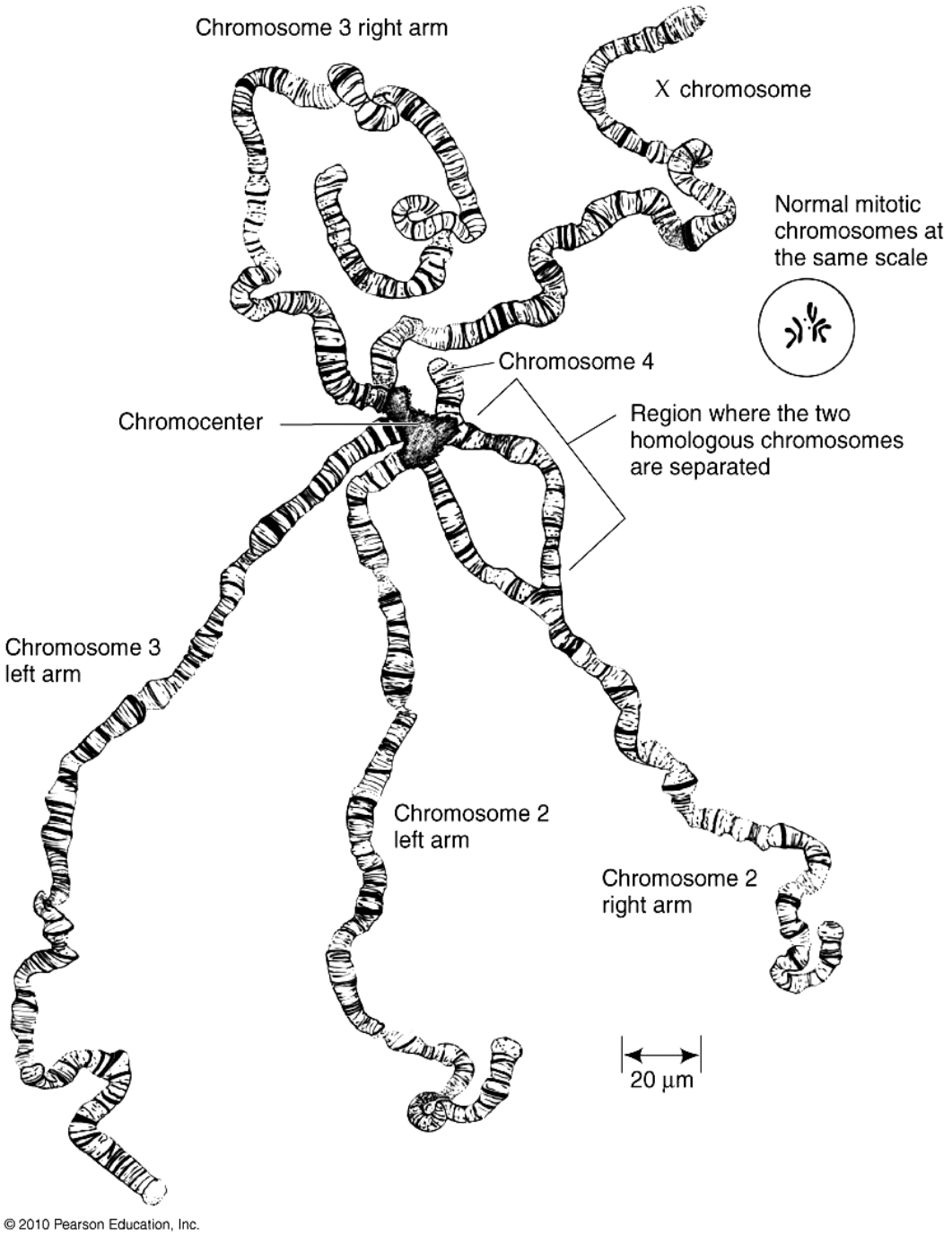


Fig. 1.3A : Polytene Chromosomes of Chironomous Larvae (from Salivary gland) (Source : mun.cal)

Fig. 1.3B : Polytene Chromosome under microscope (new.bhu.ac.in)

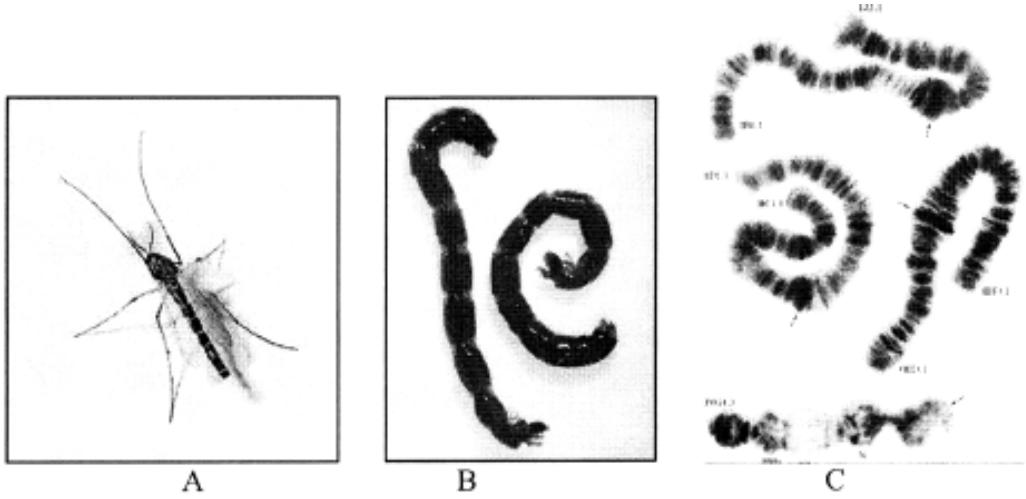


Fig. 1.4 : A. Chironomid (adult). B. Larvae C. Polytene chromosome, arrow indicates the centromere

5. The bands of polytene chromosomes become enlarge at certain times to form swellings called puffs.
6. The puffs indicate the site of active genes where mRNA synthesis takes place.

1.5 Questions

1. How the Chironomus Larvae Salivary glands have been extracted and prepared for observing polytene chromosome?
2. Describe the process of preparing and staining of polytene chromosome from Chironomus Larvae Salivary gland to observe under light microscope.
3. State the characteristics of the polytene chromose isolated from Chironomus Larvae observed under microscope?
4. Mention the reagents used to observe polytene chromosome from Chironomus Larvae.

Unit 2 □ Cell Division

Structure

2.0 Objectives

Cell division is one of the basic biological phenomene of living organism through which the life forms maintain their self-propagation and growth. In this unit learners are introduced with the following ideas.

1. Evidences of cell divisions among different organisms under light microscope.
2. Studying structural changes of a cell, its nucleus and chromosomes during the process of cell division.
3. Observing and studying different types and stages of the cell division processes.
4. Understanding the significances of different types and stages of cell divisions with their biological significances.
5. To provide practical knowledge and expertize to observe cell divisions.
6. Overall, this unit will provide a clear concept and understanding on cell division in enkaryotes and practical methodology to visualize and analyse them at the primary level.

2.1 Introduction

All cells of the living body follows the rules of the cell cycle and will divide either to increase of cell numbers for growth and development purposes or for the production of specialized reproductive cells. Some cells will never divide and they are known as super specialised cells like nerve cells. In the cell cycle there are four phases which are S phase, M phase, G_1 phase and G_2 phase. The divisional phase is M phase where the cell will divide either through mitosis or through meiosis. Mitosis is also known as equational division that means the daughter cells produced are quite alike with that of the mother cells particularly in respect to the chromosomal number. All the somatic cells will divide through mitosis. Meiosis cell division will occur only in the germ line cells for the production male and female gamete. This cell division is also known as reduction division that means the daughter cells produced are containing the half of the chromosome number in comparison to the mother cells and these daughter cells are also known as haploid cells. Although there

are two kinds of cell divisions but the mechanism of division is more or less same in both the cases. The only difference is that mitosis cell division consists of only one phase of cell division and after division there are only two daughter cells produced while in case meiosis it consists of two phases of cell divisions, meiosis-I and meiosis-Il, and produced four number of daughter cells from one mother cell. The mitosis cell division will require less time than meiosis cell division to complete the division. Both the cell division will occur in two major phases which are karyokinesis and cytokinesis. Karyokinesis means the division of nucleus while cytokinesis means the division of cytoplasm. Karyokinesis can again be divided into four sub phases which are prophase, metaphase, anaphase and telophase. These divisions of karyokinesis phase is done by the morphological changes of the cell during the time of cell division. In true sense there is no compartmentalisation during the time of cell division.

2.2 Study of Mitosis from Bone Marrow of Goat (*Capra hircus*)

2.2.0 Objective

To study the stages of somatic cell division in mammalian specimen.

2.2.1 Introduction

Before going into the detailed experimental procedure first we have to understand why the bone marrow? The bone marrow is the only tissue where all the cells are dividing through mitosis in all the time. Now the question Why? You must remember that bone marrow is actually a hematopoietic tissue that means here all the blood cells are formed and that's why this tissue requires mitosis cell division all the times. This is the only cause why we will take the bone marrow for the study of mitosis. Similarly in case of plant the root and shoot tips can be taken for the study of mitosis. Not only that the cells from the epithelium of fish gills and also from tail of the growing tadpole larvae of frog are also good sources for the study of mitosis.

2.2.2 Materials and Methods

The common Bengal variety goat, *Capra hircus* (Class: Mammalia; Order: Artiodactyla; Family: Bovidae; Subfamily: Caprinae; Genus: *Capra;* Species: *Capra hircus)* constituted the material for the present study.

1. Small 4 inch pieces of rib bones were collected from just slaughtered healthy goat as material and kept in cold (8°C) HBSS in an ice box and transported to the laboratory as quickly as possible.
2. The marrow was extracted by pressing the tips of the rib bones with the help of a bone cutter.
3. The bone marrow was flushed gently in HBSS along with 0.04% colchicines (0.5m g/l0 ml HBSS) as metaphase arrester and incubated for an hour in an incubator at 38°C.
4. The suspension was then centrifuged at 1000 rpm. for 10 min. The supematant was discarded and the palette was re-suspended in 75 mM potassium chloride (Analar) solution for hypotonic exposure and kept in an incubator at 37°C for 20 min.
5. The material was then centrifuged, supernatant was discarded and the palette was fixed slowly in chilled methanol-acetic acid (3:1 v/v) fixative and kept in refrigerator for 20 min. The process was repeated for 5 rounds.
6. The properly fixed cells were spread on clean grease-free slides by conventional air dry technique.
7. The cells spread on the slide were stained in Giemsa stain, diluted in phosphate buffer at pH 6.8. The entire process has been depicted in the form of flow chart.
8. Properly stained cells were then observed under microscope.
9. A careful observation of diploid cells revealed that all the chromosomes were of identical shape with a near terminal centromere.
10. Male animals with 58 autosomes and a single X chromosome and only one Y chromosome while female with the similar number of autosomes but there were 2X chromosomes.

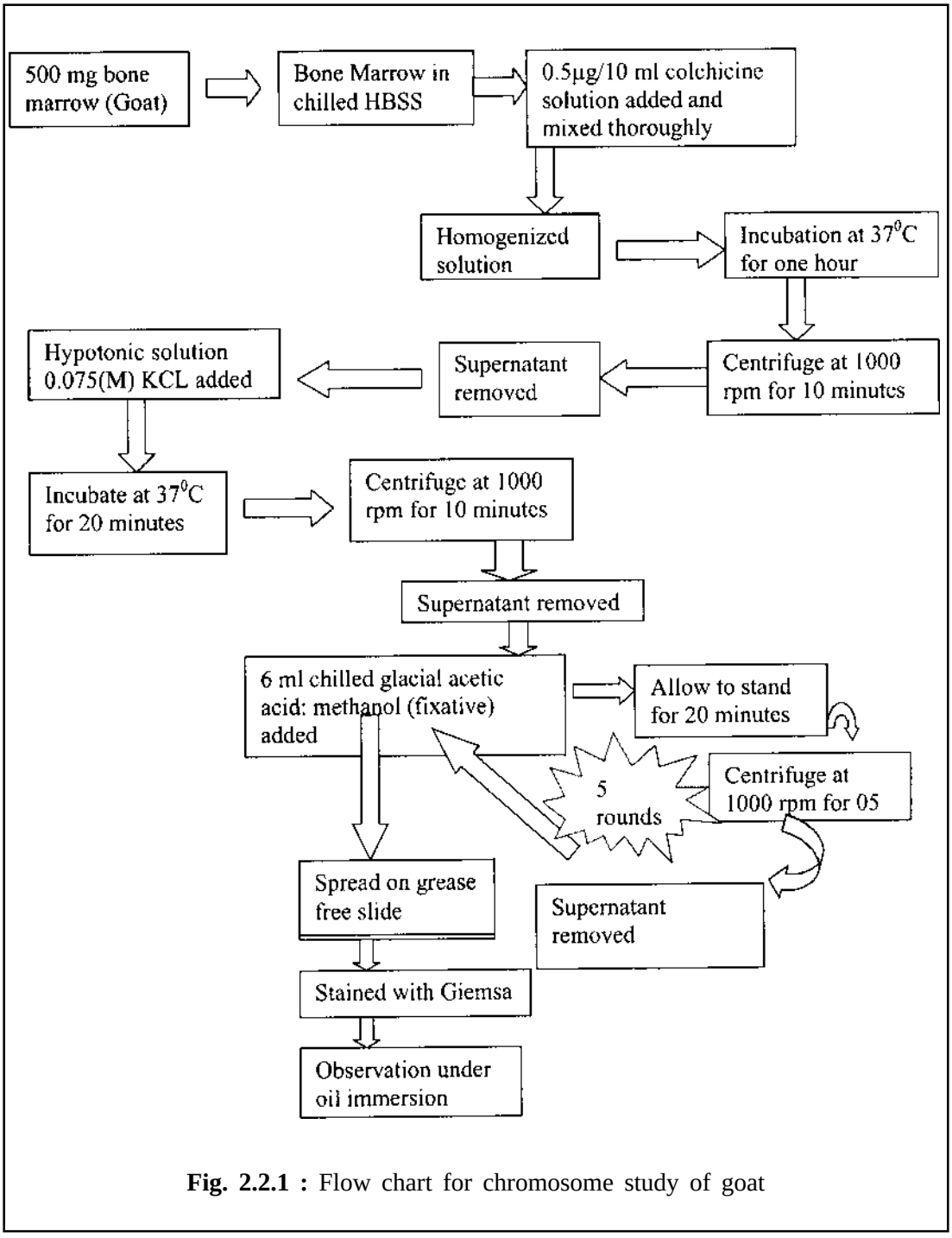


Fig. 2.2.1 : Flow chart for chromosome study of goat

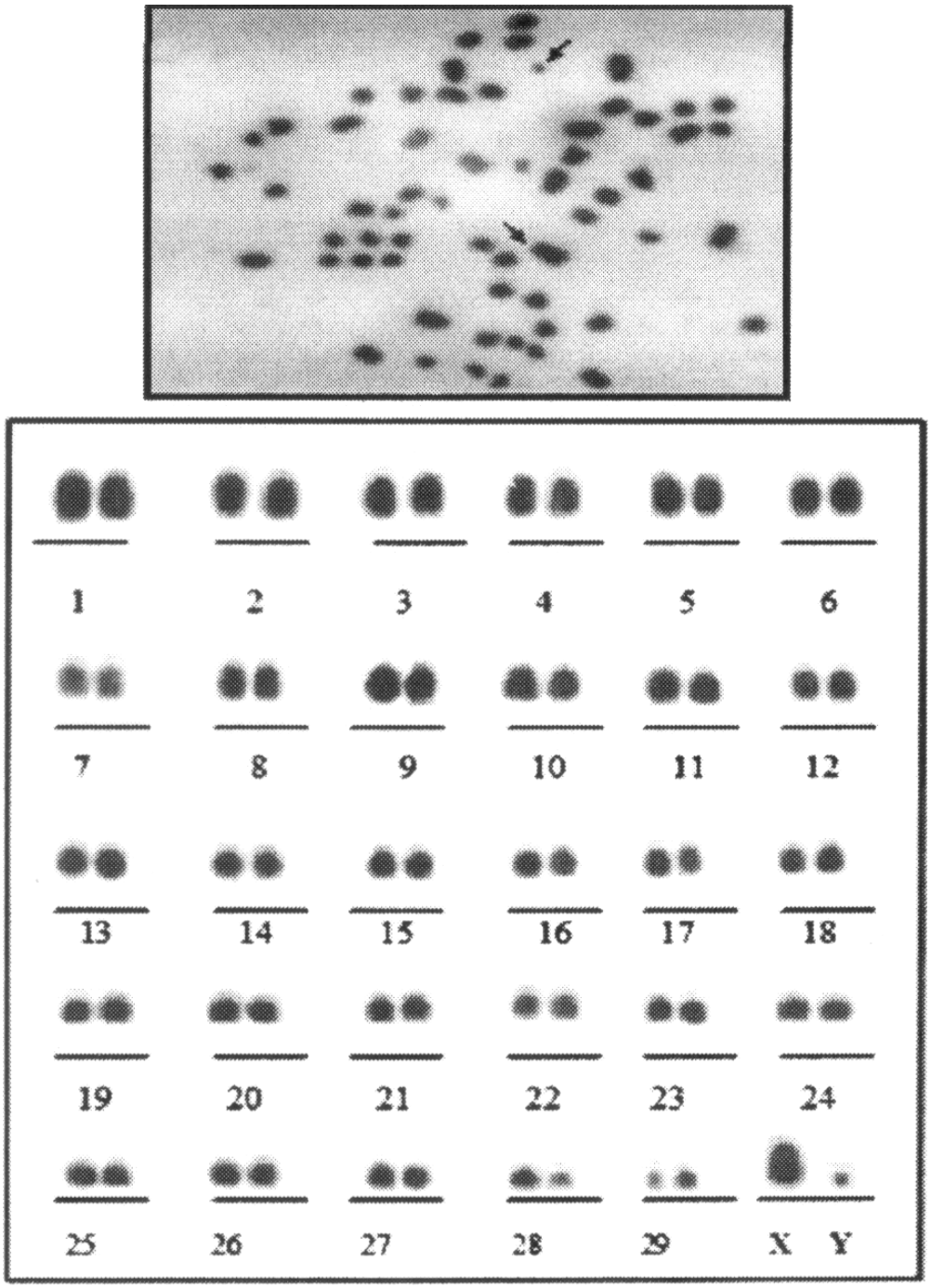


Fig. 2.2.2 : Metaphase spread from male goat bone marros cells showing 2*n*=60 acrocentric chromosomes. The arrow indicates the position of sex chromosomes.

2.2.3 Observations

The stages of mitosis can be broadly put into two events: karyokinesis (division of nucleus) followed by cytokinesis (division of cytoplasm, and ultimately of the cell). Those cells, which are not in the phases of cell division are considered to be in interphase. You will observe that most of the cells in a particular microscopic field are in interphase.

Interphase

The cells are mostly rectangular, oval or even circular in shape, with almost centrally situated densely stained nucleus. The chromatic (colored) material of the nucleus is homogeneous and looks granular. The boundary of the nucleus is distinct. One or few nucleoli (Sing: nucleolus) can also be observed inside the nucleus.

Prophase

Intact nuclear outline is seen. The chromatin (seen as a homogeneous material in the nucleus at interphase) appears as a network of fine threads(chromosomes). Nucleoli may- or may not be visible. If the cell under observation is in the early stage of prophase then the chromatin fibres (chromosomes) are very thin. However, in the cells at late prophase, comparatively thicker chromatin fibres would be visible. Besides this, in the late prophase the nuclear membrane may not be noticed.

Metaphase

The nuclear membrane disappears. Chromosomes are thick and are seen arranged at the equatorial plane of the cell. Each chromosome at this stage has two chromatids joined together at the centromere, which can be seen by changing the resolution of the microscope. Nucleolus is not observed during metaphase.

Anaphase

This stage shows the separation of the chromatids of each chromosome. The chromatids separate due to the splitting of the centromere. Each chromatid now represents a separate chromosome as it has its own centromere. The chromosomes are found as if they have moved towards the two poles of the cell. The chromosomes at this stage may look like the shape of alphabets ‘V’,’J’ or ‘I’ depending upon the position of centromere in them. Different anaphase cells show different stages of movement of chromosomes to opposite poles, and they are designated to represent early, mid and late anaphase.

Telophase

Chromosomes reach the opposite poles, lose their individuality, and look like a mass of chromatin. Nuclear membrane appears to form the nuclei of the two future daughter cells.

2.2.4 Comments

Present method is a cheaper and easier method for observing somatic cell division stages. This method can be used as prognostic and diagnostic measures of farm animals and human diseases which are related to somatic cell division and chromosomal abnormalities.

2.2.5 Questions

1. State the materials used to prepare the bone marrow of goat for observing mitosis.
2. Describe the methodology to observe mitosis from goat bone marrow. Use a schematic diagram or flowchart to state the protocal.
3. What is the appearance of interphase of mitotic cells from goat bone marrow?
4. Who the metaphase stage can be differentiated from anaphase stage in mitotic cells of goat bone marrow?
5. Mention the probable applications of this practical knowledge.

2.3 Study of Mitosis from Onion (*Allium cepa*) Root Tip

2.3.0 Objective

This study is performed to observe the mitosis cell division stages in the plant cells.

- Onion is a monocoty ledonous plant which possesses large chromosomes in the meristematic cells of root tip. Hence, the cell division stages with chromosomal structure is clearly visible.

2.3.1 Introduction

Cell division in flowering plants takes place in particular regions of the plant called meristems. Cells in meristems are not specialized for any particular function and divide repeatedly by mitosis. Some of the daughter cells remain meristematic others cease dividing and become differentiated into appropriate cell types depending on their position. The root tip meristem is usually a denser white and more rounded than the cut end.

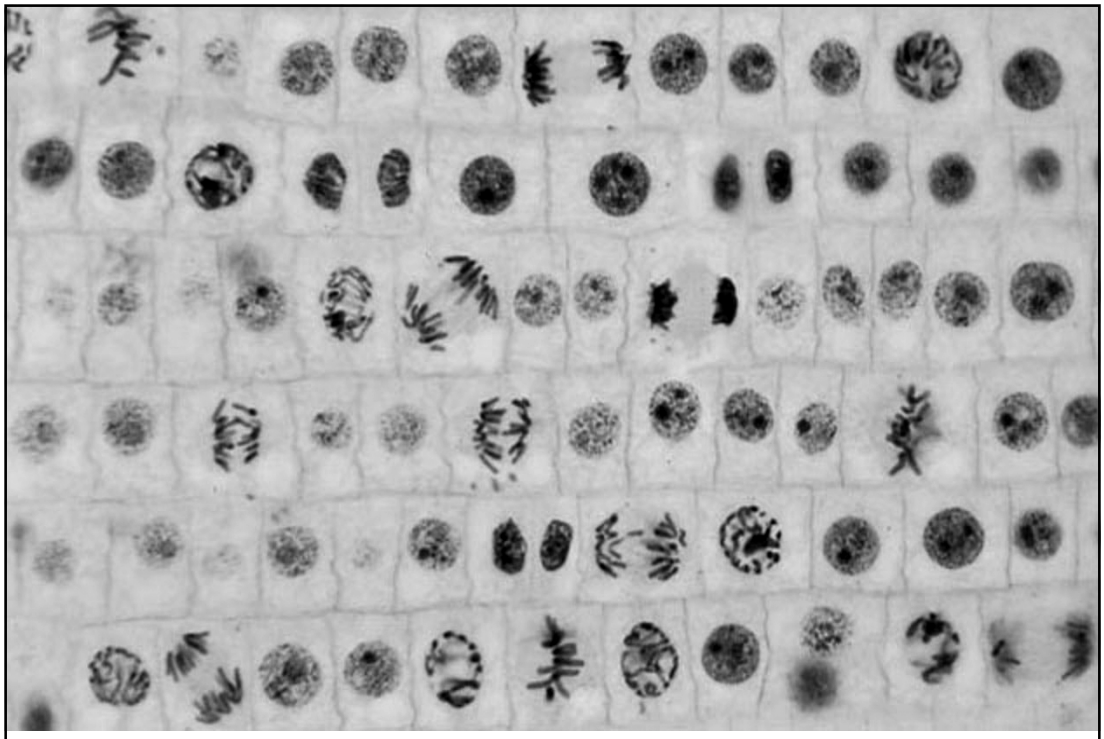


Fig. 2.3.1A : Cell division stages of onion root tip tissue (alamy.com)

Chromosomes in root tip tissue are made visible with the stain. Dividing cells (if present) will show up clearly with chromosomes in different forms according to the stage of mitosis. Individual chromosomes (as tightly-coiled threads) are visible during anaphase. The links between the cellulose walls of plant cells are broken down by the treatment with hydrochloric acid. This ensures that the stain can penetrate the cells and allows the tissue to be squashed out one cell thick.

An onion root tip is a rapidly growing part of the onion and thus many cells will be in different stages of mitosis. The onion root tips can be prepared and squashed in a way that allows them to be flattened on a microscopic slide, so that the chromosomes of individual cells can be observed easily. A process by which a parent cell divides into two or more daughter cells is called cell division. Cell division is a small part of the cell cycle. In normal eukaryotic cells, the type of cell division is known as mitosis. Another type of cell division is present in reproductive cells of eukaryotes and is known as meiosis. Cell cycle is mainly classified into two segments: M-phase and Interphase. Interphase is the longer period of cell division. During this phase the cell prepares for its next stage. This

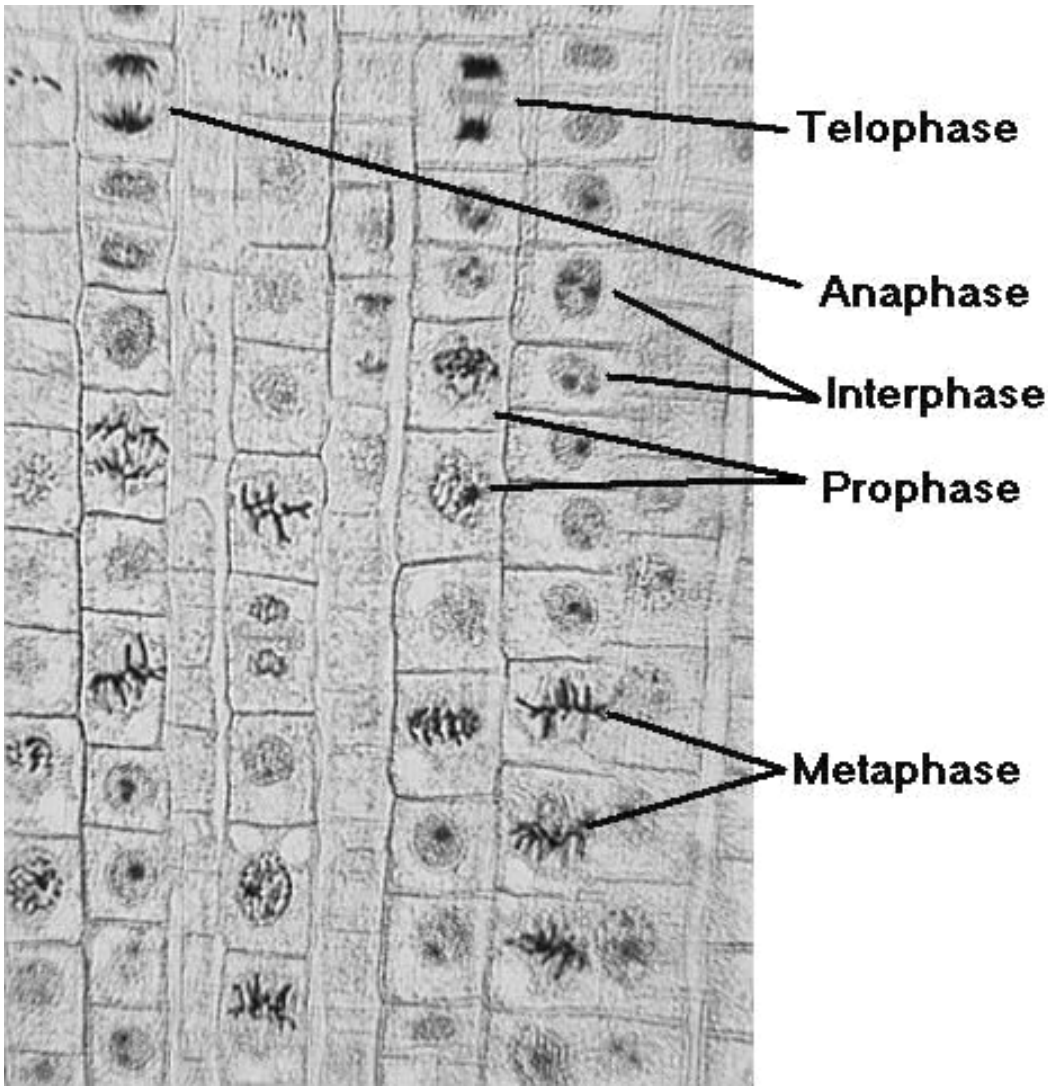


Fig. 2.3.1B : Cell division stages of onion root tip tissue (quora.com)

is a period of diverse activities and these activities are a prerequisite for the next mitotic phase. Interphase is mainly divided into three phases: G1 phase, S phase and G2 phase. S phase is the period of replication. G1 and G2 are the two gap phases during which the cell grows, producing proteins and preparing the cells. These phases also have certain check points and the whole cell cycle is strictly regulated. M phase of the cell cycle stands for Mitosis or nuclear division. In eukaryotes, DNA replication is followed by a process called mitosis which separates the chromosomes in its cell nucleus into

two identical sets, in two individual nuclei. Mitosis is followed by cytokinesis. The process of Mitosis is divided into four phases: Prophase, Metaphase, Anaphase and Telophase.

2.3.2 Materials and Method

1. Cut off the last 6 mm (1/4 inch) of root tip from sprouting onions. Place 5 of them in the labeled Eppendorf-tube.
2. Add 01 ml Carnoy's fixative and make sure that all tips are immersed.
3. Close tube and incubate for 24 hours.
4. Remove your root tips from the Carnoy's fixative and immerse in a new tube filled with 1 ml 1N HCl. Incubate for 12 minutes at 60°C.
5. Remove the HCI with a Pasteur pipette and discard in the drain with running cold tap water.
6. Add 0.5 ml Feulgen stain. (Watch out: this stain does not look brightly colored, but stains strongly - keep it away from your clothes, books, etc)
7. Let the root tip stain in Feulgen for about 10 minutes, or until the very tip of the root shows distinct dark coloring.
8. Put one drop of 45% Glacial Acetic Acid on the slide.
9. Place the root tip in the Glacial Acetic Acid on the slide. With a scalpel or razor blade, remove all but the red-stained very tip of the root.
10. Add the cover slip on top of the root tip.
11. Place the slide on a white piece of paper on your bench. Tap gently and straight down with the eraser of a pencil until the stained tip is spread out to a faint purple monolayer. Do not smear the cover slip sideways - this will shear the chromosomes.
12. Examine your spread under the microscope at low power to ensure that the cells are spread to a monolayer. If not, squish the cover slip some more.
13. Once you have spread your cells into a nice monolayer, switch to oil immersion.
14. Spend some time identifying the different stages of the cell cycle visible in your root section squashes. Illustrate examples of each mitotic stage (prophase, metaphase, anaphase, and telophase).

The stages of mitosis can be broadly categorised into two parts: karyokinesis(division of nucleus) followed by cytokinesis (division of cytoplasm, and ultimately of the cell). Those cells, which are not in the phases of cell division are considered to be in interphase.

2.3.3 Observations

The following stages can be observed with following characters under the microscope.

Interphase

1. The cells are mostly rectangular, oval or even circular in shape, with almost centrally situated densely stained nucleus.
2. The chromatic (coloured) material of the nucleus is homogeneous and looks granular. The boundary of the nucleus is distinct. One or few nucleoli (sing: nucleolus) can also be observed inside the nucleus

Prophase

1. Intact nuclear outline is seen.
2. The chromatin (seen as a homogeneous material in the nucleus at interphase) appears as a network of fine threads (chromosomes).
3. Nucleoli may-or may not be visible

Metaphase

1. The nuclear membrane disappears.
2. Chromosomes are thick and are seen arranged at the equatorial plane of the cell.
3. Each chromosome at this stage has two chromatids joined together at the centromere.
4. Nucleolus is not observed during metaphase.

Anaphase

1. Separation of the chromatids of each chromosome occurs. The chromatids separate due to the splitting of the centromere.
2. Each chromatid now represents a separate chromosome as it has its own centromere. The chromosomes are found as if they have moved towards the two poles of the cell.

3. The chromosomes at this stage may look like the shape of alphabets 'V','J' or 'I' depending upon the position of centromere in them.
4. Different anaphase cells show different stages of movement of chromosomes to opposite poles, and they are designated to represent early, mid and late anaphase.

Telophase

l. Chromosomes reach the opposite poles, lose their individuality, and look like a mass of chromatin.
2. Nuclear membrane appears to form the nuclei of the two future daughter cells.

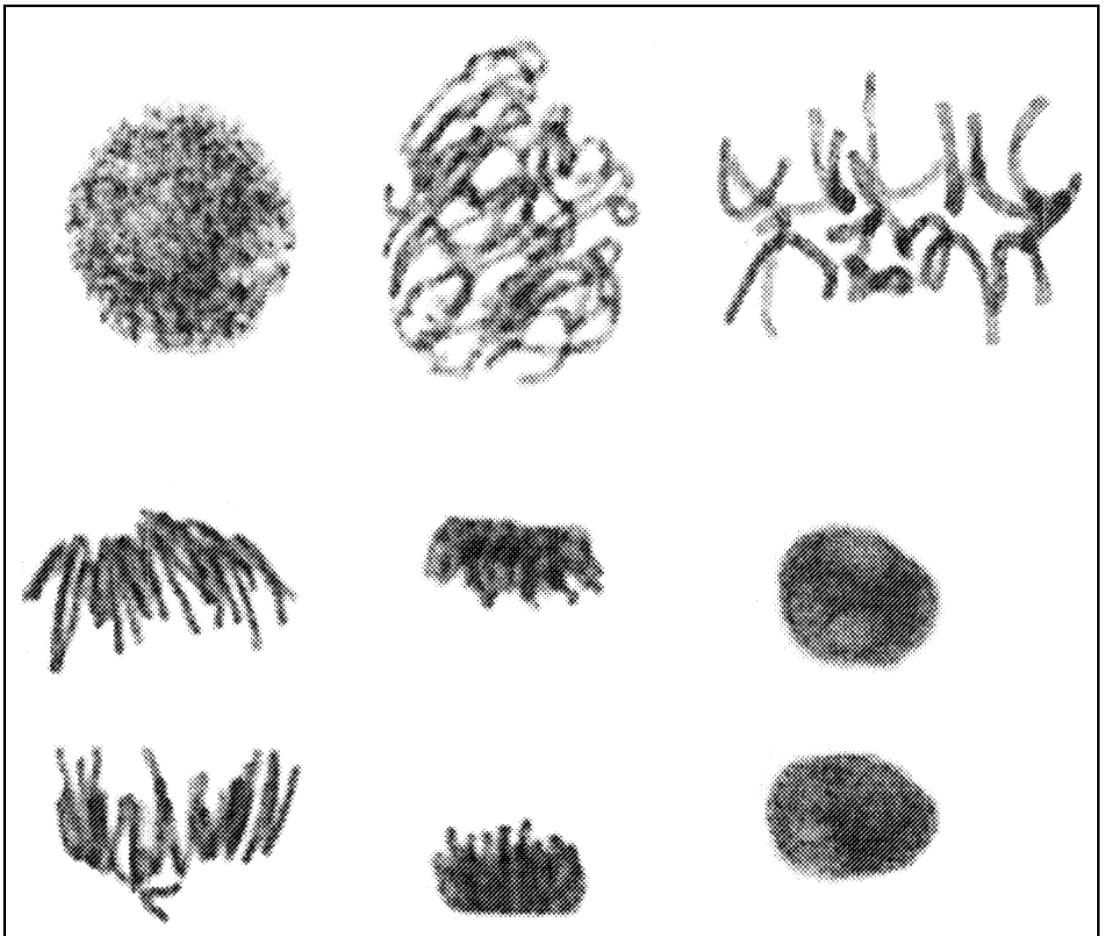


Fig. 2.3.2 : From top left Inetrphase, Prophase, Metaphase
From bottom left Anaphase, Telophase and Late telophase

Cytokinesis

1. In plants, a cell plate is formed in the middle after telophase. The plate can be seen to extend outwards to ultimately reach the margin of the cell and divide the cell into two.

2.3.4 Comments

1. Onion root-tip cells have a cell cycle of approximately 24-hour duration, i.e., they divide once in 24 hours, and this division usually takes place about two hours after sunrise. Therefore, roots grown on water should be cut only at that time to score maximum number of dividing cells.
2. Cell plates are characteristic of plant cells. However, in an animal cell, the two sides of the cell show in pushings or constrictions formed from the peripheral region in the middle of the cell, which grow inward and meet to divide the cell into two daughter cells.

2.3.5 Questions

1. State the method of preparing onion root tip to observe the cell division stages.
2. What are the observable features at different stages of onion root tip division—state with diagramatic representations.
3. Why onion root tip and goat bone marrow are prepared for observing mitotic cell division—justify with reasons.
4. Suggest names of a few tissues, which are suitable for the study of mitosis.
5. Why is mitosis also known as equational division?
6. What shape would a metacentric and a sub-metacentric chromosome exhibit during the anaphase stage?
7. How does cytokynesis differ in plant and animal cells.

2.4 Study of Various Stages of Meiosis from Grasshopper Testis

2.4.0 Objectives

- Meiosis cell division is a reduction division where two of the division stages, i.e., Meiosis I and Meiosis II is observed. Such division stages with chromosomal positions and morphology are clearly visible in the grasshopper testis preparation. Therefore, this practical is done—
- to visualize the process of meiosis
- to make the squash preparation of sample tissue, and
- to observe different stages of Meiosis I and Meiosis II.

2.4.1 Introduction

Meiosis cell division occurs only in germ line cells i.e. within testis and ovary in animals. In plants it occurs in stigma of androlcium and gynoecium of flower in phanerogamic plants. Now the question why we used the grasshopper testis for the study of meiosis. Since the beginning of this century the chromosomes of the short horned grasshoppers (Family Acrididae) have been used for a vast number of cytological studies. These chromosomes present a number of advantages to the cytologist which are :

- ➢ They are large and relatively few in number.
- ➢ The range of chromosome lengths in the complement is such that each bivalent formed at meiosis can usually be individually identified according to its length.
- ➢ Chiasmata are very clear during diplotene and diakinesis thus allowing analyses of their structure, frequency, distribution and movement.
- ➢ Often the position of the centromere is marked by relatively denser staining (precocious condensation) at early diplotene.
- ➢ Besides these cytological advantages, the techniques involved in the preparation of slides of this material are quick and simple and therefore it is ideal for demonstrating the stages of meiosis to students.

2.4.1.1 Systematic position of Grasshopper

Phylum: Arthropoda

Subphylum: Mandibulata

Class: Insecta

Subclass: Pterygota

Order: Orthoptera

Family: Acrididae

Genus: *Gesonula*

Species: *punctifrons*

2.4.2 Materials and Methods

2.4.2.1 Materials

Aceto-carmine or acetoorcein stain,

Glass slide

Cover slip

Lab needle

Sample (Grasshopper testis follicles)

Antopipette

Pasteur pipettes

Watch glass

Razor blades

Dissection box

2.4.2.2 Methods/Protocols

1. The insects are chloroformed or etherised and then dissected in insect saline. The testes lie in a dorsal position in the anterior half of the abdomen and can be easily located by making a dorsal, longitudinal, abdominal cut. They can be identified by the orange-yellow fatty tissue that cover them. Once this is removed with dissecting needles (while still in the insect saline) each testis can be seen to consist of many follicles.

2. The best fixative for these preparations is 1:3 aceto-alcohol. The testis material is left in the fixative for at 15 minutes.
3. The material can also be stored before being stained either in the fixative or by transferring it to 70 per cent ethyl alcohol after fixation. It will keep satisfactorily for at least a year either at room temperature or, preferably, in a refrigerator.
4. Place a small drop (about 5 mm. in diameter) of the stain in the middle of a clean slide.
5. Take three or four testis follicles from the fixative (or alcohol), drain off excess moisture on a piece of blotting paper, and leave in the stain for 35minutes.
6. After this time the follicles are broken up by firmly tapping them with a metal or glass rod until there is only a suspension of small particles in the stain.
7. After any remaining large pieces of material have been removed, a clean cover-slip can be applied.
8. Heating the slide gently over the flame of a spirit lamp at this stage will flatten and spread the chromosomes. The stain must not boil.
9. The slide should then be placed between two pieces of blotting paper which is firstly pressed down lightly so that excess stain around the edges of the cover-slip is absorbed, and then the preparation is squashed by firm, vertical, thumb pressure.
10. Seal with proper sealing material (molten wax, nail polish)

2.4.3 Observations

The cells of testes are undergoing spermatogenesis which involves the process of meiosis. Meiosis-I and meiosis-II are continuous processes and have sub-stages. Hence, all the different meiotic divisions are observed such as Prophase-I which includes leptotene, zygotene, pachytene, diplotene, and diakinesis. Other stages of meiosis are Metaphase-I, Anaphase-I, Telophase-I, followed by second meiotic division i.e. Prophase-II, Metaphase-Il, Anaphase-H and Telophase-H.

2.4.3.1 Characteristic feature of Prophase-I sub phases

Leptotene

a. The chromosomes become distinct and appear as long and thin thread in the nucleus.
b. The condensation and thickening of chromosomes is marked by coiling of the thin thread.

c. Each chromosome consists of two chromatids held together by centromere but these are not easily visible.

Zygotene

a. Homologous chromosomes start pairing from one end. This pairing is known as synapsis.

b. Each pair of homologous chromosomes is called bivalent.

c. The chromosomes appear as loosely coiled threads.

d. Nuclear membrane and nucleolus both visible.

Pachytene

a. The chromosomes become shorter and thicker due to contraction.

b. Each paired unit the bivalent consists of four chromatids (hence known as tetrads).

c. Crossing-over occurs by the end of this stages i.e. break and exchange of partners occurs between non-sister chromatids.

d. The point of interchange and rejoining appears as (X-shaped) and is known as chiasma.

Diplotene

a. The homologous chromosomes begin to separate.

b. The two non sister chromatids of a homologous pair remain attached at one or two points (the chiasmata).

c. It is at the chiasmata that exchange of segment of chromatids (genes) between homologous chromosomes takes place. The process is known as genetic recombination.

Diakinesis

a. The homologous chromosomes of a bivalent move apart from each other and become more compact.

b. Nuclear membrane and nucleolus disappears.

c. Spindle formation is completed.

Metaphase-I

a. The Nuclear membrane and nucleolus have disappeared.

b. The bivalents arrange themselves at the equator.

c. The spindle fibres are attached at the centromere of the chromosomes.

Anaphase-I

a. The spindle fibres shorten.

b. The centromeres of homologous chromosomes are pulled along by the spindle fibres towards the opposite pole.

c. There is no division of centromere. Thus, half of the chromosomes of the parent nucleus go to one pole and the remaining half in the opposite pole.

d. Each set of chromosomes that moves to one pole consists of a mixture of parental and maternal chromosome parts.

Telophase-I

a. The separated chromosomes form the nuclei.

b. The daughter nuclei have half the number of chromosomes of the parent nucleus which had one pair of each chromosome (i.e diploid 2n).

c. The daughter nuclei are now called haploid (i.e having n no. of chromosome).

d. The nucleolus reappears and nuclear membrane is formed around each group of chromosomes.

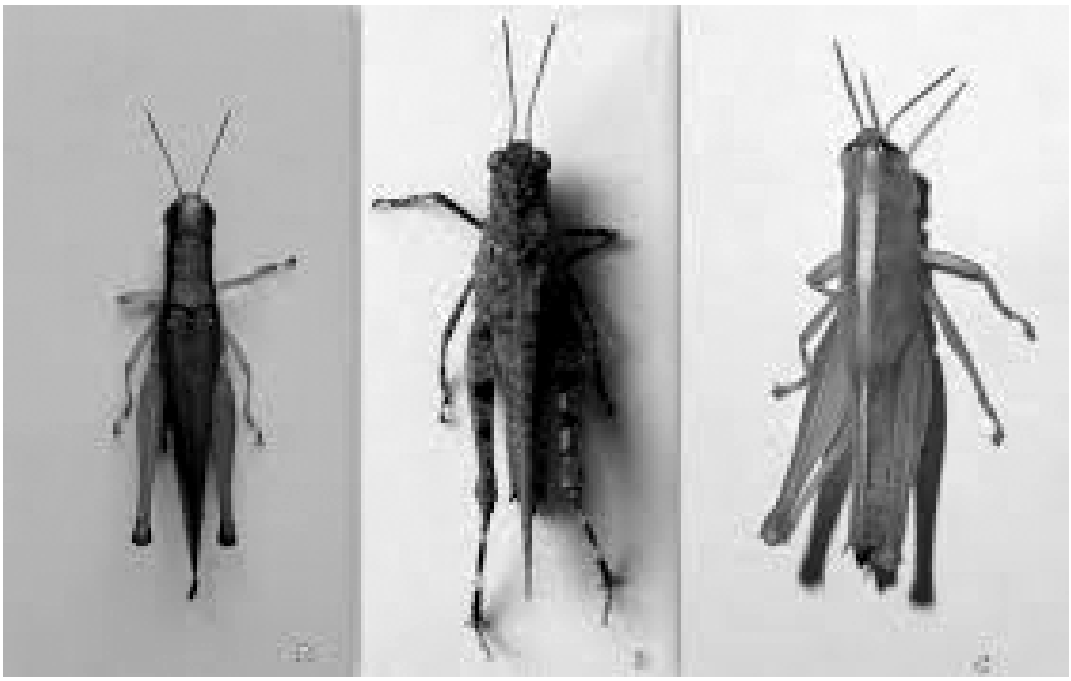


Fig. 2.4.1 : Different stages of Meiosis under microscope—
a) Zygotene, b) Pachytene, c) Diplotene, d) Diakinesis, e) Metaphase I

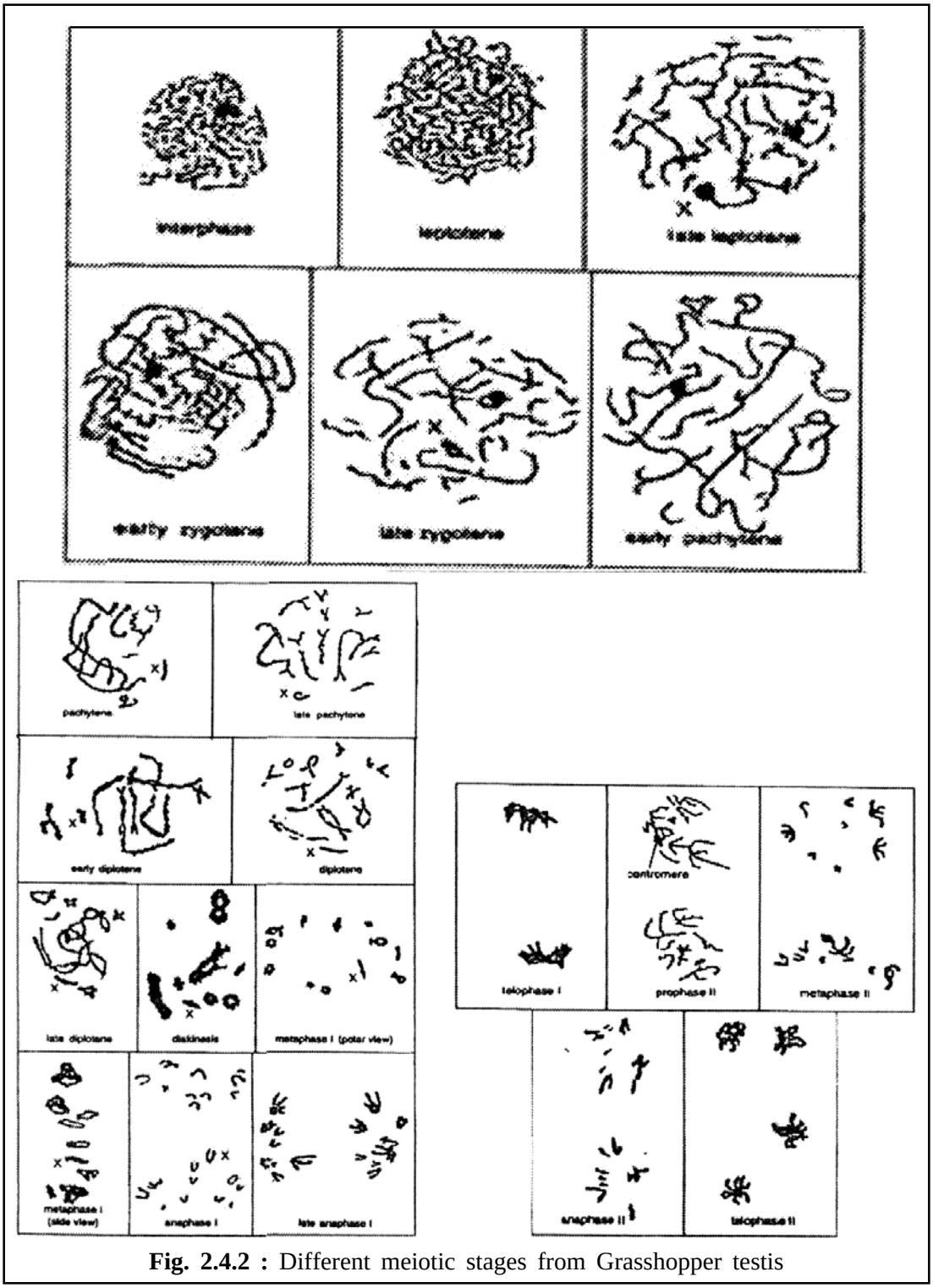


Fig. 2.4.2 : Different meiotic stages from Grasshopper testis

2.4.3.2 Characteristics feature of Meiosis-II cell divisions

Prophase II

a. The chromosomes shorten and reappear.

b. Formation of spindle starts.

c. The two chromatids are attached to the single centromere.

d. Nucleolus and nuclear membrane begin to disappear.

Metaphase II

a. The chromosomes arrange themselves along the equator.

b. Formation of spindle apparatus is completed.

c. The centromere of each chromosome is attached to the spindle fibre.

d. The centromere in each chromosome divides.

Anaphase II

a. The centromere splits and the sister chromatids move towards the poles along the spindle fibres (due to the shortening of fibres).

Telophase-II

a. On reaching the poles, the chromosomes organize themselves into haploid daughter nuclei.

b. The nucleolus and the nuclear membrane reappear.

Cytokinesis

May occur in two successive stages, once after meiosis-I and then after meiosis-II, or in some instances it occurs only after meiosis-II. This results in four haploid cells.

2.4.4 Comments

In this practical grasshopper has been selected for several reasons. Firstly, grasshopper is easily available in nature and can be captured and handled easily. Secondly and more importantly, it possesses few number of diploid chromosomes which are large and metacentric. Also with proper squash preparation the chromosomes are very clearly seen with clear view of chiasmata in diplotene and diakinesis.

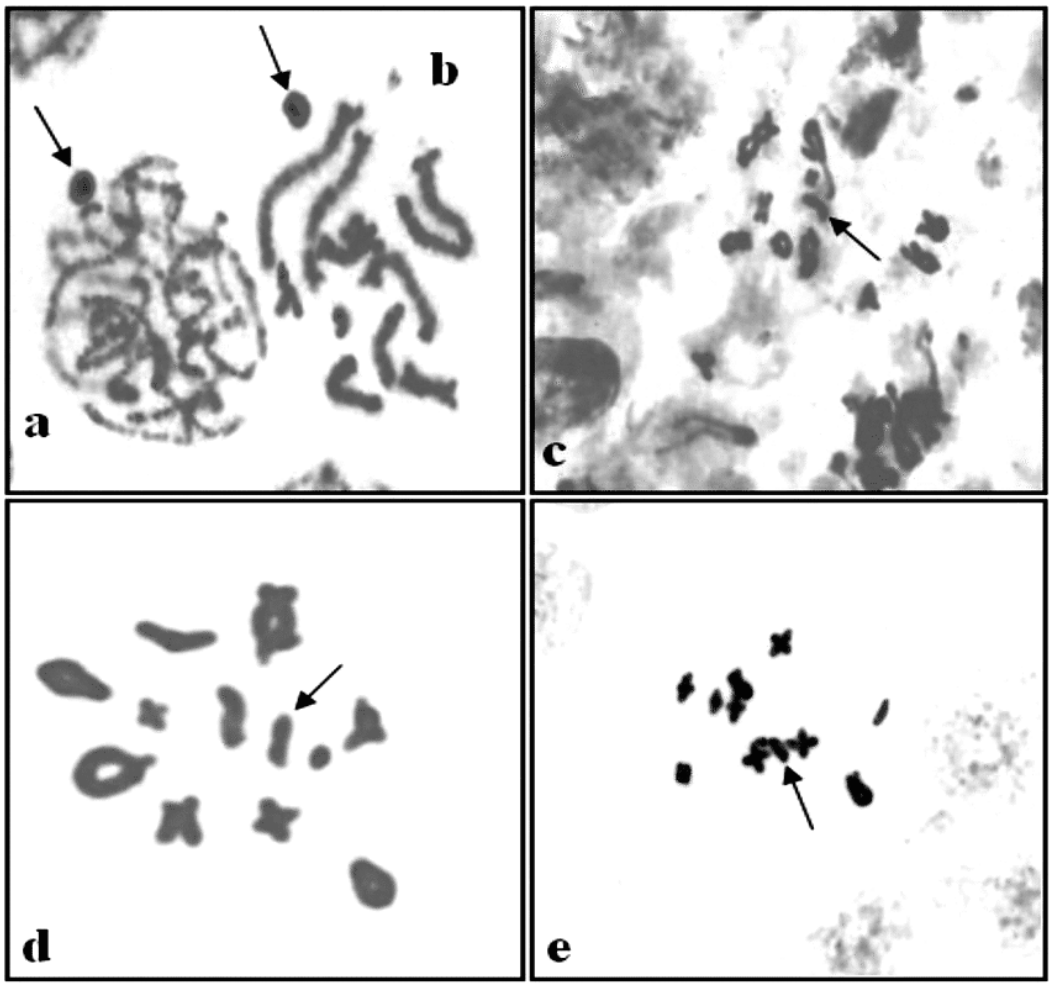


Fig. 2.4.3 : Different stages of Meiosis under microscope—
a) Zygotene, b) Pachytene, c) Diplotene, d) Diakinesis, e) Metaphase I

2.4.5 Ouestions

1. What are the fixative and preservative reagents used to prepar the grasshopper testis? Specify their activity.
2. State the procedure of grasshopper testis preparation to observe meiosis stages.
3. Mention the differences observed between different stages of 1st and 2nd meiotic divisions.
4. What are the observable differences between diplotene and pachytene?
5. Show with diagram that how metaphase I and II look different in polar and equatorial views?

6. What are the stages of Meiotic cell division.
7. What is Chiasmata? Where and when it is found?
8. Name the fixativs and stains used to visualize meiotic cell division stages from grasshopper testis.
9. Differentiate between : a) Metaphase I and II,
   b) Anaphase I and II.
10. How can you distinguish between diplotene and diakinesis.
11. Why grasshopper is a suitable model to study the meiotic cell division stages?
12. How the grasshopper testis is prepared to observe the meiotic stages?

2.5 Study of Various Stages of Meiosis from Mouse

2.5.0 Objective

- Present protocal is to study mammalian meosis that occure in the germ cells.
- This will help to leran the process of isolating and spreading spermatocytes onto the slides to prepare and observe meosis cell division stages.
- Preparation and staining chromosomal with Gimsa stain will be learnt.
- Cellular and chromosomal morphology of germ cells during meosis will be observed.

2.5.1 Introduction

Cell division is the basis for reproduction in all organisms. Cell divisions are of two types' mitosis and meiosis. Mitosis is the process of cell reproduction which involves the division of the nucleus to form two identical nuclei, followed by a division of the cytoplasm (i.e cytokinesis), which results in the two nuclei being separated into different cells. Meiosis occurs in sexually reproducing organism; a special division process called meiosis takes place. Unlike mitosis, meiosis results in the reduction of chromosome number; so that each daughter nucleus has half the number of chromosomes found in the somatic cells. Each haploid (n) nucleus produced through meiosis contains one chromosome of each pair found in diploid (2n) cells of the same organism.

In Meiosis, which is also known as 'reduction division' from the mice testis the normal chromosome number (2n) of parent cell is reduced to half (i.e n no. of chromosomes) in the daughter cells. It occurs in reproductive cells, e.g in the testis of male organism and in the ovaries of females to produce gamates. In meiosis the chromosome number is reduced to half in gamates so that when doubled at fertilization it once again becomes 2n or normal. If these gametes were produced by mitosis, the offspring developing from zygote would then have double the number of chromosome in the next generation. Every living organism has a definite number of chromosomes in its body cells (e.g. **Onion cell-16, potato-48, grasshopper-24, horse-64, man-46, mice-40, rat-42).**

Therefore, to keep the chromosome number constant the reproductive cells of the parents divide by this reduction division called Meiosis. Table showing different meiotic stages.

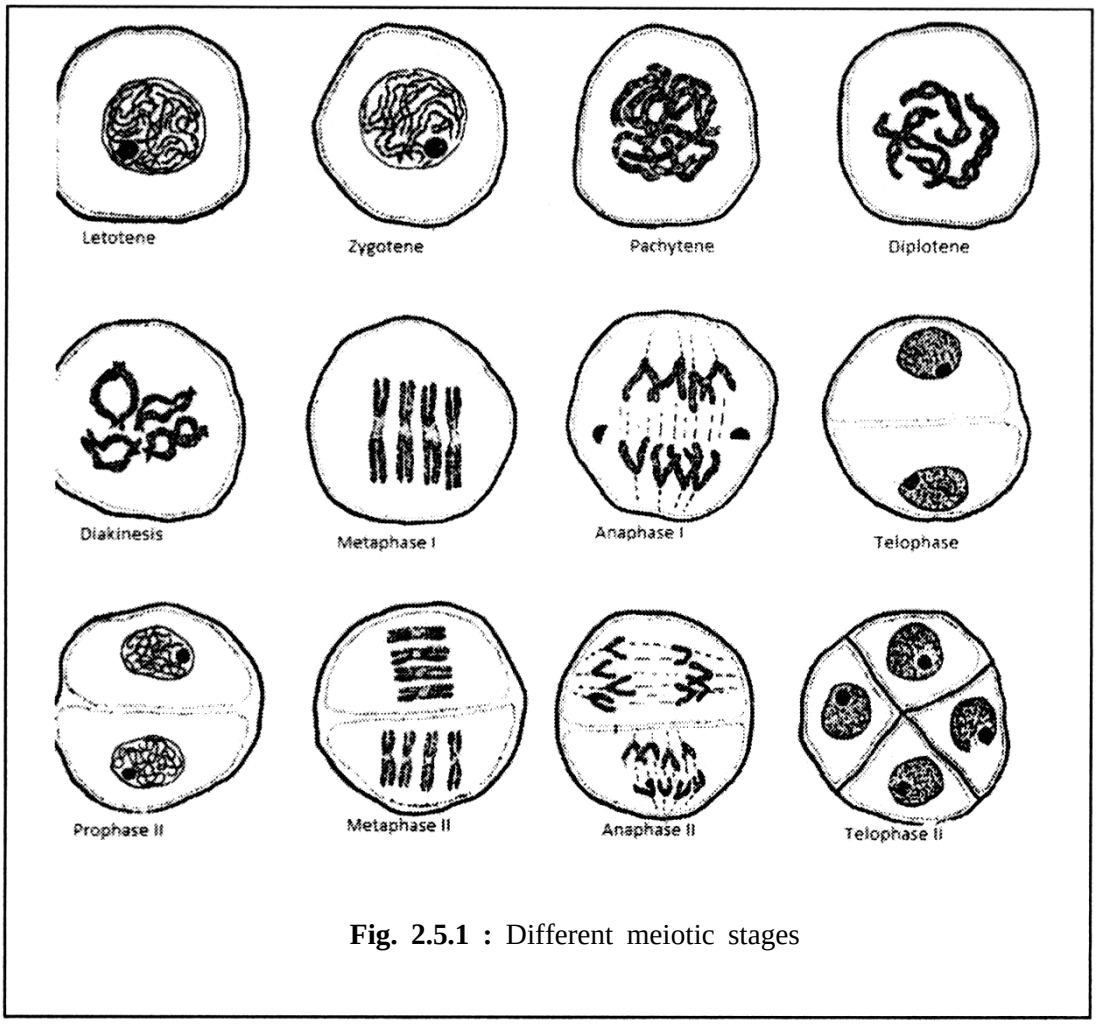


Fig. 2.5.1 : Different meiotic stages

2.5.2 Materials and Methods

2.5.2.1 Materials

1. Mice
2. 1.1% Sodium citrate (Hypotonic solution). l.lgms sodium citrate powder is dissolved in 100ml distilled water.
3. 2.2 % Sodium citrate (Isotonic solution). 2.2gms sodium citrate powder is dissolved in 100ml distilled water.
4. Fixative (l:3 Aceto-rnethanol)
5. 2% Giemsa stain. Take 19 mg Giemsa powder in 54ml glycerol. Stir and heat at 60°C for 1 hour. Cool and add 86ml Methanol to it and put the solution for

overnight in dark. Next day pour the solution in amber colour bottle. 1 ml of this Stock solution is taken in 50ml-distilled water to get 2% giemsa stain

6. Pasteur pipettes slides etc.

2.5.2.2 Methods

1. Dissect out the testis from rat or mice. Place the testis into cuvette containing isotonic solution i.e. 2.2% sodium citrate.
2. Clear out fat bodies and connective tissue layer i.e. tunica albuginea. With the help of scissors separate and tease out semineferous tubules.
3. After the separation step, allow the larger tissue pieces to settle.
4. Transfer the supernatant from cuvette to the centrifuge tube and avoid taking larger particles.
5. Add a little amount of isotonic solution and are centrifuged at 2000rpm for 15 minutes at room temperature.
6. Remove the supernatant is carefully using a Pasteur pipette without disturbing the pellet.
7. Add 5ml of hypotonic solution to the pellet with the Pasteur pipette.
8. Incubate the tube in water-bath for 20 minutes at 37°C. After that add 5ml of the fixative l:3 aceto-methanol to the tube and agitate thoroughly with the help of the pipette, so that bubbles appear.
9. Centrifuge at 2000 rpm for 15 minutes. Remove the supernatant carefully and discard it.
10. Again add fresh chilled fixative. Agitate the contents properly and centrifuge at 2000 rpm for 15 minutes.
11. Repeat for 2-3 washes of fixative with thorough mixing till the pellet is clear.
12. Add some more fixative to the pellet to prepare a proper cell suspension and agitate thoroughly with the help of the pipette, so that bubbles appear.

Slide preparation

1. Take 3-4 drops of cell suspension on a wet, clean, chilled slide the slide and flame dry it. Drain the extra solution on the slide and label it appropriately.
2. Let the slides cool at room temperature.
3. Now keep them in 2% giemsa for 3-5 minutes. Rinse in distilled water and observe under the microscope with oil immersion lens.

2.5.3 Observations

The cells of testes are undergoing spermatogenesis which involves the process of meiosis. Meiosis-I and meiosis-II are continuous processes and have sub-stages. Hence, all the different meiotic divisions are observed such as Prophase-I which includes leptotene, zygotene, pachytene, diplotene, and diakinesis. Other stages of meiosis are Metaphase-I, Anaphase-I, Telophase-I, followed by second meiotic division i.e. Prophase-II, Metaphase-II, Anaphase-II and Telophase-II.

Prophase-I

Leptotene or Leptonema

a. The chromosomes become distinct and appear as long and thin thread in the nucleus.

b. The condensation and thickening of chromosomes is marked by coiling of the thin thread.

c. Each chromosome consists of two chromatids held together by centromere but these are not easily visible.

Zygotene or Zygonema

a. Homologous chromosomes start pairing from one end. This pairing is known as synapsis.

b. Each pair of homologous chromosomes is called bivalent.

c. The chromosomes appear as loosely coiled threads.

d. Nuclear membrane and nucleolus both visible.

Pachytene or Pachynema

a. The chromosomes become shorter and thicker due to contraction.

b. Each paired unit the bivalent consists of-four chromatids (hence known as tetrads).

c. Crossing-over occurs by the end of this stages i.e. break and exchange of partners occurs between non-sister chromatids.

d. The point of interchange and rejoining appears as (X-shaped) and is known as chiasma.

Diplotene or Diplonema

a. The homologous chromosomes begin to separate.

b. The two non sister chromatids of a homologous pair remain attached at one or two points (the chiasmata).

c. It is at the chiasmata that exchange of segment of chromatids (genes) between homologous chromosomes takes place. The process is known as genetic recombination.

Diakinesis

a. The homologous chromosomes of a bivalent move apart from each other and become more compact.

b. Nuclear membrane and nucleolus disappears.

c. Spindle formation is completed.

Metaphase-I

a. The Nuclear membrane and nucleolus have disappeared.

b. The bivalents arrange themselves at the equator.

c. The spindle fibres are attached at the centromere of the chromosomes.

Anaphase-I

a. The spindle fibres shorten.

b. The centromeres of homologous chromosomes are pulled along by the spindle fibres towards the opposite pole.

c. There is no division of centromere. Thus, half of the chromosomes of the parent nucleus go to one pole and the remaining half in the opposite pole.

d. Each set of chromosomes that moves to one pole consists of a mixture of parental and maternal chromosome parts.

Telophase-I

a. The separated chromosomes form the nuclei.

b. The daughter nuclei have half the number of chromosomes of the parent nucleus which had one pair of each chromosome (i.e diploid 2n).

c. The daughter nuclei are now called haploid (i.e having n no. of chromosome).

d. The nucleolus reappears and nuclear membrane is formed around each group of chromosomes.

Meiosis-II

Prophase II

a. The chromosomes shorten and reappear.

b. Formation of spindle starts.

c. The two chromatids are attached to the single centromere.

d. Nucleolus and nuclear membrane begin to disappear.

Metaphase 11

a. The chromosomes arrange themselves along the equator.

b. Formation of spindle apparatus is completed.

c. The centromere of each chromosome is attached to the spindle fibre.

d. The centromere in each chromosome divides.

Anaphase 11

a. The centromere splits and the sister chromatids move towards the poles along the spindle fibres (due to the shortening of fibres).

Telophase-II

a. On reaching the poles, the chromosomes organize themselves into haploid daughter nuclei.

b. The nucleolus and the nuclear membrane reappear.

Cytokinesis

May occur in two successive stages, once after meiosis-I and then after meiosis-II, or in some instances it occurs only after meiosis-II. This results in four haploid cells.

2.5.4 Comments

2.5.4.1 Significance

It helps to maintain constant number of chromosomes in a species in sexual reproduction. Meiosis occurs during gamete formation (gametogenesis) and reduces the number of chromosomes from diploid (2n) to haploid (n) in the gametes. These haploid gametes fuse to form diploid zygote during fertilization. The diploid zygote develops into a normal diploid individual. Meiosis establishes new combination of characters due to:

1. Mixing of paternal and maternal chromosomes.

2. Crossing over during prophase 1. As a result, the progeny inherits traits of both mother and the father in a new combination.

2.5.4.2 Precautionary measure

1. The temperature of water-bath should be maintained at 37°C and hence should be checked periodically.
2. The fixative used here is, which should always be freshly prepared and chilled.

2.5.5 Questions

1. What are the reagents and stains used for observing meosis stages from grasshopper testis?
2. Mention the methodology of slide preparation through a flow-chart for observing meosis stages from grasshopper testis.
3. What is the cell type to observe meiosis stages from grass hopper testis tissue?
4. How can you distinguish between pachytene and diakinesis?
5. State the differences between telophase I and II.
6. What is the significance of prophase I in meiosis? —Explain in the light of your observation in the mouse testis preparation.

Unit 3 □ Study of Life stages of some Human Parasites

3.0 Objectives

The objectives of this unit are as follows—

- To develop ideas about the parasitic association of some organisms with the human body.
- To identify the parasites and their injective stages.
- To learn about parasitic behaviour and consequences of the organisms with proper identification.

3.1 Introduction

Parasitology is an important part of biology where the association between two organisms, that is, host and parasite, are being discussed. Parasitism is a form of ecological interaction, in which a member, the parasite, benifits from the use of resources gathered by another member, the host. Parasites have significant influences on host which affect its state of health, reproductive capacity and ability to obtain resources and survival quality. In contrary, host is like the habitat of a parasite, sustainability of which is dependent on the utilization or exploitation of parasite. Such animal association is dynamic and evolving.

Parasitic diseases are long been considered as serious public health problem around the world. They are particularly very relevant in the tropical countries like us. For this reason, etiological agents, biological life cycles, processes of infection and propagation as well as pathological manifestation are to be studied. Medical parasitology traditionally has included the study of three major groups of animals : parasitic protozoa, parasitic helminths (worms), and those arthropodes that directly cause disease or act as vactors of various pathogens. Many of the parasitic diseases may cause epidemic problems locally regionally or globally. Therefore, sufficient ideas an these parasitic diseases may also help to develop knowledge to create social awareness to improve human health.

In Unit 3 different protozoan and helminth parasites has been discussed which are endoparasitic in nature. In the next unit, i.e. Unit 4 some arthropode parasites, which are ectoparasitic in nature, has been presented to study.

3.2 *Entamoeba histolytica*

Entamoeba histolytica is an invasive, pathogenic protozoan, causing amoebiasis, and an important cause of diarrhoea in developing countries. Our understanding of its epidemiology has dramatically changed since this amoeba was distinguished from another morphologically similar one, *Entamoeba dispar*, a non-pathogenic and commensal parasite. These two species can now be distinguished mainly through molecular and immunological procedures. The life cycle of the parasite is represented by two forms: the cyst and the trophozoite. The cyst is the infective and non-motile form of the parasite. It is excreted in the faces and can survive for weeks in the environment. Mature cysts possess 4 nuclei and average 20 μm in diameter. The trophozoite is the motile form, with a size ranging from 10 to 60 μm. It colonizes the intestinal tract leading mainly to tissue destruction and secretory bloody diarrhoea.

Amoebiasis is basically an acute disease acquired by:

(i) ingestion of cysts present in contaminated food, water, or plants,

(ii) through person to person contact,

(iii) exposure in endemic areas, and

(iv) swimming in contaminated water. Clinical manifestations range from the asymptomatic carrier state to dysenteric symptoms represented by abdominal pain and bloody diarrhoea

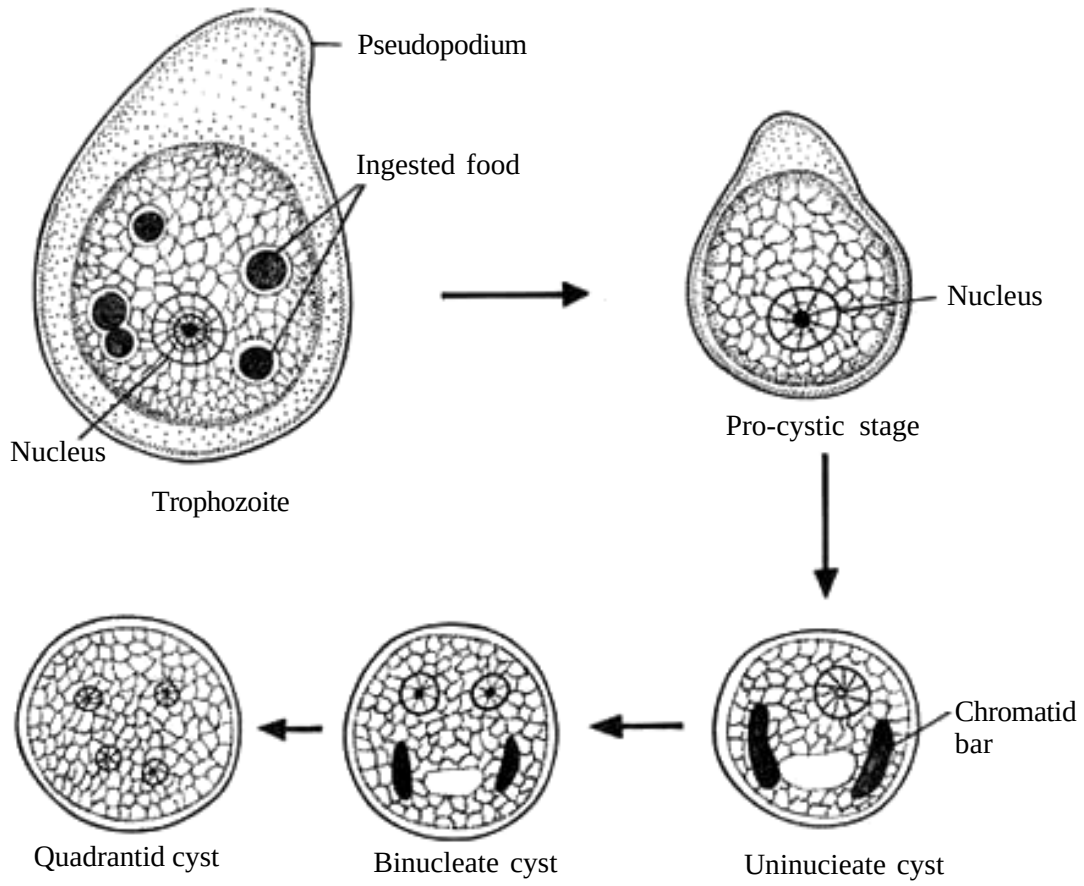


Fig. 3.1 : *Entamoeba histolytica* life stages (bramkart.com)

The organism can be prevalent in cold regions as well as tropical and subtropical regions that have contaminated water. In fact, *E. histolytica* is an important cause of morbidity and/or mortality wherever sewage facilities are inadequate. As is the case for other intestinal protozoan pathogens, wastewater treatment techniques are reported not to be very efficient for *E. histolytica* elimination possibly because of their resistance to disinfectants and the small size of the cysts. Stabilization ponds have been reported to be more effective than activated sludge for their abatement. Sedimentation and filtration can enhance the removal of cysts from wastewater. *Entamoeba* species are single cell organisms with two life cycle stages. Cysts are directly excreted in the stool and spread through the environment via contaminated water, soil, and fresh vegetables as well as unsanitary household conditions. Species cannot be differentiated based on cyst or trophozoite morphology. Following ingestion, cysts transform into vegetative forms or

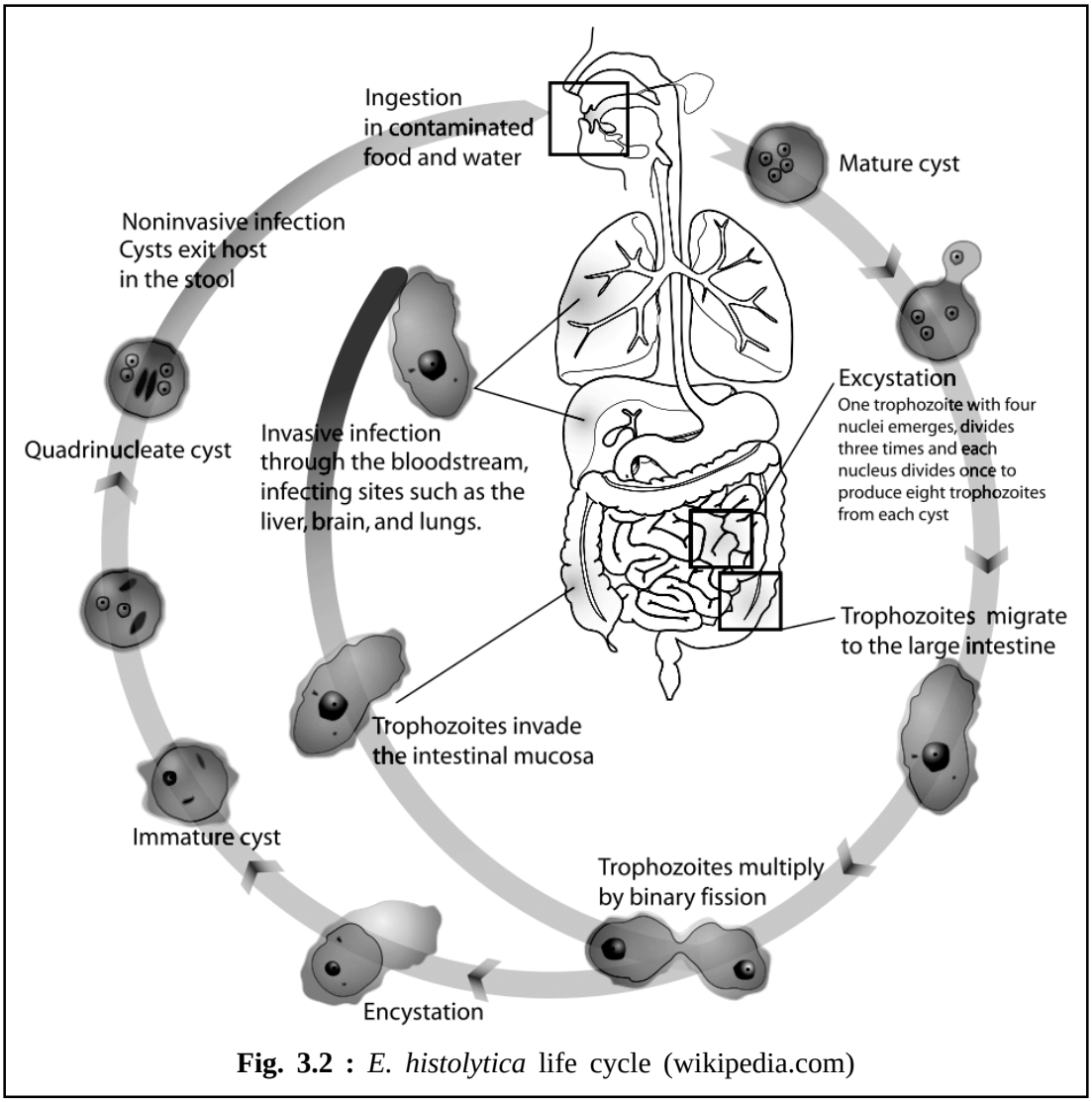


Fig. 3.2 : *E. histolytica* life cycle (wikipedia.com)

trophozoites, the motile stage that moves with the aid of pseudopodia and colonize the intestinal mucosa of the large bowel. Damage to the colon is caused by neutrophils that respond to infection from *E. histolytica*. Trophozoites can also invade the intestinal mucosal barrier and, via the bloodstream, disseminate to the liver, lung, and other sites with resultant pathologic manifestations. Drug treatments are available. Lagoons and constructed wetlands, sedimentation, filtration, flocculation, chemical and ultraviolet disinfection have all been employed for removal of cysts from water with varying degrees of success. *E. histolytica* presence and the kind of amebiasis manifestations are cosmopolitan in their distribution: amoebic liver abscess is the major form of amebiasis in South Africa, while,

in Egypt, in central and South America, Africa and Asia, intestinal invasive manifestation is the predominant form.

Systematic position

Subkingdom: Protozoa

Phylum: Sarcomastigophora

Subphylum: Sarcodina

Superclass: Rhizhopoda

Class: Lobosea

Order: Amoebida

Genus: *Entamoeba*

Species: *histolytica*

Trophozoites

Identifying Characters

1. Trophozoites vary remarkably in size-from 10 to 60 μm or more in diameter, actively motile by pseudopodia.
2. Amebas are anaerobic organisms and do not have mitochondria.
3. Shape changes constantly due to pseudopodial movement.
4. Cytoplasm is divided into clear outer ectoplasm and inner granular endoplasm.
5. Endoplasm contains the nucleus and food vacuoles.
6. The nucleus has a distinctive central karyosome and a rim of finely beaded chromatin lining the nuclear membrane. The food vacuoles contain may contain bacteria or red blood cells.

Cyst

Identifying Characters

1. The cyst is spherical, 10-20 μm in diameter, with a thin transparent wall.
2. Fully mature cysts contain four nuclei. The nuclei have fine evenly distributed uniform granular peripheral chromatin, with small discrete central karyosome.
3. Chromatoidal bars, crystallized ribonucleo-proteins, are present variably, and are more common in immature cysts. They are elongated bars with bluntly rounded ends.

4. Inclusions in the form of glycogen masses also may be present. Usually diffuse concentrated masses often present young cysts. They stain reddish brown with iodine.

3.3 *Leishmania donovani*

Leishmaniasis is a vectorborne disease that is transmitted by sand flies and caused by obligate intracellular protozoa of the genus *Leishmania.Leishmania* species are intracellular parasites in the white blood cells, liver cells and spleen cells Human infection is caused by more than 20 species. These include the *L. donovani* complex with 2 species *(L. donovani, L. infantum* [also known as *L. chagasi* in the New World]); the *L. mexicana* complex with 3 main species *(L. mexicana, L. amazonensis,* and *L. venezuelensis); L. tropica; L. major; L. aethiopica;* and the sub genus *Viannia* with 4 main species *(L. [V.] braziliensis, L. [V] guyanensis, L. [V] panamensis,* and *L. [V] peruviana).* The different species are morphologically indistinguishable, but they can be differentiated by isozyme analysis, molecular methods, or mono clonal antibodies. *Leishmania donovani* is known to infect man in India, China, South America, parts of Africa and Mediterranean countries. This genus was created by Ronald Ross in 1903. The species was simultaneously reported by Leishman in London and Donovan from Madras (India), hence the name given as *Leishmania donovani.*

Systematic position

Subkingdom: Protozoa

Phylum: Sarcomastigophora

Subphylum: Mastogophora

Class: Zoomastigophora

Order: Kinetoplastida

Genus: *Leishmania*

Species: *donovani*

Amastigote stage (leishmanial form):

Identifying Characters

1. Amastigote forms are intracellular, non-flagellate, oval 2 to 5 μm in length and 1 to 2.5 μm in breadth. They contain a central nucleus measuring less than urn in

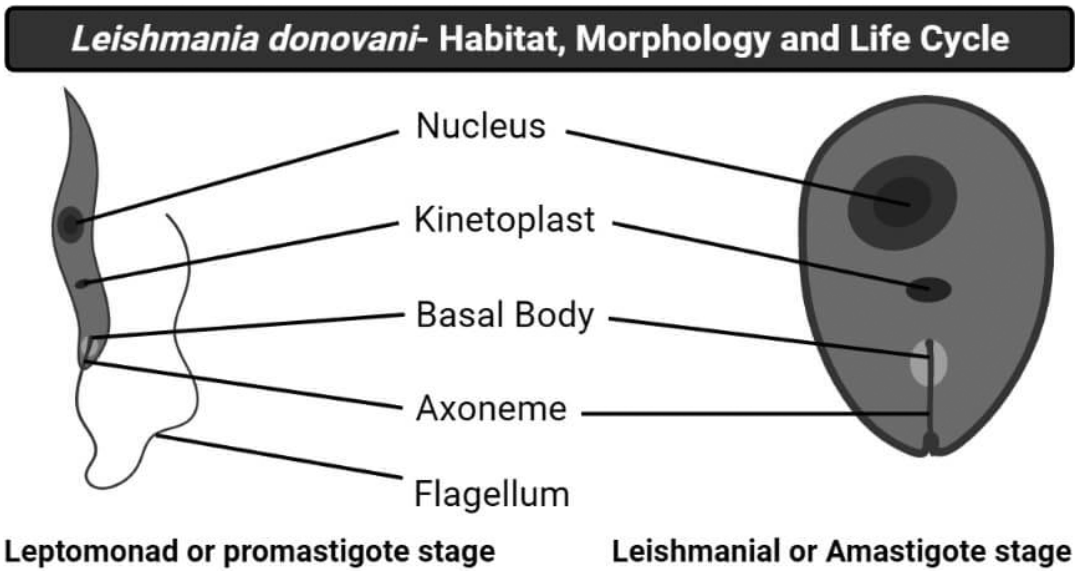


Fig. 3.3 : *L. donovani* life cycle stages (microbenotes.com)

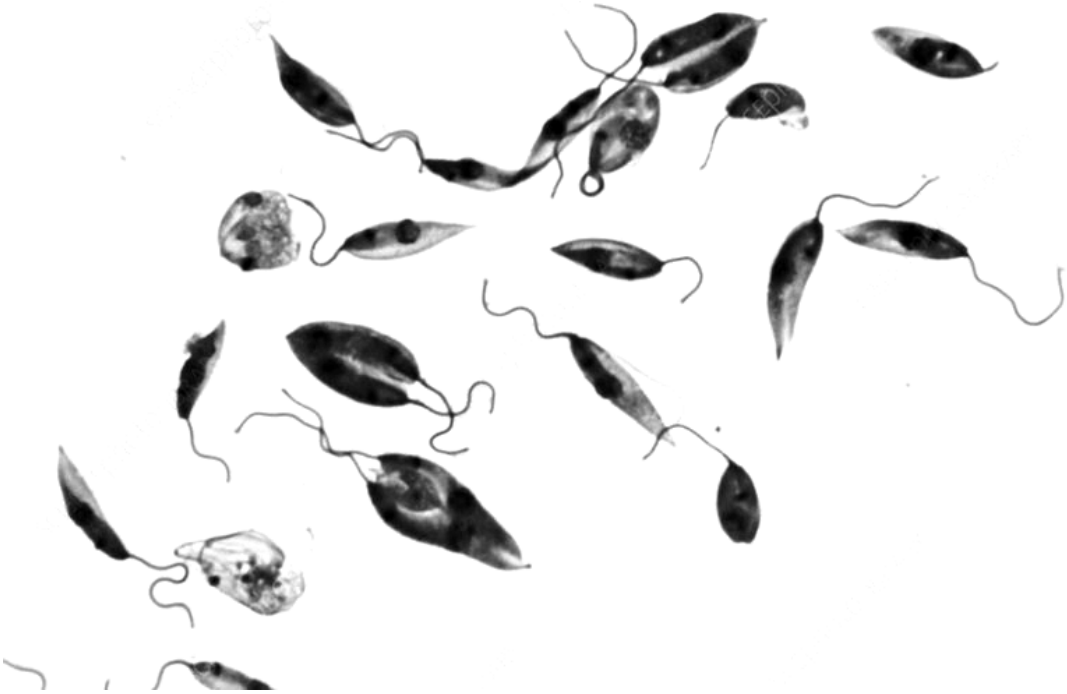


Fig. 3.4 : *L. donovani* under microscope (sciencephoto.com)

diameter. During this stage, the parasite resides inside the cells of the reticulo-endothelml system of man.

2. A minute structure, called kinetoplast lies at right angle to the nucleus. Kinetoplast contains a DNA containing body and a mitochondrial structure.
3. A delicate filament extending from kinetoplast to the margin of the body is axoneme (rhizoplast). Axonemes represent the root of the flagellum. A clear vacuole surrounds the axoneme.

Promastigote stage (Leptomonad form):

Identifying Characters

1. It is also known as the flagellar form. In the gut of the sand-fly, the mastigote form transforms into the pro mastigote form.
2. A fully developed promastigote is long, slender; spindle shaped measuring 15 to 20 μm in length and I to 2 μm in breadth.
3. A single nucleus lies in the centre while kinetoplast (Basal body) near the anterior end. In front of the kinetoplast is found an eosinophilic vacuole, over which the root of flagellum runs.
4. A single long flagellum orginates from the basal body which protrudes outside the body. Flagellum is almost of the same length of the body or is even longer.

3.4 *Plasmodium vivax*

Life cycle of *Plasmodium vivax* is digenetic and the malarial parasite has a complex, multi stage life cycle occurring within two living beings, the vector mosquitoes and the vertebrate hosts.

1. Primary host or definitive host: Female Anopheles mosquito is the primary host. The organism which contains sexual phase of the parasite and is regarded as definitive host.
2. Secondary host or intermediate host: human is the secondary host. Human contains asexual phase of the parasite and develops symptoms of disease due to the presence of parasite and is termed as secondary host.

The survival and development of the parasite within the invertebrate and vertebrate hosts, in intracellular and extracellular environments, is made possible by a toolkit of more

than 5,000 genes and their specialized proteins that help the parasite to invade and grow within multiple cell types and to evade host immune responses. The parasite passes through several stages of development such as the **sporozoites** (Gr. *Sporos* = seeds; the infectious form injected by the mosquito), **merozoites** (Gr. *Meros* = piece; the stage invading the erythrocytes), **trophozoites** (Gr. *Trophes* = nourishment; the form multiplying in erythrocytes), and **gametocytes** (sexual stages) and all these stages have their own unique shapes and structures and protein complements. The sexual phase of malarial parasite is called ***sporogony*** and results in the development of innumerable infecting forms of the parasite within the mosquito that induce disease in the human host following their injection with the mosquito bite. After the sporogonic phase of 8-15 days, the oocyst bursts and releases

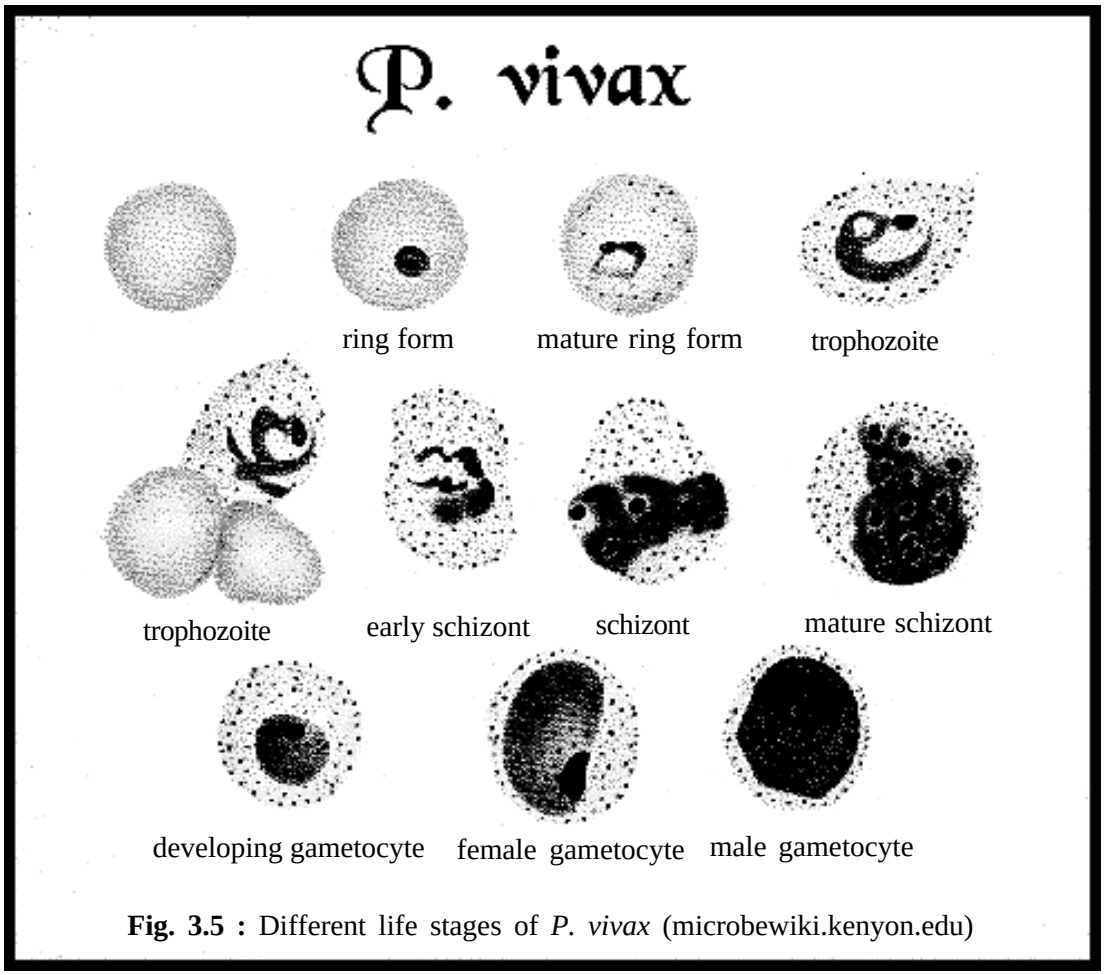


Fig. 3.5 : Different life stages of *P. vivax* (microbewiki.kenyon.edu)

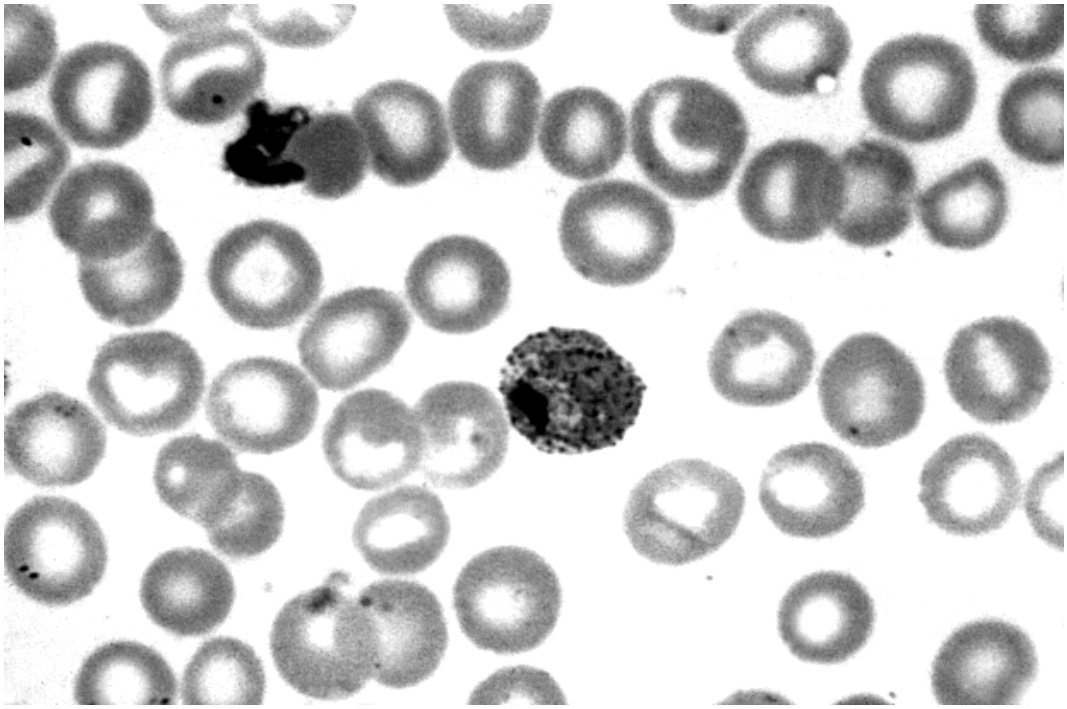


Fig. 3.6 : P. vivax infected RBC. Trophozoites visible in enlarged RBC (wilcimedia commons)

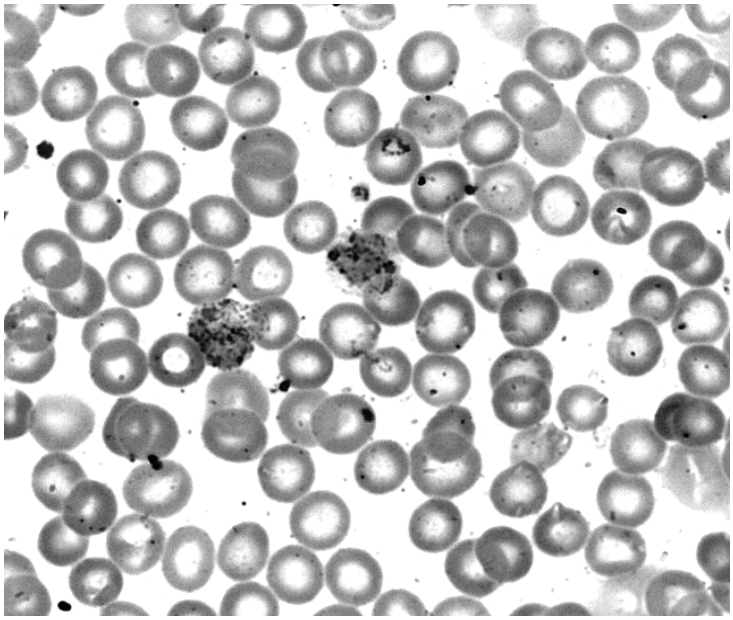


Fig. 3.7 : Peripheral smear showing schizont stage of P. vivax (wilcimedia commons)

sporozoites into the body cavity of the mosquito, from where they travel to and invade the mosquito salivary glands. When the mosquito thus loaded with sporozoites takes another blood meal, the sporozoites get injected from its salivary glands into the human blood-stream, causing malaria infection in the human host. The sporozoites inoculated by the infested mosquito initiate this phase of the cycle from the liver, and the latter part continues within the red blood cells, which results in the various clinical manifestations of the disease. Each sporozoite develop into a schizont containing 10,000-30,000 merozoites. The merozoites that develop within the hepatocyte are contained inside host cell-derived vesicles called merosomes that exit the liver intact, thereby protecting the merozoites from phagocytosis by Kupffer cells. In *P. vivax* malaria, some of the sporozoites may remain dormant for months within the liver. Termed as hypnozoites, these forms develop into schizonts after some latent period, usually of a few weeks to months. Each merozoite grows and divides within the vacuole into 8-32 (average 10) fresh merozoites, through the stages of ring, trophozoite, and schizont.

Systematic position

Subkingdom: Protozoa

Phylum: Apicomplexa

Class: Sporozoa

Subclass: Coccidia

Genus: *Plasmodium*

Species: *vivax*

Trophozoite Stage:-

Adult stage or feeding stage of parasitic protozoa is known as trophozoite stage. Human blood film containing trophozoites of *P. vivax* stained with Leishman stain show the following characters.

Identifying Characters

1. Within RBC the parasite appears as a half ring form, known as signet ring. Diameter varies from 2.5 μm to 3 μm.
2. Cytoplasm of the trophozoite stains blue surrounding a vacuole.
3. The cytoplasm on the one side of the vacuole is thick and broad while on the other side is narrow and thin.

4. The redish chromatin granules, the nucleus lies on this thin side.
5. The haemozoin granules, the undigested waste haematin materials, are deposited in the broader part of the cytoplasm.
6. Cytoplasm contains characteristic Schuffner's dot.
7. Matured trophozoite is irregular shaped with pseupodia like projections.

3.5 *Taenia solium*

Life cycle of *Taenia solium* or cysticercosis. Cysticercosis is an infection of both humans and pigs with the larval stages of the parasitic cestode, *Taenia solium.* This infection is caused by ingestion of eggs shed in the feces of a human tapeworm carner. Pigs and humans become infected by ingesting eggs or gravid proglottids. Humans are infected either by ingestion of food contaminated with feces, or by auto infection.

Human infected with adult *T solium* can ingest eggs produced by that tapeworm, either through fecal contamination or, possibly, from proglottids carried into the stomach by reverse peristalsis.

Once eggs are ingested, oncospheres hatch in the intestine invade the intestinal wall, and migrate to striated muscles, as well as the brain, liver, and other tissues, where they develop into cysticerci.

In humans, cysts can cause serious sequellae if they localize in the brain, resulting in neuro cysticercosis.

The parasite life cycle is completed, resulting in human tapeworm infection, when humans ingest under-cooked pork containing cysticerci.

Cysts evaginate and attach to the small intestine by their scolex.

Adult tapeworms develop, (up to 2 to 7 m in length and produce less than 1000 proglottids, each with approximately 50,000 eggs) and reside in the small intestine for years.

Humans infected with adult *T. solium* worms are asymptomatic or have mild Gastro Intestinal complaints.

Cysticerci may also infect the spinal cord, muscles, subcutaneous tissues, and eyes.

Systematic position

Phylum: Platyhelminthes

Class: Cestoda

Subclass: Eucestoda

Order: Cyclophyllida

Genus: *Taenia*

Species: *solium*

Identifying Characters

1. Body divisible into scolex, neck and strobila.
2. Scolex bears four cup-shaped suckers and a terminal rostellum crowned with double rows of hooks.
3. Hermaphroditic, sex organs are fully developed in posterior segments
4. Mature uterus with a central stem and lateral branches
5. Yellowis white in colour
6. Scolex with 4 suckers and rostellum with 25-30 hooks.
7. Uterus bears 7-12 lateral branches on each side.

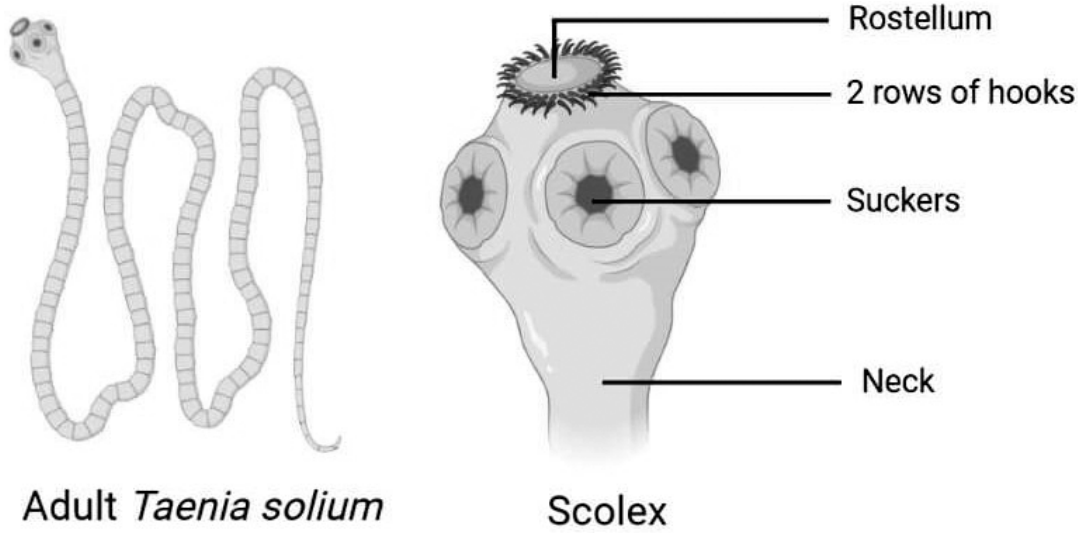


Fig. 3.8 : *Taenia solium*

3.6 *Ascaris lumbricoides*

Ascaris lumbricoides is the largest nematode (roundworm) parasitizing the human intestine. (Adult females: 20 to 35 cm; adult male: 15 to 30 cm.)

Human is the definitive host. There is no intermediate host.

Tramission is human—feces—human.

Adult ascarids live in the small intestines.

Females produce 200 000 eggs per day. Eggs are deposited in the lumen, passed in faces, and must embryonate for 3 weeks in the soil before becoming infectious.

Ingestion of infective eggs by another human from contaminated soil results in infection.

After ingestion, the hatched larvae penetrate intestinal mucosa and invade portal venules.

They are carried to the liver, and travel via the hepatic vein to the right heart and into the lungs.

Larvage enlarge and rupture into alveoli, are coughed up and subsequently swallowed.

Upon reaching the small bowel, they mature, mate and deposit eggs.

The incubation period is prolonged.

The interval between ingestion of the egg and the evelopment of egg-laying adults is approximately 8 weeks.

Systematic position

Phylum: Aschelminthes

Class: Nematoda

Order: Ascaroidea

Genus: *Ascaris*

Species: *lumbricoides*

Identifying Characters

1. The body of the roundworms is elongated and cylindrical. It gradually tapers at both ends. The anterior end is more slender than that of the posterior end.

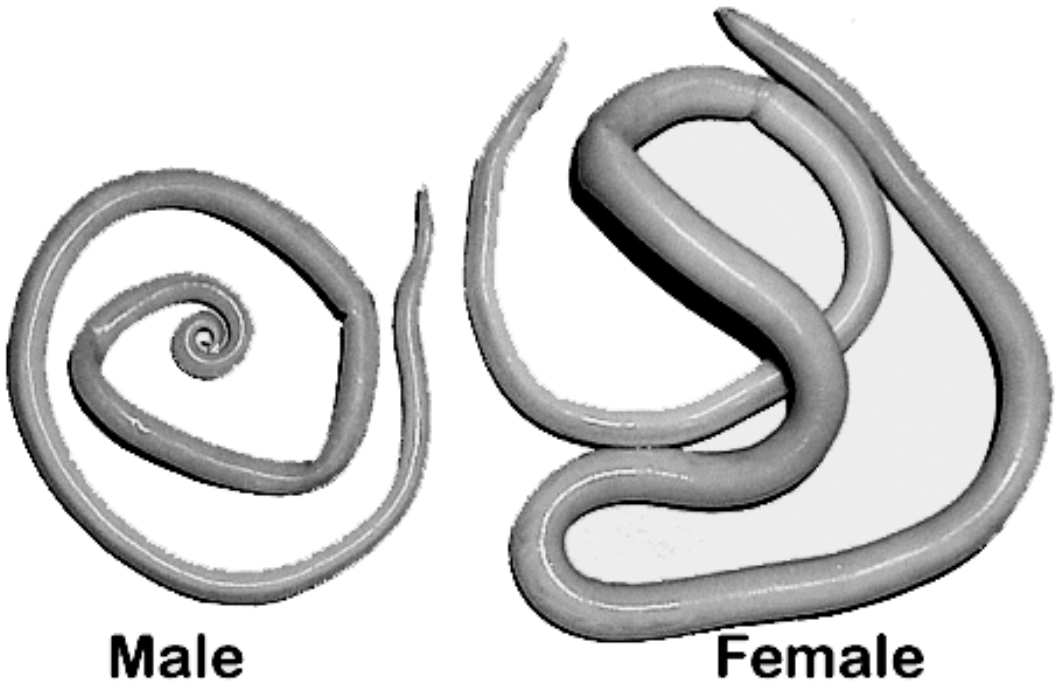


Fig. 3.9 : Ascaris lumbricoides—male & female (biologyeducare.com)

2. The body of these worms is covered by cuticle, which has minute striations which imparts a pseudo segmented appearance to the worms.
3. The fresh specimens are light yellow to light pink in color.
4. The sex of the roundworms is separate with sexual dimorphism.
5. Female roundworm measures about 20-40 cm in length and 4-6 mm in diameter, the posterior end of the female round worm is straight compared to that of the male.
6. Male roundworms measure upto 20 cm in length and 2-4 mm in diameter.

The males are smaller compared to the females. The posterior end of the male roundworms is curved with a pair of equal pineal setae.

7. Body cavity pseudocoel.
8. Alimentary canal straight with mouth and anus at opposite end.
9. Excretory pore is at the anterior end.

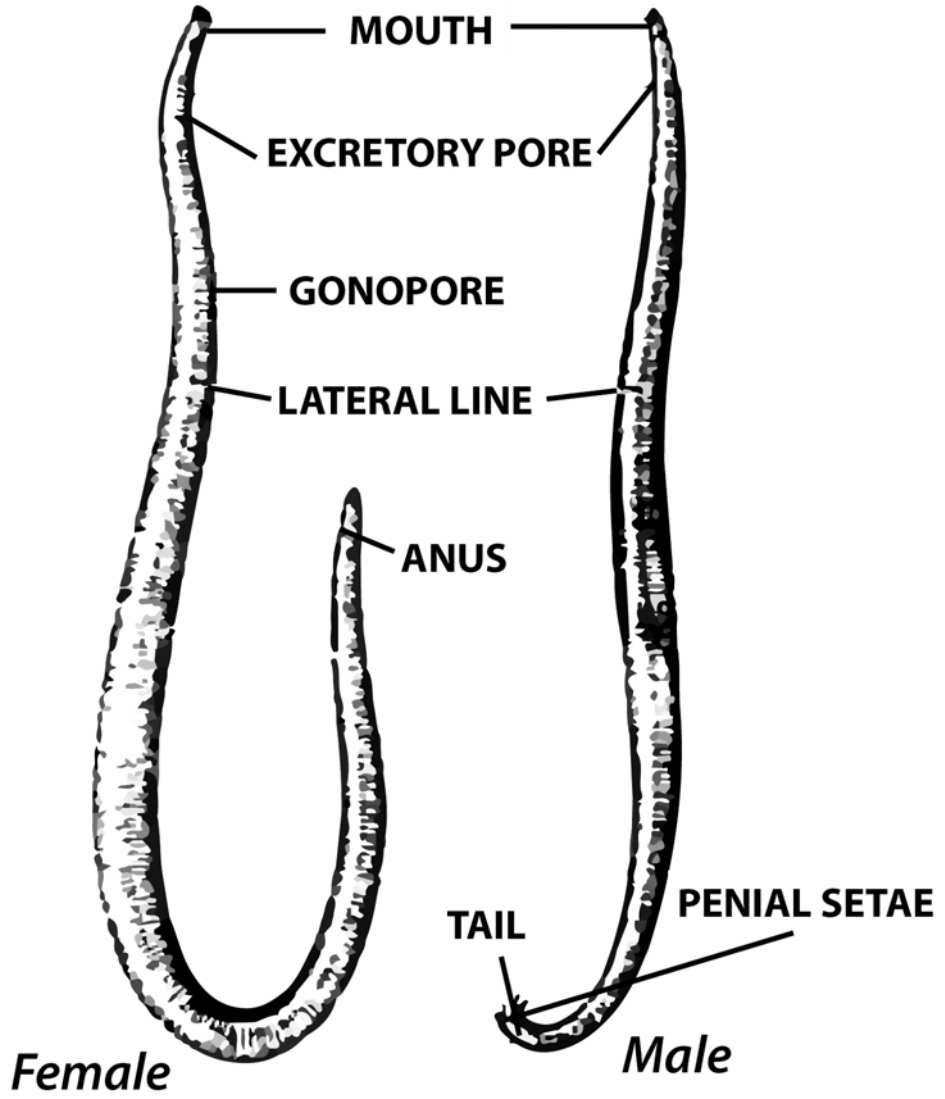


Fig. 3.10 : Ascaris lumbricoides—male & female (vedantu.com)

3.7 *Ancylostoma duodenale*

Ancylostoma duodenale is a nematode endoparasite, inhabiting the small intestine of man particularly in the jejunum, causing a disease called ancylostomiasis. *A. duodena le* is commonly known as "hook worm". They are especially prevalent in the areas where the humidity and temperature of the soil is favourable for the growth and

development their larvae. Regions of the world like Europe, North Africa, Egypt, Sri Lanka Central and Northern China, South America, Pacific islands and India are the endemic areas. In India Punjab and Uttar Pradesh are favourable belts for this parasite. *A. duodenale* is a monogenetic parasite. Man is the only host. Adult worms live inside the jejunum part of the small intestine of man. The worms are small, cylindrical, greyish-white nematode. Female laid eggs in the lumen of the gut of the infected person from where they pass out the body of the host along with the faeces. The eggs are colourless, oval in shape, measuring 65 μm in length and 40 μm in breadth. At the time of lying, the eggs are unsegmented and remain surrounded by a hyaline shell membrane. During their passage to outside, the zygote inside the egg divides twice to reach 4-celIed stage. A female lay about 25,000 eggs each day. The eggs arrive in the soil along with faeces. At this time it is non-infective. Within 48 hours a "rhabditiform larva" emerges out from each egg. Each larva measures about 250 μm in length. Inside soil, the rhabdatiform larva moults twice, once on the 3rd day and then on the 5th day to develop into a "filariform larva", measuring 500 to 600 μm in length. The filariform is the infective stage larva. Eight to ten days are required to develop the egg into the infective stage larva. When a bare footed man moves on the soil containing the

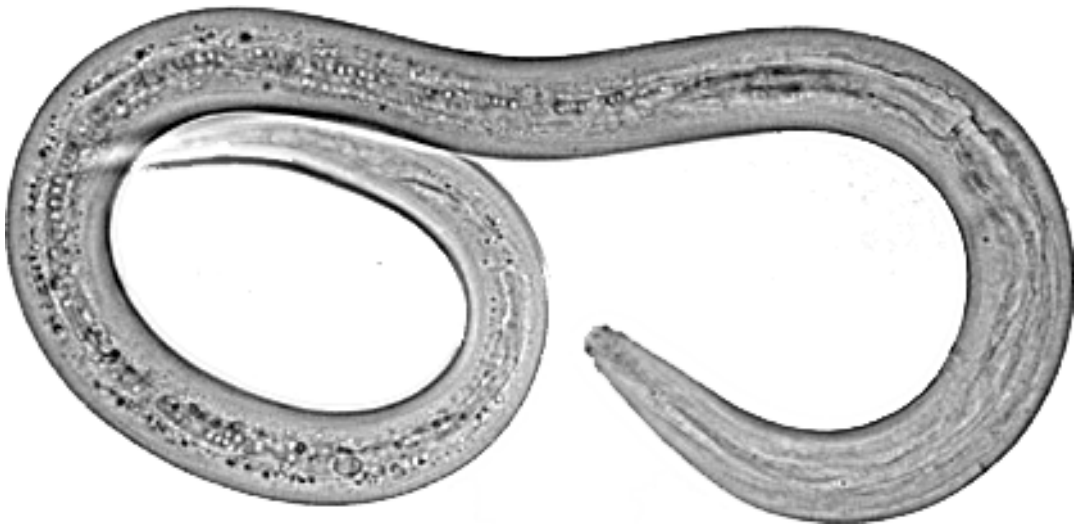


Fig. 3.11A : *Ancylostoma* sp. under microscope (cdc.gov)

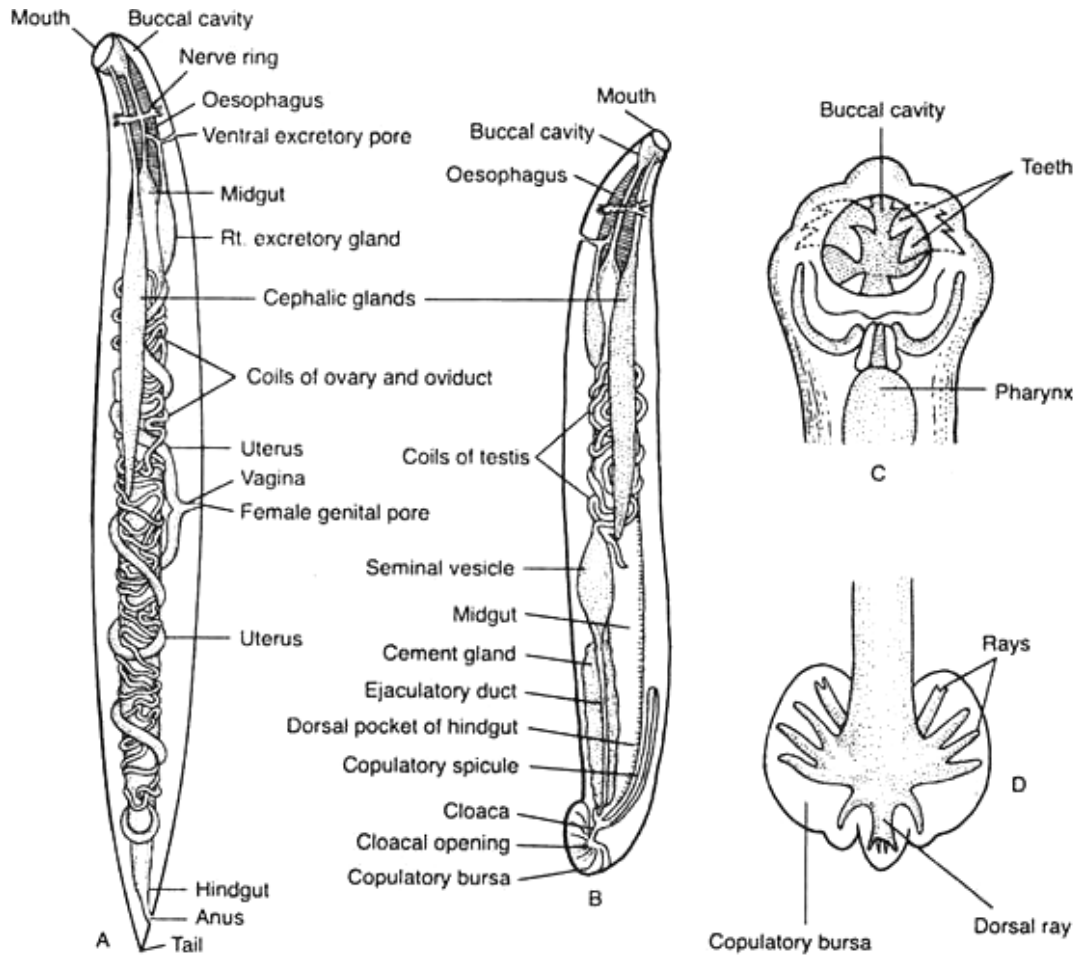


Fig. 3.11B : *Ancylostoma duodenale* A. Adult ♀, B. Adult ♂, C. Anterior end D. Posterior end of ♂(biologydiscussion.com)

infective larvae, the larvae casts off their outer covering sheath and penetrate the skin of the host to enter into its body. After entering into the body of the host, the larvae migrates through the different organs and parts of body. A filariform larva after penetrating through the skin reaches the sub-cutaneous tissues, from where it enters into the lymphatic vessels and through venous circulation reaches the right heart. From heart, through pulmonary artery the larvae reach into lungs where, after breaking the capillary wall they get freed into the alveolar spaces. Through, bronchi, trachea the larva enters into the pharynx and ultimately is swallowed to reach the small intestine.

Systematic position

Phylum: Nematoda

Class: Secernentia

Subclass: Rhabditia

Order: Strongylida

Genus: *Ancylostoma*

Species: *duodenale*

Identifying Characters

1. The worm is pinkish-white, cylindrical, dimorphic.
2. S-shaped worm because of its flexure at the frontal end.
3. Adult male hookworms range in size from 8-11 mm long, whereas adult females range in size from 10-l3 mm long.
4. The males having copulatory bursa made up of three lobes, of which one is dorsal and two are lateral, l3 chitinous rays support it.
5. Females have a vulva located approximately one-third of the body length from the posterior end.
6. Both male and female hookworms have two powerful ventral teeth in the adult forms of the parasite, one along each side of the buccal capsule; smaller pairs of teeth are located deeper in the capsule.

3.8 *Wuchereria bancrofti*

Wuchereria bancrofti is a nematode causing lymphatic filariasis throughout the tropics and subtropics. There are two strains of *W bancrofti;* the nocturnal periodic strain which is widely distributed in endemic regions, the microfilariae being in their highest concentrations between the hours of 10pm and 2am, and the sub-periodic strain which is found in the Pacific region, and has a microfilaraemia all the time with the highest numbers being detected between noon and 8pm. Humans are the only known reservoir host of *W bancrofti*.

Lymphatic filariasis is caused by three species of parasitic worm, ***Wuchereria bancrofti. Brugia malayi*** and ***B. timori***, which have generally similar life cycle. In the human body, adult worms (male and female) live in nodules in the lymphatic system and, after mating, produce numerous microfilariae, which circulate in the bloodstream. The lifespan of adult worms is 4-6 years. Microfilariae migrate between the lymph system and blood channels to reach the peripheral blood vessels, often at times of the day that coincide with the peak biting activity of local vectors. When female mosquitoes ingest a blood meal, they consume microfilariae with the blood. In the mosquitoes' stomachs, they lose their sheath, and some of the parasites migrate through the stomach wall to reach the thoracic flight muscles, where they develop into **first-stage larvae** (L1). The larvae grow and moult into **second-stage larvae** (L2) and moult again to produce highly active infective **third-stage larvae** (L3), a process that takes 10-12 days from the LI stage to the L3

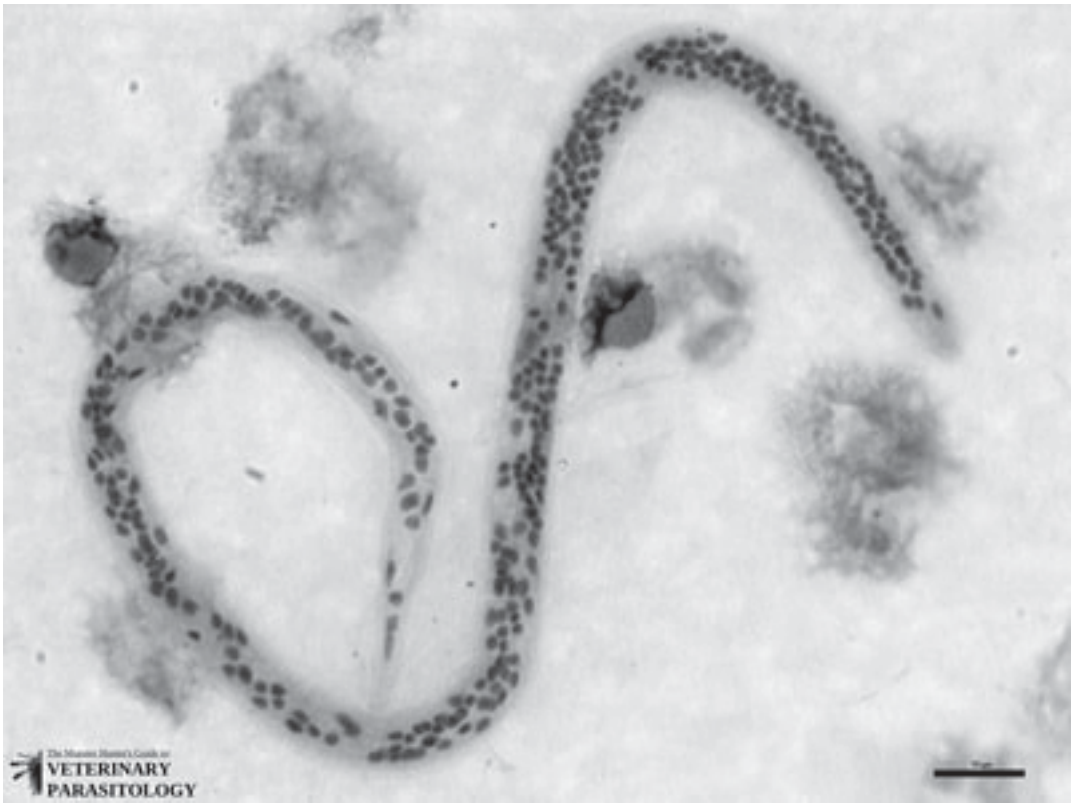


Fig. 3.12A : *Wuchereria bancrofti* microfileria is blood smear (veterinaryparasitology.com)

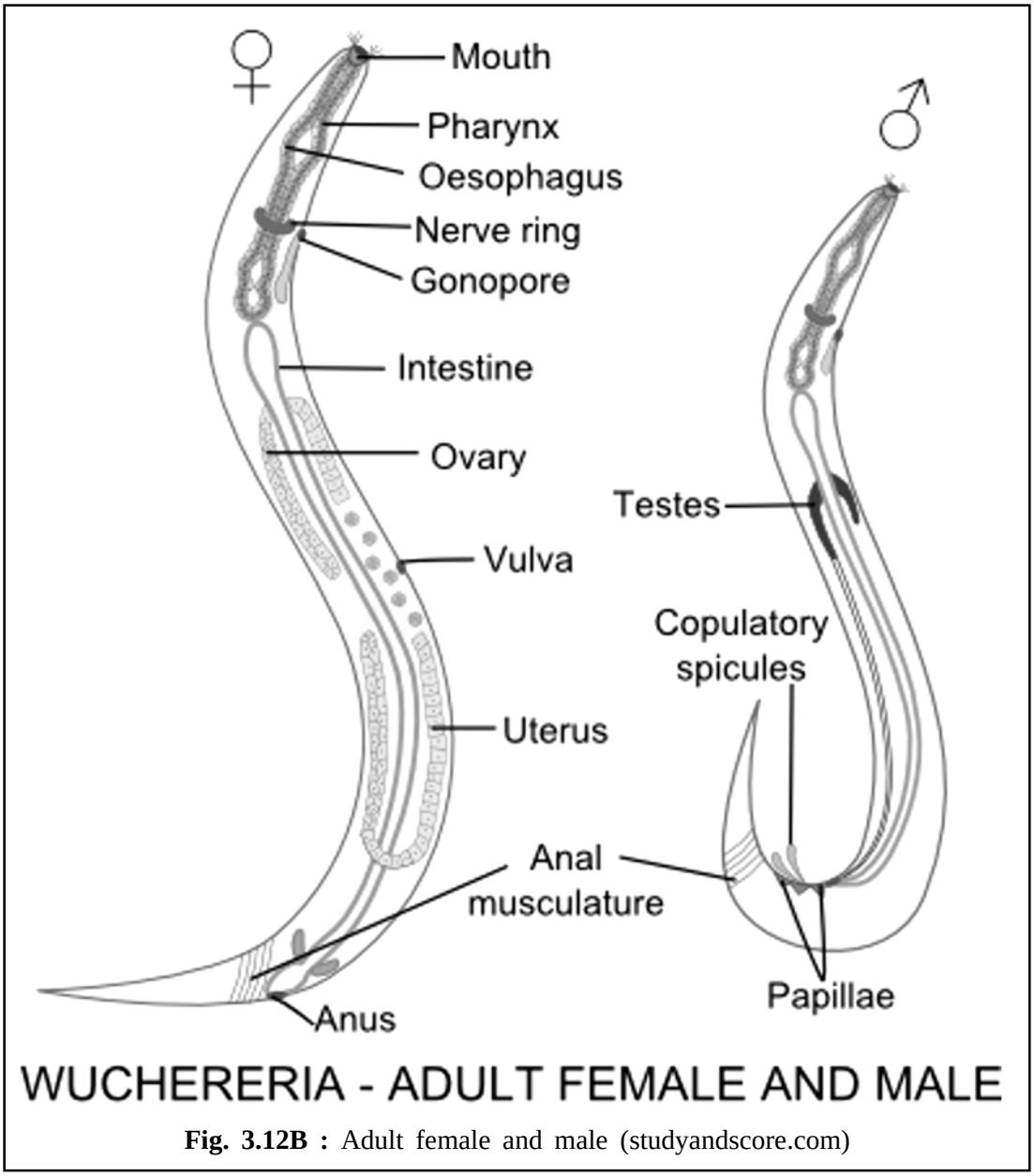


Fig. 3.12B : Adult female and male (studyandscore.com)

stage. The infective larvae migrate to the mosquito's proboscis, where they can infect another human host when the mosquito takes a blood meal. The L3 are deposited on the skin and find their way through a bite wound. The L3 develop to **fourth-stage larvae** (L4) as they migrate through the human body to the lymphatic vessels and lymph nodes, where they develop into adult worms.

The rate of uptake of microfilariae by a mosquito vector from a human host depends on the prevalence and intensity of infection in the community and the biting rate of the mosquito. In general, the greater the number of infectious hosts available in a community with a moderate-to-high density of circulating microfilariae in their peripheral blood and the higher the biting rate, the higher the chance of a mosquito picking up microfilariae from a human host and causing transmission. Extremely high levels of microfilariae in the blood may, however, result in a substantial number of mosquito deaths as the larvae develop.

Many factors contribute to the inefficient transmission of lymphatic filariasis.

- Firstly, microfilariae do not multiply in the mosquito body; hence, the number of L3 is limited by the number of microfilariae ingested.
- Second, only those mosquitoes that survive more than 10 days will contribute to transmission of the parasites. Those mosquitoes that die before the L3 develop cannot play a role in the transmission cycle.
- Third, the L3 are deposited on the skin and have to find their way into the bite wound (rather than being injected with the mosquito saliva like malaria sporozoites).

In view of all these factors, the transmission of lymphatic filariasis parasites is considered to be less efficient than that of other vector-borne parasites, such as malaria and dengue.

Systematic position

Phylum: Nematoda

Class: Secernentia

Subclass: Spiruria

Order: Spirurida

Genus: *Wuchereria*

Species: *bancrofti*

Identifying Characters

Adult

1. Hair like transparent worms.
2. Filiform and both ends are tapering.
3. Body curved without kinks
4. The posterior end of male is curved ventrally and with two unequal spicules.
5. The posterior end of-female is narrow, straight and abruptly pointed.
6. Male and females usually remain coiled.

Microfilaria

1. Each microfilaria is about 0.2 to 0.3 mm in length. It is surrounded by loose cuticular sheath. This cuticular sheath is also known as egg membrane.
2. The surface of this larva is covered by flattened epidermal cells.
3. Its body contains columns of cytoplasm with a number of nuclei.
4. An oral style is present at the anterior end where the mouth develops in the future.
5. Microfilaria also bears nerve ring around the pharynx, excretory pore, renette cells, four large germinal cells, future anal pore, inner cell mass, and somatic cell mass.

3.9 Questions

1. What is amoebiasis? What are the causative agents and symptoms for such disease.
2. State the identifying characters of the infective stage of *Entamoeba histolytica.*
3. Name some types of *Leishmania* sp. parasite. What is the stage of *Leishmanie* sp. which lies inside the human cells?
4. What is the stage of *Leishmania* found in the vector. State their characters for identification.

5. Mention different stages of material parasite. Which stage is found where to identify the infection in human body? State the identifying characters of that stage.
6. What is cysticercosis? What is the causative agent and stage of cysticercosis? State its systematic position and identifying character.
7. Identify the causative agent of Ascariasis with characters. Distinguish between male and female worm. State its systematic position.
8. State the identifying characters and systematic position of 'hook worm'.
9. What is 'filariasis'? What is the causative agent and symptoms of the disease? How can we identify the causative agent?

Unit 4 □ Study of *Pediculus humanus, Ctenocephalides spp.* and *Cimex lectularis*

Structure

4.0 Objectives

This unit is designed to introduce the learners with different arthropodes which act either as parasite (ecto) on vector of some other pathogens. After stydying this unit learners will be able to identify such harmful arthropodes and their mode of action to develop disease or pathogenicity in human.

4.1 Introduction

Ectoparasites are organism which are mostly arthropoda in nature. They generally include lice, fleas, bedbugs, mites and ticks and act as human ectoparasites. In many occasion these organisms act as vectors prother pathogens. They are typically regarded as vexing disorders initially, therefore attain less clinical attention. However, depending or socio-economic status and population setup such infections may cause significant morbidity and affect large portion of population. Infestation of these arthropodes are more prominent in developing countries and related with population density, weather condition and poor personal hygiene. Human body has some distinct ectoparasite defence system that includes cutancous sensory mechanisms, itch-generation mechanism and grooming behaviours. Overall, a general idea about these organisms will help us to understand better about our living, health and hygiene.

4.2 *Pediculus humanus*

The 3 types of human lice include the head louse *(Pediculus humanu scapitis)*, the body louse *(Pediculus humanus corporis)*, and the crab louse *(Pthirus pubis)*. Body lice infest clothing, laying their eggs on fibers in the fabric seams. Head and pubic lice infest hair, laying their eggs at the base of hair fibers.

Lice have simple or gradual metamorphosis. The immatures and adults look similar, except for size. Lice do not have wings or powerful jumping legs so they move about by clinging to hairs with their claw-like legs. Head lice prefer to live on the hair of the head although they have been known to wander to other parts of the body. Head lice do not normally live within rugs, carpet, or school buses. Body lice live in the seams of clothing, generally where it touches the skin, and only contact the body to feed, usually holding on to the clothing while they do this. However, sometimes they will move to the body itself.

The eggs of lice are called nits. They are oval white cylinders (1/16 inch long). The eggs of head lice are usually glued to hairs of the head near the scalp. The favourite areas for females to glue their eggs are near the ears and back of the head. The eggs of body lice are laid on clothing fibers and occasionally on human body hairs.

Under normal conditions the eggs will hatch in seven to 11 days. The young lice which escape from the egg must feed within 24 hours or they will die. Newly hatched lice will periodically take blood meals and moult three times before becoming sexually mature adults. Normally a young louse will mature in 10 to 12 days to an adult (1/8 inch in length). Adults range in colour from white to brown to dark grey.

Female lice lay six to seven eggs (nits) per day and may lay a total of 50 to 100 eggs during their life which may last up to to days. Adults can only survive one to two days without a blood meal. The nymphs and adults all have piercing-sucking mouthparts which pierce the skin for a blood meal. The reaction of humans to louse bites can vary considerably. Persons previously unexposed to lice experience little irritation from their first bite. After a short time, individuals may become sensitized to the bites, and may react with a general allergic reaction including reddening of the skin, itching, and overall inflammation.

Both the immature or nymphal forms and adult lice feed on human blood. To feed, the louse bites through the skin and injects saliva which prevents blood from clotting; it then sucks blood into its digestive tract. Bloodsucking may continue for a long period if the louse is not disturbed. While feeding, lice may excrete dark red faeces onto the skin.

The body louse is the vector of three human diseases—eqidemic or louse-borne typhus, caused by ***Rickettsia prowazeki*** de Rocha-Lima; trench fever, caused by ***Rochalimaea quintana*** (Schmincke) Krieg (long known as ***Rickettsia quintana***); and louse-borne relapsing fever, caused by ***Borrellia recurrentis*** (Lebert).

Systematic position

Phylum: Arthropoda

Subphylum: Mandibulata

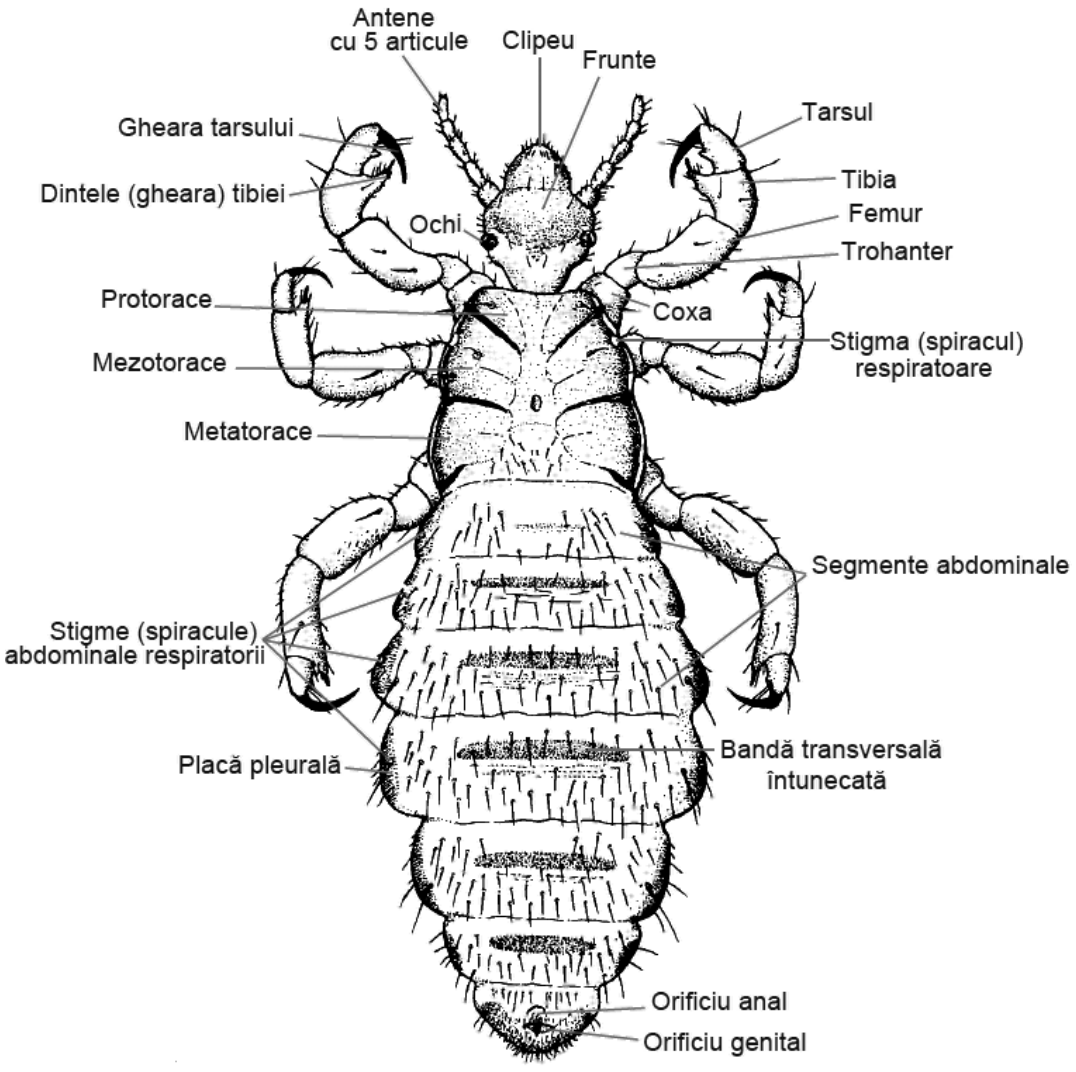


Fig. 4.1 : *Pediculus humanus*, male (wikimedia commons)

Class: Insecta

Subclass: Pterygota

Order: Siphunculata

Genus: *Pediculus*

Species: *humanus*

Identifying Characters

Phylum-Arthropoda

1. Bilaterally symmetrical and metamerically segmented
2. Majority of the segments bear a pair of jointed appendages.
3. Body is covered with a thick chitinous exo-skeleton.

Sub-phylum-Mandibulata

1. Body usually divided into head, thorax and abdomen.
2. One or two pairs of antennae present.
3. One pair of mandibles present.

Class-Insecta

1. Body is divided into head, thorax and abdomen
2. Three pairs of thoracie legs
3. Antenna one pair.

Sub-class-Pterygota

1. With wings or secondarily wingless.

Order-Siphunculata

1. Small wingless insect.
2. Dorsoventrally flattened with dark markings along the side.
3. Antennae short.
4. Thoracic segments fused and contains clawed legs.
5. Abdomen swollen.
6. Eyes reduced or absent.

Characters

1. Small head bears a pair of antennae and a pair of feebly developed compound eyes. Normally 1-2 mm long.

2. Piercing and sucking mouthparts for digging into the skin for blood.
3. Abdomen sowlen with small bristles on the side.
4. Legs equipped with sharp claws.

Habit and Habitat

P. humanus are transmitted by direct contact, by clothes or a brush, or by fallen hair. They prefer to lay them behind the ears or on the back of the head, near the neck line. The habitat of the human louse is solely on the human body or in the clothes and can only survive away from the host for a few days.

Lice are obligate ectoparasites and live off of the blood of humans. They have specially designed mouth parts for piercing the skin of human and retrieving the blood that is present.

Economic Importance for Humans:

P. humanus has relatively little direct effect on its hosts and can be the vectors for important diseases. The three most important diseases they can carry are typhus, trench fever (both caused by bacteria in the genus *Rickettsia*), and relapsing fever (caused by another bacteria species *Borrelia recurrentis*).

7.2 *Ctenocephalides sp*

Fleas are tiny, wingless insects arc capable of long jumps and hitch hiking a ride around the world sticking to an animal's fur belongs to the Order - Siphonoptera. Sucking on blood like tiny vampires, fleas are able to transfer many serious diseases to their host. There are different types who survive by sucking the blood of mammals and birds. Let us learn about these flea types.

Fleas are divided according to the type of host species they live on. However, some fleas are very well capable of jumping over from one animal species to another. Till date, there are over 2,000 species of tleas found in the world. Some of the important fleas are;-

Dog Flea *(Ctenocephalides canis)*

These fleas feed on the blood of dogs. They are capable of feeding on cat blood too and are able to bite humans sometimes. These fleas can survive for months without a meal. Their mouth parts are well adapted to be able to pierce the skin and suck the blood of the dog. Their body is flat from side to side and is covered in spines and hair. This helps them get a grip on the dog hair while traveling. Their hind legs are very long, that help them to jump onto a suitable host body.

Cat Flea *(Ctenocephalides-felis)*

These are the most found fleas on the earth. These fleas feed on the blood of their feline host, but may even enjoy a dog blood meal. Cat fleas can bite humans, but cannot cause an infestation. These fleas can transmit diseases like murine typhus as well as parasites like tapeworms into the host body.

Moorhen Flea *(Dasypsyllus gallinulae)*

These are large fleas that are found in birds. They are easy to identify due to the horn-like spines on the genital flaps of male fleas. These are common parasites for many birds that include the grouse, European Robin, Willow Tit, common Moorhen, etc.

Northern Rat Flea *(Nosopsyllus-fasciatus)*

This is a flea type that inhabits the fur of domestic rats and house mice. These fleas have an elongated body that is about 4 mm in length. They are normally parasites of the Norway rat. But, they may occasionally feed on the blood of wild rodents as well as humans. They are known to be carriers of the rat tapeworm as well as serve as a vector for plague.

Oriental Rat Flea *(Xenopsylla cheopis)*

Also called the tropical rat flea, they are one of the most dangerous fleas in the world. They are the primary vectors for murine typhus and bubonic plague. When this flea feeds on the blood of the infected rat and then bites a human, it spreads the diseases. These fleas look very similar to the dog and cat fleas. The oriental rat fleas are responsible for the spread of Black Plague.

Human Flea *(Pulex irritans)*

They prefer feeding on human blood. However, it has no qualms feeding on other mammals like cats, dogs and in particular swine blood. They can bite anyone and everyone at home. These are brownish black fleas that are about 4 mm long. They are a nuisance for the human population and can transmit diseases like tularaemia, murine typhus, diseases caused by tapeworm, etc.

Systematic position

Phulum: Arthropoda

Sub-phylum: Mandibulata

Class: Insecta

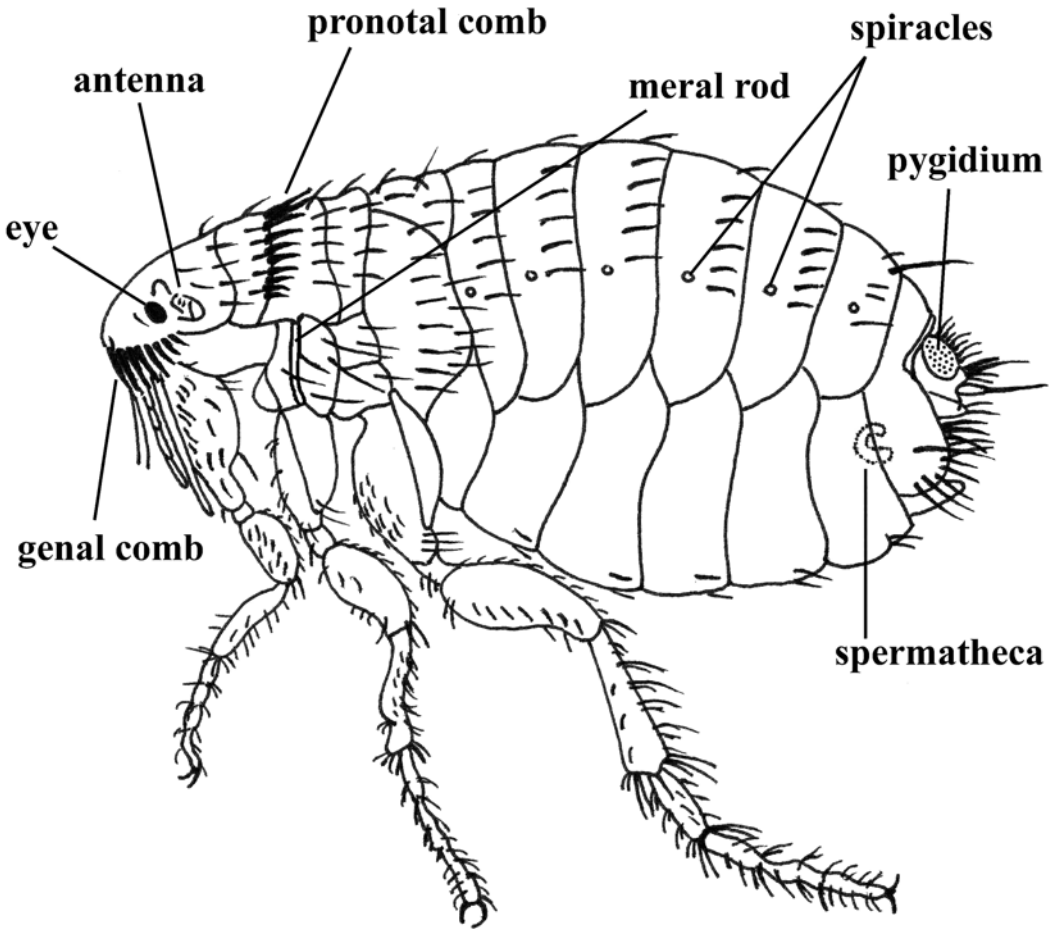


Fig. 4.2 : *Ctenocephalides felis* (pinterest.com)

Sub-Class: Pterygota

Order: Siphonaptera

Genus: *Ctenocephalides*

Order-Siphonaptera

1. Ectoparasite on birds and mammals.
2. Mouthparts piercing and sucking type.
3. Antennae short, eyes simple, no ocelli.
4. Long legs, adopted for jumping.

5. Wingless.
6. Abdomen without cerci.

Identifying Characters

1. Laterally compressed and wingless
3. It has a pronotal ctenidium and a genal ctenidium with more than 5 teeth.
4. The hind tibia lacks an outer apical tooth.
5. Presence of complex, snail-shaped genitalia in males.
6. Two mm long and reddish-brown to black, with the females being a bit larger than males and a slightly different in color.

Habits & Habitat

After piercing the skin of the host, adult fleas use their mouth parts to suck up blood. The blood-meal then passes through epithelial cells in the gut that are elongated into spines, collectively called the proventriculus, where it is broken up. They are known to bite humans in the absence of other hosts. In contrast to adult *Ctenocephalides* sp., larvae feed on the faeces of the adult fleas and detritus in the environment.

Cosmopolitan, live in the nests and resting places of their hosts (cats, dogs, rabbits, horses, skunks, foxes, mongooses, koalas, poultry) when they are not feeding, and on their hosts when they are feeding. They live in just about any type of habitat, as long as it is warm and humid enough to promote development.

Economic Importance for Humans:

Ctencephalides sp. is a vector of murine typhus in humans, caused by *Rickettsia mooseri.*

7.3 *Cimex lectularis* (Bed bug)

Common bed bugs, *Cimex lectularius* L., and a few closely-related species of blood feeding true bugs (Hemiptera: Cimicidae) have been persistent pests of humans throughout recorded history. They may have evolved from cave-dwelling ectoparasites of mammals (especially bats). As humans moved from caves to tents and then into houses, the bugs went with them. Bed bugs have been mentioned in the literature and folk remedies of many cultures and countries since the times of ancient Greece. Bed bugs became very rare in many industrialized countries soon after World War II because of the widespread use of synthetic insecticides. By 1997, they were so scarce in the United States that it was hard

to find specimens to use in college entomology classes. Adult bugs are 6-7 mm long, broadly oval, flat, brown to reddish-brown true bugs, with a 3-segmented beak, 4-segmented antennae, and vestigial wings. They have dorso-ventrally flattened bodies covered with short, golden-coloured hairs. They give off a distinctive, musty, sweetish odour containing various aldehydes which are produced by glands located in the ventral metathorax. They deposit undigested parts of prior blood meals in their harbourages as tarry or "rusty" spots. The tips of the abdomen are usually pointed in males and more rounded in females. Bed bugs feed only on the blood of mammals or birds and mate by traumatic insemination. One life cycle from egg to egg is 5 weeks at 75-80% RH and 28-32°C. They can survive and remain active at temperatures as low as 7°C. Bed bugs are nocturnal but they can feed in day time if they are starved. Bed bug bites can cause physical and psychological discomfort. Although their feeding usually is never felt, the saliva contains biologically active proteins, which may cause progressive, allergenic, visible symptomatic skin reactions to repeated bites. Typical symptoms include a raised, inflamed, reddish weal at each bite site, which may itch intensely for several days. Immediate reactions may appear from 1 to 24 h after a bite and may last 1-2 d; delayed reactions usually appear 1-3 d (or more) after a bite and may last 2-5 d. There can also be a social stigma attached with having a bed bug infestation.

Systematic position

Phulum: Arthropoda

Sub-phylum: Mandibulata

Class: Insecta

Sub-Class: Pterygota

Order: Hemiptera

Genus: *Cimex*

Species: *Cimex lectularis*

Identifying Characters

Order-Hemiptera

1. Usually two pairs of wings, fore wing modified as hemi elytra, with membranous apex and leathery base to cover membranous hind wings.
2. Legs absent or adopted for running, jumping, digging, grasping pray or swimming.
3. Mouth parts piercing or sucking type.

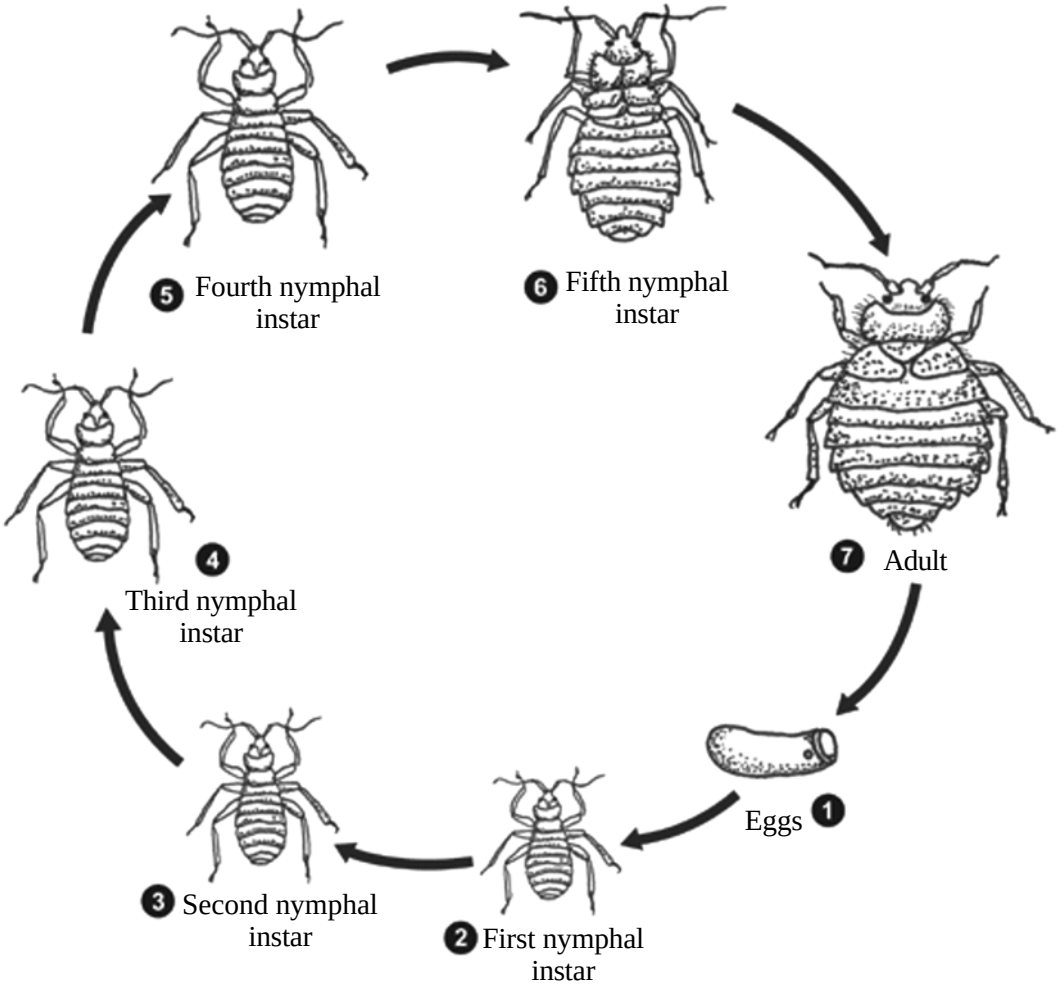


Fig. 4.3A : *Cimex lectularis life-cycle* (cdc.gov)

Characters

1. Body flat and oval covered with bristle and hairs.
2. Head small and bears distinct, paired compound eyes and a pair of four jointed antennae.
3. Piercing and sucking type of mouth parts
4. Prothorax semilunar, mesothorax triangular
5. Fore wing present as atrophied condition
6. Abdomen flat and eight segmented

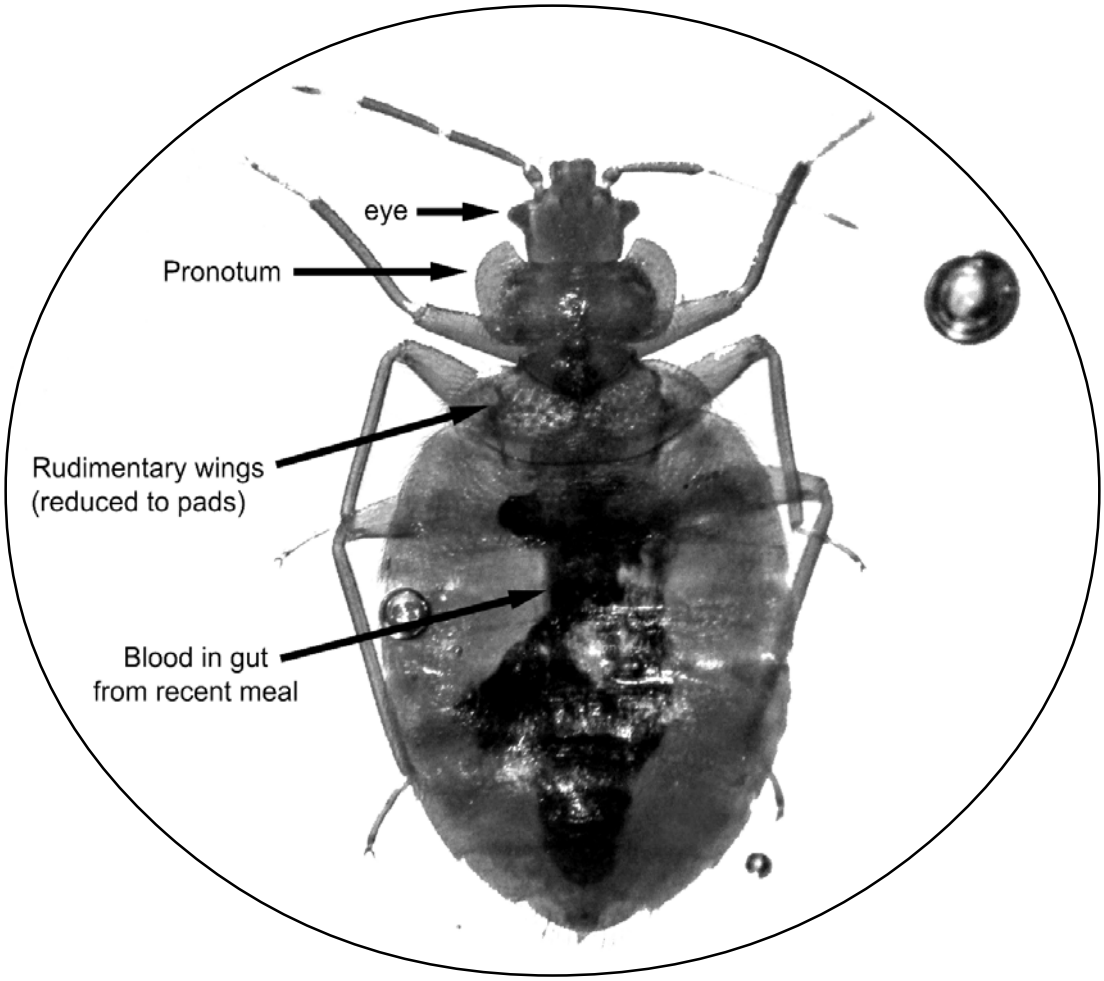


Fig. 4.3B : *Cimex lectularis life-cycle* (pathologyantlines.com)

Habit and habitat

Bed bugs are obligatory bloodsuckers. They have mouth parts that saw through the skin, and inject saliva with anticoagulants and painkillers. Sensitivity of humans varies from extreme allergic reaction to no reaction at all (about 20%). The bite usually produces a swelling with no red spot, but when many bugs feed on a small area, reddish spots may appear after the swelling subsides. Bedbugs prefer exposed skin, preferably the face, neck, and arms of a sleeping person.

Economic importance for humans

Bed bugs bites are not known to transmit any infectious disease.

4.5 Questions

1. State the scientific name and systematic position of human lice. Identify the specimen with characters. Name few disease and pathogens where human lice acts as vector.
2. What are fleas and how they cause harm to human and other associated species?
3. Identify the human flea with characters. State its systematic position, habit and habitats. How it can cause harm to human?
4. What is common bed bug? Mention its identifying character and systematic position. How they are harmful to human?

Unit 5 □ Study of Nematode/Cestode Parasites from the intestine of Poultry Bird

Structure

5.0 Objectives

The objective of the present unit is to aware the learners about the common causes of parasitic infections in the intestine of poultry birds. They can identify the infective parasites in poultry birds. Also such knowledge will help us to take remedial measures when farming.

5.1 Introduction

Poultry birds are important farm animal and rich source of protein supply to human population. Therefore, the health issues of poultry birds are important for us. It has been found that different parasites infect these birds and affect their health and many of which reside in their intestine. In intensive and semi-intensive poultry farming, the control of such parasite infection is important to maintain the quality of poultony products. Mostly, these birds are infected in Helminthiasis, the infection by the members of Nematoda (round worm) and cestoda (tape worms on flat worms).

The common internal parasitic infections occur in poultry include cestodes, nematodes and coccidia. These worm infections may cause considerable damage and great economic loss to the poultry industry due to malnutrition, decreased feed conversion ratio, weight loss, lowered egg production and death in young birds. Generally, four species of cestodes namely ***Raillietina echinobothridia, R. tetragona, R. cesticillus*** and ***Choanotaenia infundibulum*** and two species of nematodes namely, ***Ascaridia galli*** and ***Heterakis gallinarum*** are the causative agents.

5.2 Materials and Method

5.2.1 From intestine (Protocol)

1. For the collection of endoparsites from the body of the hosts need to dissect open midventrally and different organs including alimentary canal were removed and kept in separate desired size petridishes where these organs were teased and cut open to search for parasites if any.
2. Cestodes may be collected by the help of dropper and preserved in 10% formalin or conoy's fluid for the identification.
3. Morphology of cestodes was studied by preparing permanent slide.
4. After washing, nematodes were collected by the help of curved needle and kept in glycerin alcohol. Nematodes were best killed in steaming hot 70% alcohol, and stored in the same solution. Later, a few drops of glycerin are to be added.
5. Thorough morphological study of nematodes may performed by the preparation of sub-permanent slide by adding one drop of lactophenoI. The other steps in this

were fixation, staining, dehydration, de-alcoholisation and clearing, mounting and labeling.

5.2.2 From faecal matter (Protocol)

1. Freshly passed out faecal matter are collected and a small amount is placed on 3-4 glass slides precoated with mayer's albumen.
2. Few drops of normal saline is put on the faecal matter and mixed thoroughly to obtain a thin suspension.
3. The suspension is spread uniformly over a small area of the glass slide.
4. Schaudin's fluid is put over it immediately and then transferred to 90% alcohol in a coupling jar for 15 minutes.
5. The slide is then passed through 70% and 50% alcohol and then to distilled water.
6. Then dipped into haematoxylin and then into eosin.
7. It is then dehydrated through 50%, 70%,90% and 100% alcohol and mounted in DPX.

5.3 Common Nematodes

5.3.1 *Ascaridia galli*

Systematic position

Phylum: Nematoda

Class: Secernentia

Order: Ascaridida

Genus: *Ascaridia*

Species: *Ascaridia galli*

5.3.2 Identifying Characters

1. The body is semitransparent, creamy-white, and cylindrical, body is entirely covered with a thick cuticle. The cuticle is striated transversely through the length of the body

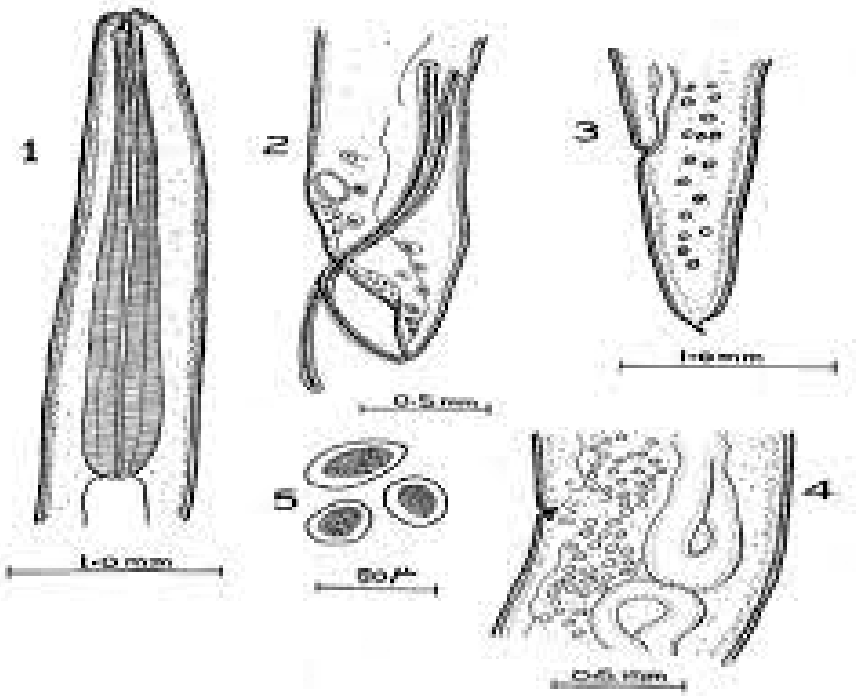


Fig. 5.1 : Camera Lucida drawing of *A galli*

2. The anterior end is characterized by a prominent mouth, which is surrounded by three large, trilobed lips. The edges of the lips bear teeth- like denticles.
3. Two conspicuous papillae on the dorsal lip and one on each of the sub ventral lips.
4. Distinct sexual dimorphism. Females are considerably longer measuring 72 to 112 mm long with a vulva opening at the middle portion of the body and anus at the posterior end of the body. The tail end of females is characteristically blunt and straight.
5. Males are relatively shorter and smaller, measuring 50 to 76 mm long, with a distinct pointed and curved tail. Ten pairs of caudal papillae are found towards the tail region of the body.

5.3.3 Pathogenicity

The nematode infects fowl of all ages, but the greatest degree of damage is often found in birds less than 12 weeks of age. Heavy infection is the major cause of weight depression and reduced egg production in poultry husbandry, adult worms may move up the oviduct and be found in hens' eggs, and sometimes they are also found in the birds' feces.

5.4 *Heterakis gallinarum*

5.4.1 Systematic position

Phylum: Nematoda

Class: Secementia

Order:Ascaridida

Genus: *Heterakis*

Species: *Heterakis gallinarum*

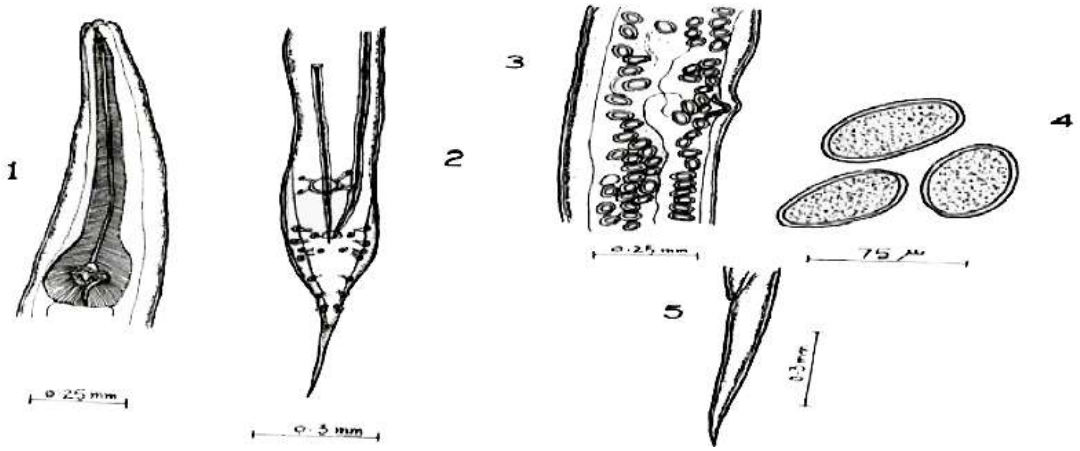


Fig. 5.2 : *Heterakis gallinarum* camera lucida drawing.
1. Anterior end of female (ventral view);
2. Posterior end of male (dorsal view);
3. Valval region of female (lateral view);
4. eggs;
5. Posterior end of female (lateral view) (omiesonline.org).

5.4.2 Identifying Characters

1. Typical roundworm, body covered with cuticle, three papillae-lined lips and alae, are ridges formed by the thickening of the cuticle that may act as receptors.
2. The parasite is a dioecious species with marked sexual dimorphism.
3. Males are smaller and shorter, measuring around 9 mm in length, with a unique bent tail, having a pre cloacal sucker at the posterior end

4. Females are stouter and longer, measuring roughly 13 mm in length, with a straight tail end.

5.4.3 Pathogenicity

H. gallinarum infection is itself is mildly pathogenic. However, ***H. gallinarum*** plays the role of carrier in the lifecycle of ***Histomonas meleagridis***, the causal pathogen of entero hepatitis "blackhead" of turkeys. Heavy infection indicated gross lesions characterized by congestion, thickening, petechial haemorrhages of the mucosa, and nodules in the caecal wall.

5.5 Common cestode

5.5.1 Systematic position

Phylum: Platyhelminthes

Class: Cestoda

Order: Cyclophyllidea

Genus: *Raillietina*

Species: *Raillietina tetragona*

5.5.2 Identifying Characters

1. It is whitish in colour, highly elongated, dorso-ventrally flattened, and entirely covered with a tegument, measuring up to 30 cm in length and 1-1.5 cm in breadth.
2. The body is divisible into the head region called 'scolex', followed by an unsegmented 'neck', and then by highly segmented body proper called strobila.
3. The strobila is composed of a series of ribbon-like body segments called proglottids, gradually enlarging from the anterior end towards the posterior.
4. The scolex bears an apical rounded rostellum, which is armed with 100 minute hooks, arranged in single row. This is surrounded by four suckers which are lined with 5-6 rows of spines.
5. Hermaphroditic having a complete reproductive system in itself.

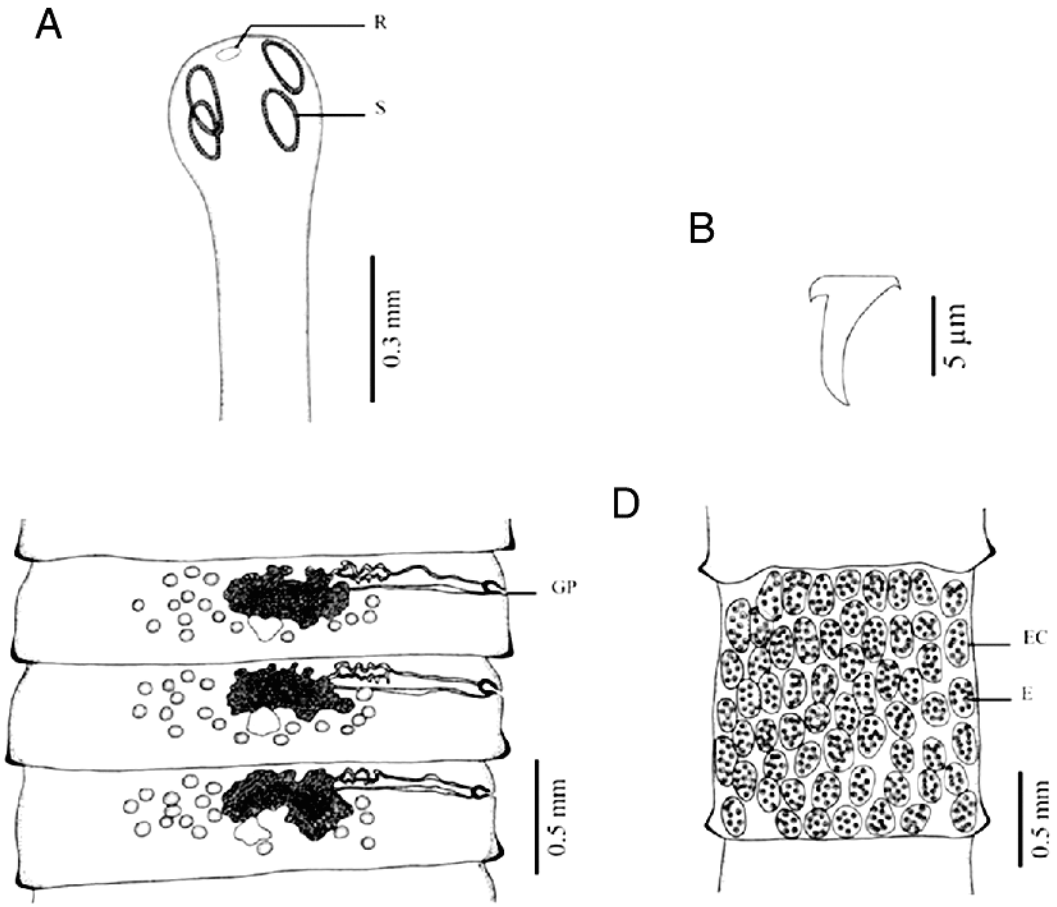


Fig. 5.3 : Morphology of *Raillietina tetragona.* (A) Scolex bearing the rostellum (R) Surrounded by 4 ovoid suckers (S). (B) Small hammershaped hook. (C) Mature proglottid showing unilateral opening of the genital pore (GP). (D) Gravid proglottid showing several eggs (E) per egg capsule (EC). (researchgate.net)

6. Each mature proglottid has a set of male and female reproductive organ and genital pores on one side.
7. Testes are located on both sides of the ovary and behind vitellarium.

5.5.3 Pathogenicity

The adult parasite infects the small intestine of fowl, from where it obtains nutrition from the digested food of the host. Generally the parasite is quite harmless, and does not cause serious lesions under natural conditions. However, instances of reduced weight loss and decreased production of eggs are observed.

5.6 Questions

1. State the method to collect and observe endoparasites from poultry bird—a) from intestine and b) from faecal matter.
2. Mention the chemicals used to preserve endoparasitic nematodes and cestodes from poultry bird intestine.
3. Give example of a common cestoda parasite which affect poultry birds. Name the causative parasites. Which may cause weight loss and decreased egg production. State their identifying characters.
4. Who is the host of the pathogen for entero-hepatitis “black head” of turkeys? From where and how such hosts can be collected? How they can be identified?
5. How can you identify *Ascaridia galli*? State its systematic position and pathogenicity.

Unit 6 □ Histological study of Spleen, Thymus and Lymph node

Structure

6.0 Objectives

The aim of this unit is—

- To give an idea about the tissue organization of some of the specific organs of our body which are directly involved in the immune system of the body.
- This will provide primary knowledge of histological architecture of these immune organs and the observable features of nascent and maturing immune cells in the tissue.
- Also learners receive the ideas about the clustering features and zonations of different immune cells in the tissue with their functional significance.

6.1 Introduction

The cells involved in the immune response of our body are organized within our body in a special system of organs and tissues. Such organization is needed to perform their functions effectively. These organs and tissue structures are collectively called Lymphoid system. With the immune cells, such system possesses some encapsulated tissues which

form organs. These oprgans are divided into primary and secondary organs. Primarily, lymphocytes are produced and matured in primary lymphoid organs and function in secondary lymphoid organs. The primary lymphoid organs are bone marrow and thymms, whereas, secondary lymphoid organs are spleen, lymph nodes etc.

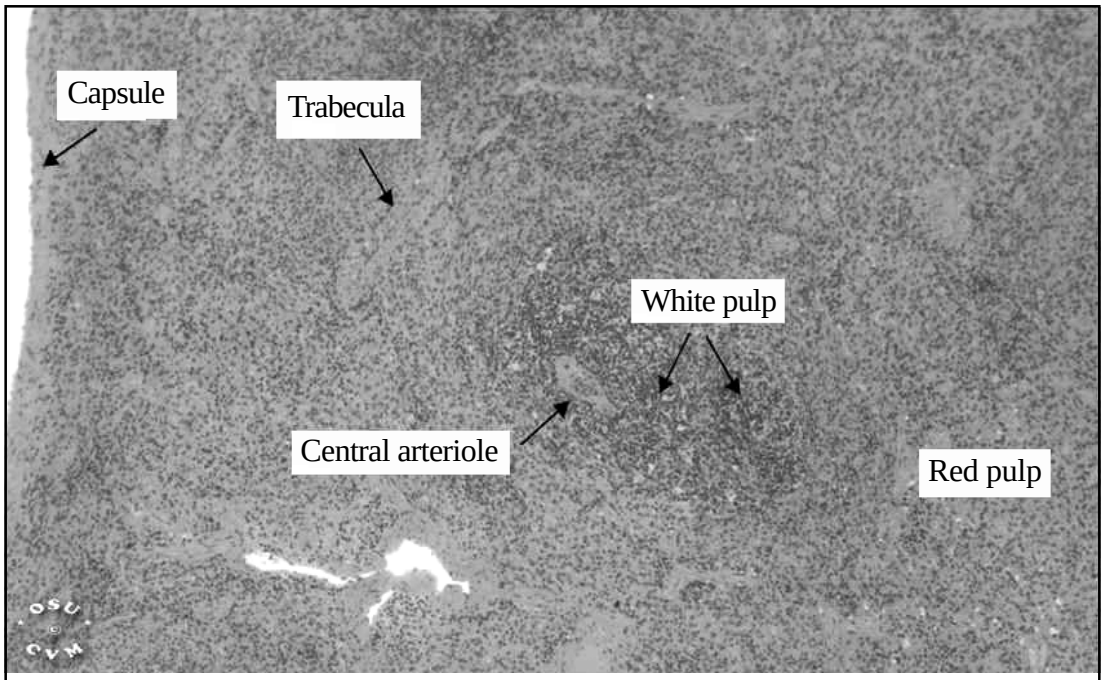


Fig. 6.1A : H/E Stained histological section of spleen [T.S] (pinterest.com)

In the following section we will discuss about the tissue organization of some of these lymphoid organs and how they appear under microscope in standard histological preparation.

6.2 Spleen

The spleen is the largest organ in the lymphatic system. It is an important organ for keeping bodily fluids balanced, but it is possible to live without it.The spleen is located under the ribcage and above the stomach in the left upper quadrant of the abdomen. A spleen is soft and generally looks purple. It is made up of two different types of tissue. The red pulp tissue filters the blood and gets rid of old or damaged red blood cells. The white pulp tissue consists of immune cells (T cells and B cells) and helps the immune system fight infection.

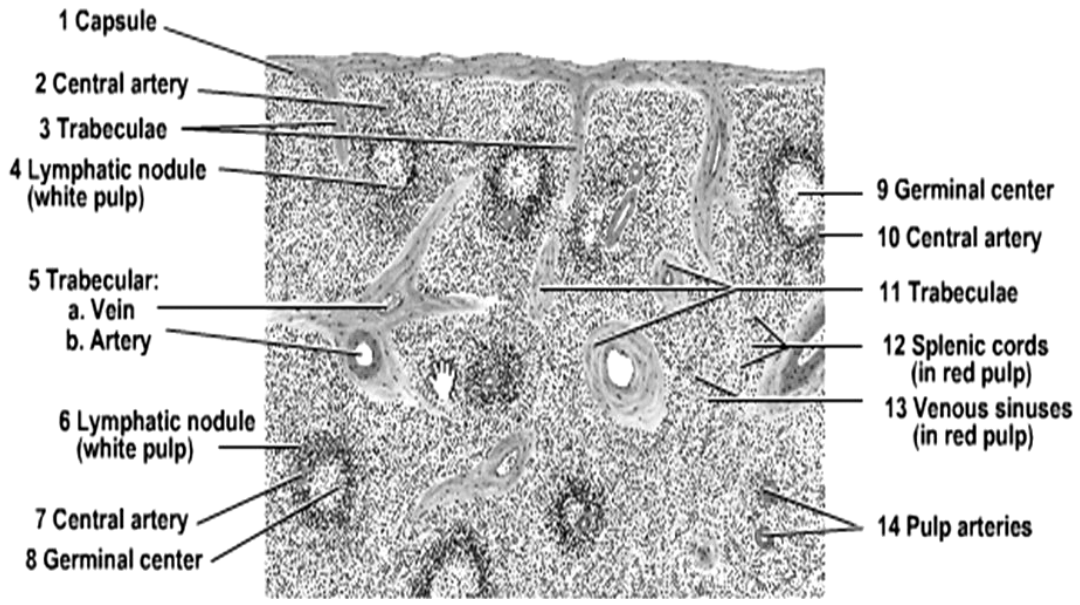


Fig. 6.1B : Histological drawing of spleen (slideplayer.com)

Although it varies in size between individuals, a spleen is typically around 3-5.5 inches long and weighs 5.3-7.1 ounces (Oz). The spleen is a soft organ with a thin outer covering of tough connective tissue, called a capsule. The spleen's primary job is to filter the blood. As blood flows into the spleen, it performs a quality control service, detecting any red blood cells that are old or damaged. Blood flows through a maze of passages in the spleen. Healthy cells flow straight through, but those considered to be unhealthy are broken down by large white blood cells called macrophages. The spleen also stores blood - the blood vessels of the spleen can expand significantly. In humans, around 1 cup of blood is kept in the spleen, ready to be released if there is a significant loss of blood, after an accident, for instance.The spleen also plays a role in the immune response by detecting pathogens (bacteria, for instance), and producing white blood cells in response. Around one-quarter of our Iymphocytes (a type of white blood cell) are stored in the spleen at anyone time.The spleen also produces compounds called opsonins, such as properdin and tufts in, that help the immune system.

6.2.1 Identifying Characters

1. The capsule enclosing the spleen is covered by single layer of mesothelial cells and consists of a dense connective tissue containing many elastic fibres and a few smooth muscle fibres.
2. Scattered throughout the spleen are lymphoid follicles separated by splenic pulp; the white and red pulp.
3. White pulp is cylindrical or fusiform in shape and consists of reticular fibres, reticular cells and lymphocytes.
4. Red pulp is a reticular network honeycombed by sinuses and consists of nongranular leucocytes, splenic cells, granular leucocytes and erythrocytes.

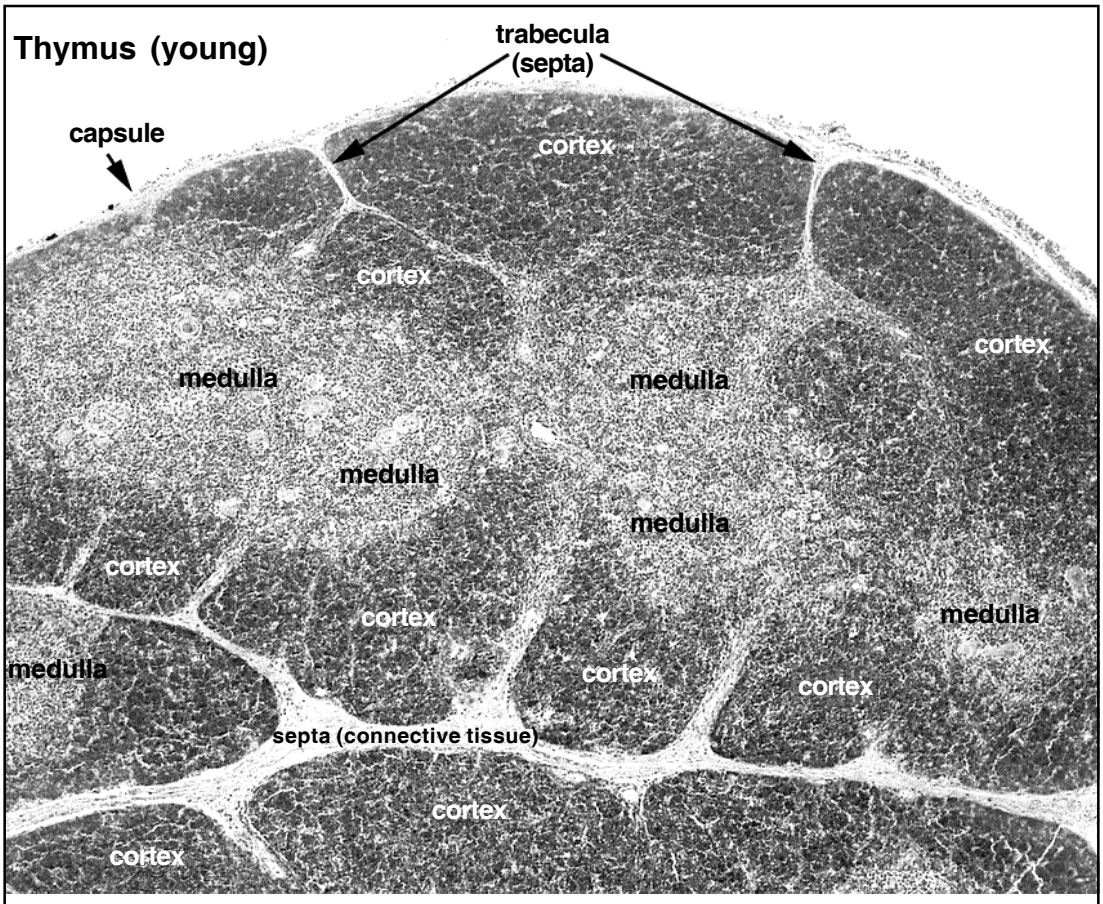


Fig. 6.2 : H/E Staining of T.S of mammalian Thymus (researchgate.net)

6.3 Thymus

The thymus is a small, irregular-shaped gland in the top part of the chest, just under the breastbone and between the lungs. It is located in an area of the body called the mediastinum. The thymus is part of both the lymphatic system and the endocrine system. The thymus is divided into 2 main parts - a right lobe and a left lobe. Each lobe is divided into smaller sections called lobules that give the thymus its bumpy appearance. Each lobule is made up of a centre part (called the medulla) and an outer layer (called the cortex). A thin covering (capsule) surrounds and protects the thymus. The thymus is mainly made up of epithelial cells, immature and mature lymphocytes and fat tissue. The thymus changes in size as you get older. It is large in new born and toddlers. It is biggest during puberty then slowly begins to shrink as adulthood approaches. The thymus is most active during childhood and youth. By late adulthood, most of the thymus is made up of fat tissue. The thymus makes T cells (T Iymphocytes) that travel throughout the body to help fight infection, disease and foreign substances. The thymus also makes hormones to help T cells develop and keep the immune system working properly.Lymphocytes travel from the bone marrow to the thymus, where they mature into T cells. Once T cells mature, they are able to leave the thymus and enter the blood so they can help the immune system. T cells also travel to lymph nodes and the spleen where they continue to mature.

6.3.1 Identifying Characters

1. It is divided into a morphologically distinct cortex and medulla separated by a vascular corticomedullary zone.
2. A thin connective tissue capsule surrounds each lobe and, in most species, gives rise to septae, that partially subdivide the thymus into interconnecting lobules of variable size and orientation.
3. The capsule is composed of an outer and inner layer of collagen and reticular fibres between which are occasional clusters of lymphocytes.
4. The darkly staining cortex contains densely packed, small, immature lymphocytes.
5. The medulla is paler staining, less densely cullular than the cortex, and contains more mature T-cells, prominent epithelial cells.

6. Plentiful blood cessels (predominantly arterioles) with scant perivascular connective tissue and mature and immature T lymphocytes characterize the corticomedullary junction.

6.4 Lymph node

Lymph nodes, also known as lymph glands, are oval-shaped masses of tissue in the body that serve an important role in protecting the body from infection and cancer. Every vertebrate animal has an extensive lymphatic system in their body, which consists of lymph nodes and lymph vessels. The lymph vessels carry a clear fluid called lymph that is collected from tissues throughout the body. Lymph contains cell wastes like cancer cells, bacteria, and viruses. This fluid then drains into lymph nodes where it's filtered by infection-fighting cells within the lymph nodes. These infection-fighting cells, also called white blood

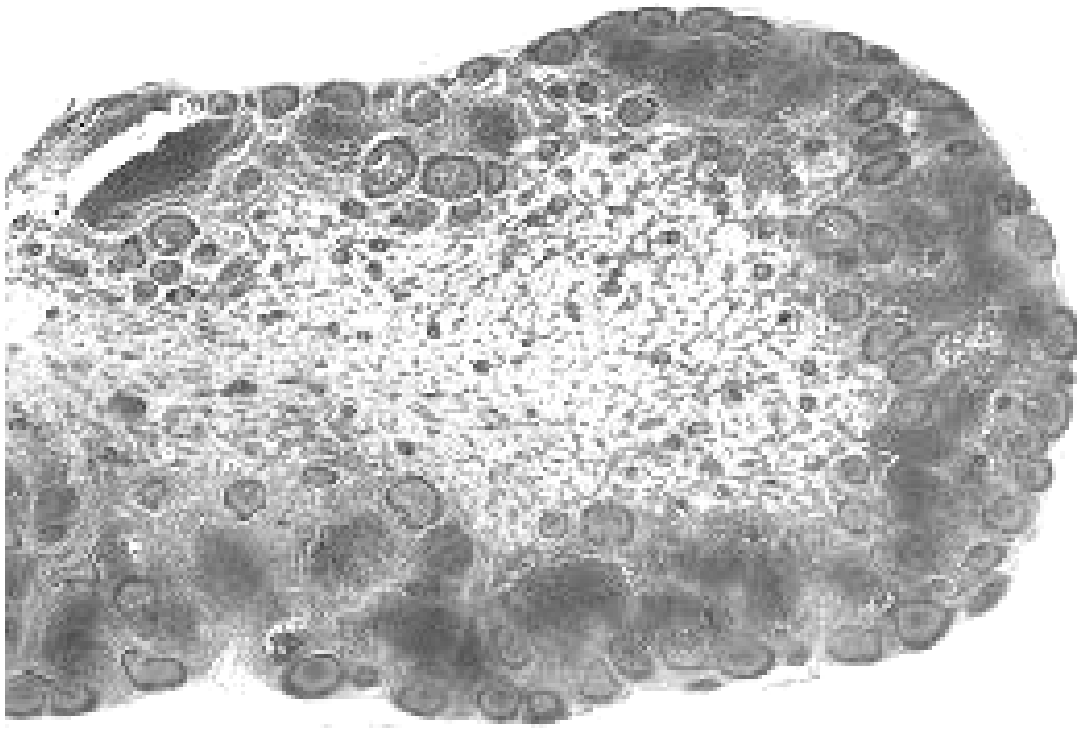


Fig. 6.3A : H/E Stained histological section of mamalian Lymph node (medcell.med.yale.edu)

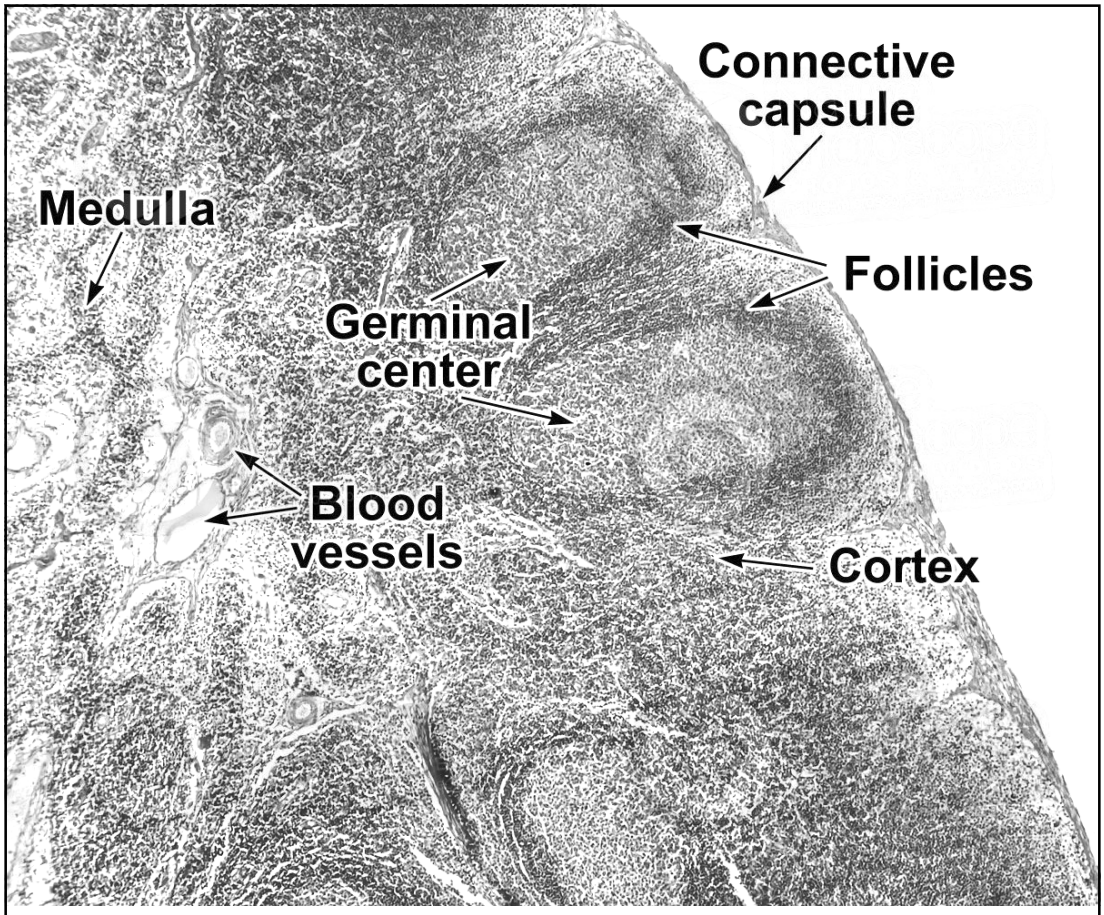


Fig. 6.3B : H/E Stained histological section of mamalian Lymph node at high resolution (nature-microscope-photo-video-com)

cells, destroy these foreign or "bad" cancer and infection-related cells. Lymph nodes are scattered throughout the body and located in groups, like in the armpit, groin, neck, pelvis, and abdomen. In some areas like the neck, the lymph nodes are located superficially and may be palpated—they feel like a pea or small bean. In other areas, like the abdomen or the chest, lymph nodes are located deeper and cannot be felt.

6.4.1 Identifying Characters

1. The structure includes the capsule, subcapsular sinus, cortex (B cell zone with follicles and germinal centers), paracortex (T cell zone), medullary sinuses, medullary cords and hilus.

2. The darker cortex at the periphery of the node has nodules, some of which show pale, mitotically active germinal centers.
3. In the middle of the lymph node is the medulla with its dark cords of dense lymphocyte population.
4. Paler lymph channels (the medullary sinuses) which have relatively fewer lymphocytes surround the medullary cords.

6.5 Questions

1. State the identifying characters of spleen. Mention their significance.
2. Mention the anatomical position of thymas. State that how could you identify a thymus tissue.
3. Discuss the general histological features of a lymph node. Mention about the zonation in the tissue.
4. What is red pulp? Differentiate between red and white pulp.
5. Differentiate between cortex and medulla of thymus.

Unit 7 □ Preparation of Stained Blood Film to study Various types of White Blood Cells

Structure

7.0 Objectives

In this unit learners will learn how to prepare a blood film to observe different blood cells.

Learnears will receive the practical knowledge of how different blood cell appear under microscope in standard staining technique.

Most importantly they will learn how different WBC appear under microscope and will be able to identify them for further analysis.

7.1 Introduction

There are two types of blood smears which are thick and thin blood smears. This two types of blood smear are prepared for different purposes. Thick blood smear is generally used for the detection of different blood parasites present in the blood.

Preparation of thick blood smear

Thick smears consist of a thick layer of dehemoglobinized (lysed) red blood cells (RBCs). The blood elements (including parasites, if any) are more concentrated (app. 30^x) than in an equal area of a thin smear. Thus, thick smears allow a more efficient detection of parasites (increased sensitivity). However, they do not permit an optimal review of

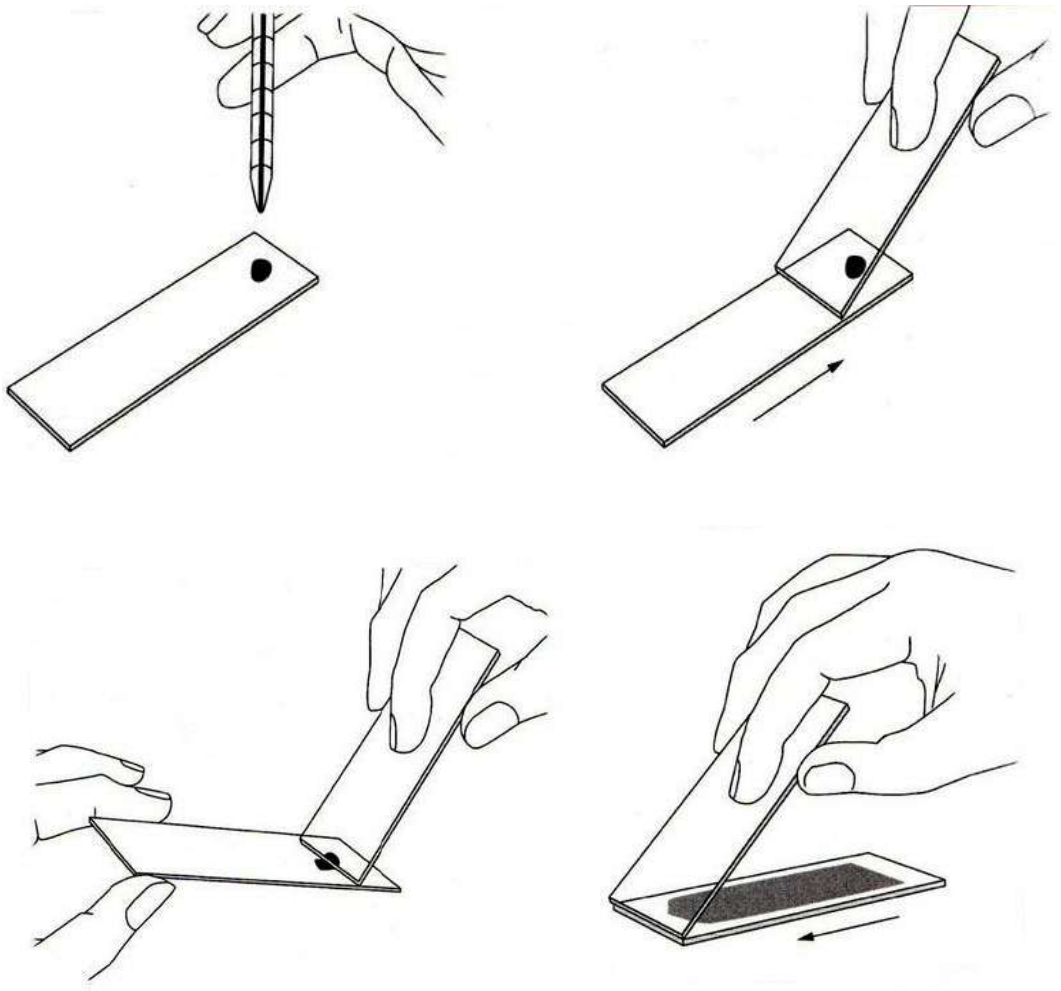


Fig. 7.1 : Procedure of blood smear preparation (researchgate.net)

parasite morphology. For example, they are often not adequate for species identification of malaria parasites: if the thick smear is positive for malaria parasites, the thin smear should be used for species identification.

1. Place a small drop of blood in the centre of the pre-cleaned, labelled slide.
2. Using the corner of another slide or an applicator stick, spread the drop in a circular pattern until it is the size of a diameter (1.5 cm^2).
3. A thick smear of proper density is one which, if placed (wet) over newsprint, allows you to barely read the words.

4. Lay the slides flat and allow the smears to dry thoroughly (protect from dust and insects). Insufficiently dried smears (and/or smears that are too thick) can detach from the slides during staining. The risk is increased in smears made with anticoagulated blood. At room temperature, drying can take several hours; 30 minutes is the minimum; in the latter case, handle the smear very delicately during staining. You can accelerate the drying by using a fan or hair dryer (use cool setting). Protect thick smears from hot environments to prevent heat-fixing the smear.
5. Do not fix thick smears with methanol or heat. If there will be a delay in staining smears, dip the thick smear briefly in water to haemolyse the RBCs.

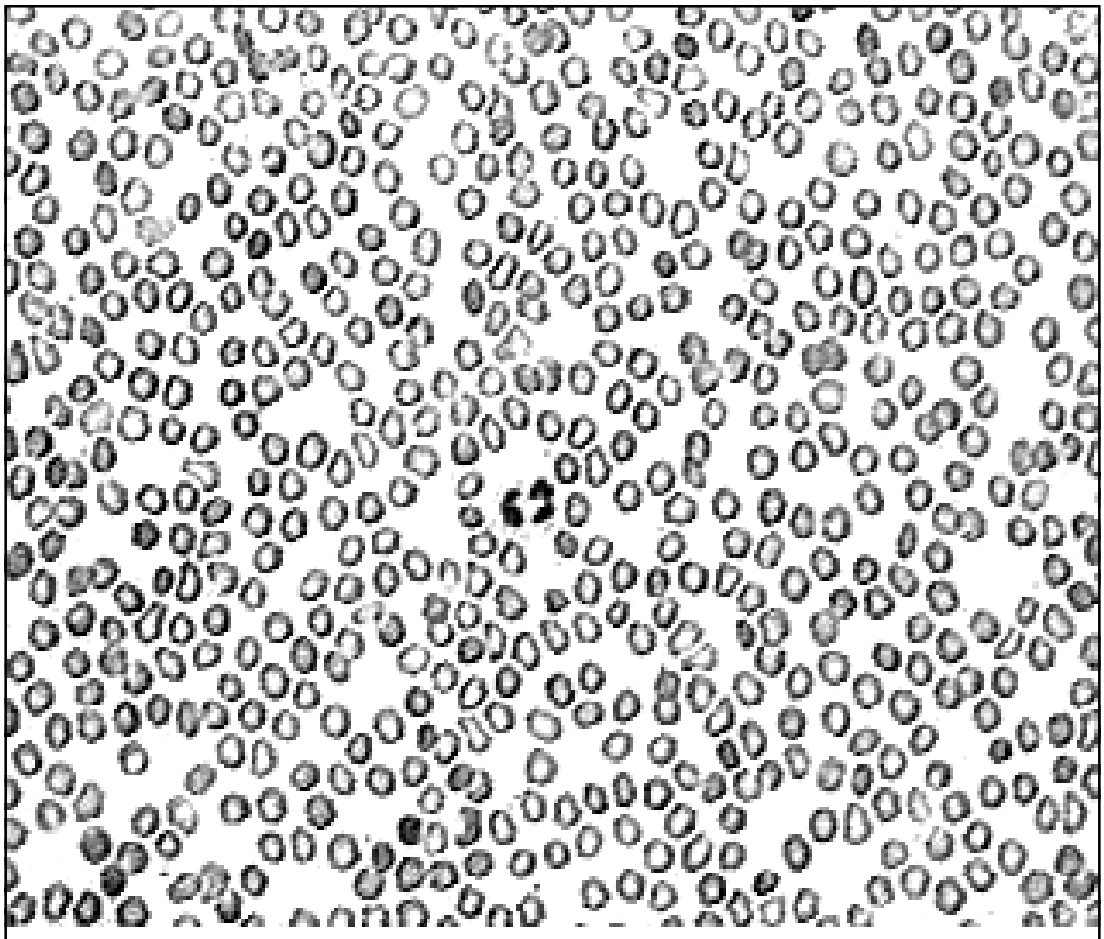


Fig. 7.2 : Blood film under microscope (at low magnification) with RBC (austince.edu)

Scratch Method for Thick smears

The scratch method is an alternate method for making thick films that allows for improved adherence and faster turnaround times. The process is similar to making a normal thick film, but instead of using a stick to spread the blood, the edge of a glass microscope slide is used, while applying firm pressure to create small scratches in the underlying slide. The scratches allow for improved adherence of the blood film to the slide without affecting the smear morphology. The smear can then be stained as soon as it is dry, generally within 20-30 minutes of smear preparation.

Preparation of thin blood smear

A well prepared thin blood smear is necessary for microscopic examination of blood. Blood smears are used to determine leukocyte differentials, to evaluate erythrocyte, platelet and leukocyte morphology, and, if necessary, to estimate platelet and leukocyte counts.

7.2 Collection of Sample

1. Finger Prick or
2. E.D.T.A. blood (within 1 hr. of collection)

7.3 Preparation of Blood Film

1. Place a small drop of blood, or one side about 1-2 cm from one end of a clean slide.
2. Without delay place a spreader at an angle of 45° from the slide and move it back to make contact with the drop. The drop should spread out quickly along the line of contact of spreader with the slide.
3. The moment this occurs, spread the film by rapid smooth forward movement of the spreader.
4. The film should be 3-4 cm in length. The ideal thickness is such that there is some overlap of R.B.C. throughout most of its length with proper separation and lack of distortion of RBC's. The end from where the spread had ended is called tail end.

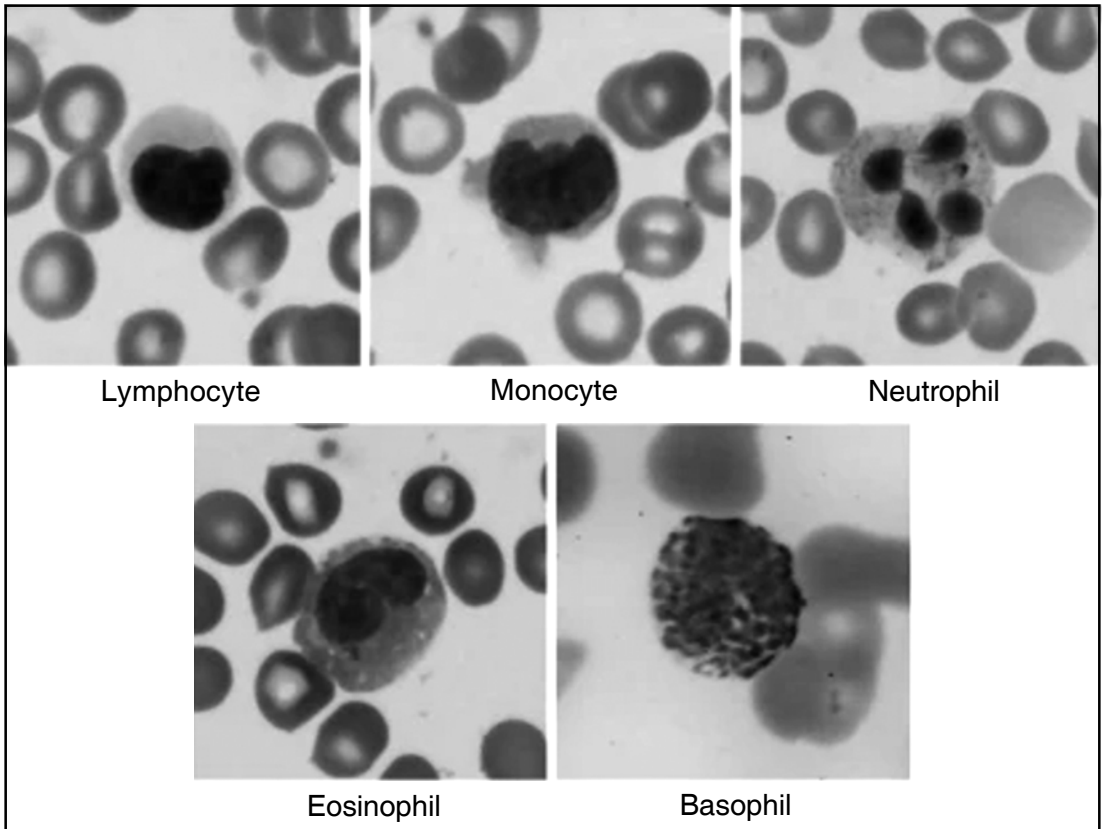


Fig. 7.3 : Different types of WBC observed under microscope at high magnification in a blood smear preparation (nature.com)

7.4 Staining

The slide is covered with Leishman stain for 2 mins. This much time is required for fixation. After 2 mins it is diluted with double the volume of buffer water. On adding buffer water a metallic shin will be formed, if the stain is dry. Allow this to stand for 15 min. after min, flood the slide with water to remove stain. Then wash under tap water wiping the back of slide with finger or cotton. Dry in air.

7.5 Precautions/Comments

1. Angle should be maintained at 45°.
2. Blood drop should be of proper size.

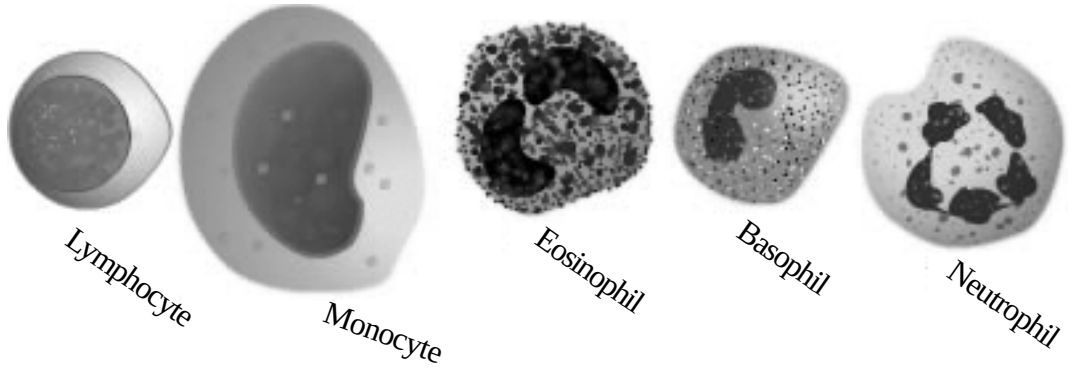


Fig. 7.4 : Different W.B.C

3. Spreader's edges should be smooth and it should be smaller than the slide on which smear is being made.
4. Pressure applied should be proper.
5. Drop should be pulled with spreader not pushed with it.
6. Preparation should be in one single stroke.
7. Initial staining time 2 minutes, is important. After dilution increase of 1-2 minutes, does not alter staining.
8. Never let the stain dry on the slide otherwise stain deposits will make it impossible to count leucocytes (DLC).
9. Staining should be deposit free.
10. For washing the smear -let the water stream replace the stain. Do not throws the stain first.

7.6 Questions

1. State the differences and utility of thick and thin smear preparation of blood.
2. Describe thick blood smear preparation and seratch method.
3. Describe thin blood smeare preparation and staining methods.
4. What precautions should be maintained in blood smear preparation to observe WBCs?
5. How can we identity—a) eosinophils, b) monocytes, c) neutrophils, d) lymphocytes.

Unit 8 □ Demonstration of ELISA

Structure

8.0 Objectives

ELISA is an immuneassay where monoclonal antibodies are used to detect specific antiges/biomolecules and prominantly used in clinical diagnosis and research. Therefore, with the practical knowledge of this technique learners will learn—

- How an immune technique can be used to detect and quantity specific target proteins of our body precisely.
- How monoclonal antibodies and their conjugates can be used in diagnosis.
- How the protocol of ELISA can be performed.

8.1 Introduction

Enzyme-linked immunosorbent assayes (ELISA) is a fundamental tool of clinical immunology, and is used as an initial screen for HIV detection. Based on the principle of antigen-antibody interaction. This test allows for easy visualization of results. ELISA utilizes the enzyme immobilized in antibodies or antigens. It is used to quantify the antigen or antibody concentration. It is the common serological test used for the detection of a specific antigen or antibody. ELISA has been used as a diagnostic tool in medicine and plant pathology, as well as quality control check in various industries.

ELISAs can be performed with a number of modifications to the basic procedure: **direct, indirect, sandwich or competitive.** The key step, immobilization of the antigen of

interest, can be accomplished by direct adsorption to the assay plate or indirectly via a capture antibody that has been attached to the plate. The antigen is then detected either directly (enzyrne-labeled primary antibody) or indirectly (enzyme-labeled secondary antibody). The detection antibodies are usually labeled with **alkaline phosphatase (AP) or horseradish peroxidase (HRP).** A large selection of substrates is available for performing the ELISA with an HRP or AP conjugate. The choice of substrate depends upon the required assay sensitivity and the instrumentation available for signal-detection **(spectrophotometer, fluorometer or luminometer).** Among the standard assay formats discussed and illustrated below, where differences in both capture and detection were the concern, it is important to differentiate between the particular strategies that exist specifically for the detection step. However an antigen is captured to the plate (by direct adsorption to the surface or through a pre-coated "capture" antibody, as in a sandwich ELISA), it is the detection step (as either direct or indirect detection) that largely determines the sensitivity of an ELISA.

1. Direct ELISA

For direct detection, an antigen coated to a multi-well plate is detected by an antibody that has been directly conjugated to an enzyme. This detection method is a good option if there is no commercially available ELISA kits for your target protein.

Advantages

- Quick because only one antibody and fewer steps are used.
- Cross-reactivity of secondary antibody is eliminated.

Disadvantages

- Immune reactivity of the primary antibody might be adversely affected by labeling with enzymes or tags.
- Labeling primary antibodies for each specific ELISA system is time-consuming and expensive.
- No flexibility in choice of primary antibody label from one experiment to another.
- Minimal signal amplification.

2. Indirect ELISA

For indirect detection, the antigen coated to a multi-well plate is detected in two stages or layers. First an unlabeled primary antibody, which is specific for the antigen, is applied.

Next, an enzyme-labeled secondary antibody is bound to the first antibody. The secondary antibody is usually an anti-species antibody and is often polyclonal. The indirect assay, the most popular format for ELISA, has the advantages and disadvantages:

Advantages

- A wide variety of labeled secondary antibodies are available commercially.
- Versatile because many primary antibodies can be made in one species and the same labeled secondary antibody can be used for detection.
- Maximum immuno-reactivity of the primary antibody is retained because it is not labeled.
- Sensitivity is increased because each primary antibody contains several epitopes that can be bound by the labeled secondary antibody, allowing for signal amplification.

Disadvantages

- Cross-reactivity might occur with the secondary antibody, resulting in nonspecific signal.
- An extra incubation step is required in the procedure.

3. Sandwich ELISA

Sandwich ELISAs typically require the use of matched antibody pairs, where each antibody is specific for a different, non-overlapping part (epitope) of the antigen molecule. A first antibody (known as capture antibody) is coated to the wells. The sample solution is then added to the well. A second antibody (known as detection antibody) follows this step in order to measure the concentration of the sample. This type of ELISA has the following advantages:

- High specificity :the antigen/analyze is specifically captured and detected
- Suitable for complex (or crude/impure) samples: the antigen does not require purification prior to measurement
- Flexibility and sensitivity: both direct or indirect detection methods can be used.

4. Competitive ELISA

The key event of competitive ELISA (also known as inhibition ELISA) is the process of competitive reaction between the sample antigen and antigen bound to the wells of a

micro titer plate with the primary antibody. First, the primary antibody is incubated with the sample antigen and the resulting antibody-antigen complexes are added to wells that have been coated with the same antigen. After an incubation period, any unbound antibody is washed off. The more antigen in the sample, the more primary antibody will be bound to the sample antigen. Therefore, there will be a smaller amount of primary antibody available to bind to the antigen coated on the well, resulting in a signal reduction. The main advantage of this type of ELISA arises from its high sensitivity to compositional differences in complex antigen mixtures, even when the specific detecting antibody is present in relatively small amounts.

8.2 Materials and Method

Sample Preparation

The procedure below provides a general guidance for the preparation of commonly tested samples for use in ELISA assays. Generally:

- Protein extract concentration is at least 1-2 mg/mL.
- Cell and tissue extracts are diluted by 50% with binding buffer.
- Samples are centrifuged at 10,000 rpm for 5 min at 4°C to remove any precipitate before use.

1. Cell Culture Supernatants

Centrifuge cell culture media at 1,500 rpm for 10 min at 4°C. Aliquot supernatant immediately and hold at -80°C, avoiding freeze/thaw cycles.

2. Cell Extracts

Place tissue culture plates on ice. Remove the media and gently wash cells once with ice-cold PBS. Remove the PBS and add 0.5 ml extraction buffer per 100 mm plate. Tilt the plate and scrape the cells into a pre-chilled tube. Vortex briefly and incubate on ice for 15-30 min. Centrifuge at 13,000 rpm for 10 min at 4°C (this creates a pellet from the insoluble content). Aliquot the supernatant into clean, chilled tubes (on ice) and store samples at -80°C, avoiding freeze/thaw cycles.

3. Conditioned Media

Plate the cells in complete growth media (with serum) until the desired level of confluence is achieved. Remove the growth media and gently wash cells using 2-3 mL of

warm PBS. Repeat the wash step. Remove the PBS and gently add serum-free growth media. Incubate for 1-2 days. Remove the media into a centrifuge tube. Centrifuge at 1,500 rpm for 10 min at 4°C. Aliquot the supematant and keep samples at -80°C, avoiding freeze/thaw cycles.

4. Tissue Extract

Mince tissue on ice in ice-cold buffer, preferably in the presence of protease inhibitors. Place the tissue in micro-centrifuge tubes and dip into liquid nitrogen to snap freeze. Keep samples at -80°C for later use or keep on ice for immediate homogenization. For every 5 mg of tissue, add 300 ul, of extraction buffer to the tube and homogenize:

- l00 mM Tris, pH 7.4
- 150 mM NaCI
- 1 mM EGTA
- 1 mM EDTA
- 1% Triton X-l00 0.5%
- 0.5% sodium deoxy cholate

(This portion of the buffer can be prepared ahead of time and stored at 4°C. Immediately before use, the buffer must be supplemented with phosphatase inhibitor cocktail [as directed by manufacturer), protease inhibitor cocktail [as directed by manufacturerjand PMSF to* 1 *mM to generate a complete extraction buffer solution.)

Rinse the blade of the homogenizer twice with 300 ul, extraction buffer.

Place the sample on a shaker at 4°C for 2 hours. Centrifuge the sample for 20 min at 13,000 rpm at 4°C.

Aliquot the supematant into pre-chilled tubes sitting in ice.

Keep the samples at -80°C, avoiding freeze/thaw cycles.

Rinse the blade of the homogenizer twice with 300 ul, extraction buffer.

Place the sample on a shaker at 4°C for 2 hours. Centrifuge the sample for 20 min at 13,000rpm at 4°C.

Aliquot the supematant into pre-chilled tubes sitting in ice. Keep the samples at –80°C, avoiding freeze/thaw cycles.

Note: Lysis buffer volume must be determined according to the amount of tissue present. Typical concentration of final protein extract is at least 1 mg/mL.

Reagent Preparation

1. Standard Solutions

- 10,000 pg/mL: Add 1 mL of sample diluents buffer into one tube of standard (10 ng per tube) and mixthoroughly. Note: Store this solution at 4°C for up to 12 hours (or – 20°C for 48 hours)and avoid freezethaw cycles.
- 5,000 pg/mL: Mix 0.3 mL of 10,000 pg/mL with 0.3 mL of sample diluent buffer and mix thoroughly.
- 2,500 pg/mL: Mix 0.3 mL of 5,000 pg/mL with 0.3 mL of sample diluent buffer and mix thoroughly.
- Perform similar dilutions until the standard solutions with these concentrations (pg/mL) are made: 1,250,625,312, 156 and 78.
- Add 100 μL of each of the diluted standard solutions to the appropriate empty wells. Repeat in duplicate or triplicate for accuracy. Note: The standard solutions are best used within 2 hours.

2. Biotinylated Antibody

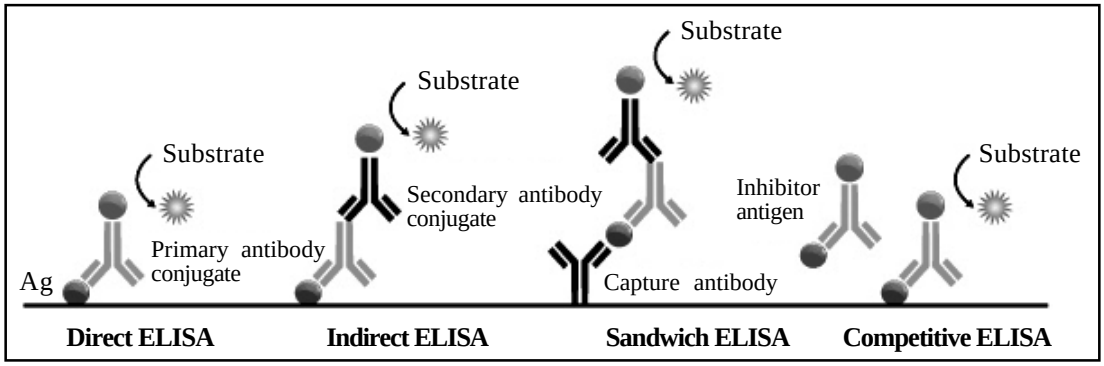
- Calculate the total volume needed for the assay by multiplying 0.1 mL/well and the number of wells required. Add 2-3 extra wells to the calculated number of wells to account for possible pipetting errors.
- Generate the required volume of diluted antibody by performing a l : 100 dilution (For each l μL concentrated antibody, add 99 μL antibody dilution buffer) and mixing thoroughly.

3. Avidin-Biotin-Peroxidase (ABC)

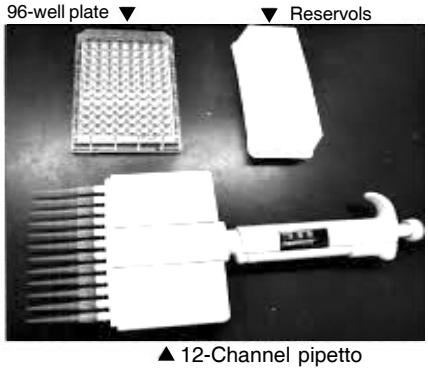
- Calculate the total volume needed for the assay by multiplying 0.1 mL/well and the number of wells required. Add 2-3 extra wells to the calculated number of wells to account for possible pipetting errors.
- Generate the required volume of diluted ABC solution by performing a 1 : 100 dilution (For each 1 μL concentrated ABC solution, add 99 μL ABC dilution buffer) and mixing thoroughly. Note: The diluted ABC solution should not be prepared more than 1 hour prior to the experiment.

8.2.1 Materials and Method Protocols

1. Add diluted antibody to each well of a 96-well ELISA plate. Seal the plate to prevent evaporation, and allow it to incubate at 4°C for 15-18 hours to immobilize the antibody.
2. Remove the diluted antibody, and wash 3 times with washing solution.
3. Add blocking buffer to each well, and allow it to incubate at 37°C for 1 hour to reduce non-specific binding of the target protein to the well.
4. Remove the blocking buffer, and wash 3 times with washing solution.
5. Dilute the samples with sample dilution buffer, and add 100 µl, of each sample to each well. For the calibration curve, prepare a dilution series of the standard on the same plate. Allow it to incubate at 37°C for 1 hour.
6. Remove the samples, and wash 5 times with washing solution.
7. Dilute the detection antibody in sample dilution buffer, and add 100 µl, to each well. Allow it to incubate at 37°C for 1 hour.
8. After reaction, remove the detection antibody, and wash 5 times with washing solution.
9. Dilute an enzyme-labeled secondary antibody with sample dilution buffer, and add 100 µl, to each well. Allow it to incubate at 37°C for 1 hour.
10. After reaction, remove the secondary antibody, and wash 5 times with washing solution.
11. Allow it to incubate as the color develops.
12. Add a stop solution to stop the reaction when the color IS sufficiently developed.
13. Measure the absorption at 450 nm by a plate reader.

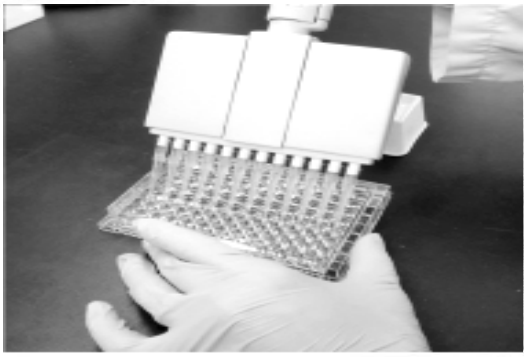


96-well plate ▼ ▼ Reservols



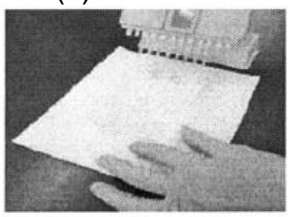
▲ 12-Channel pipetto

(1)



(2)

Adding slop solution ▶



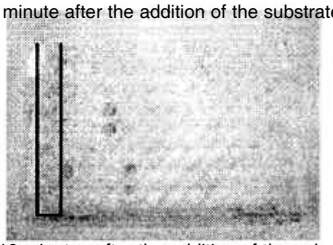
(3)

▼ After the addition of slop solution

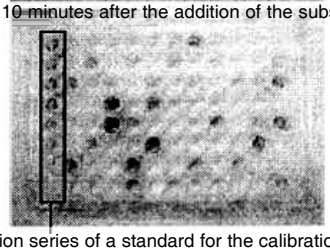
Dilution series of a standard for the calibration curve

(4-5)

1 minute after the addition of the substrate



10 minutes after the addition of the substrate



Dilution series of a standard for the calibration curve

(6-7)

Plate reader ▼

(8)

8.3 Comments

To perform ELISA of unknown samples a calibration process is needed which require dilution of standard reference serum.

Different combination of enzyme-substrate systems may be used like avidin-biotin-peroxidase or alkaline phosphatase lebelled enzyme system to develop the color reaction to detect and quantity target protein or biomolecule in the photometric reaction.

The ELISA plate-reader is the essential instrument to perform this technique which is a modified form of spectrophotometric device to read the color intensity generated in each well of ELISA plate or microtiter plate.

8.4 Questions

1. Describe the methodology to perform sandwich ELISA.
2. Briefly state the procedure for preparation of tissue extract to perform ELISA.
3. Why standard solution is necessary. Describe its preparation and use in ELISA.
4. State the procedure of preparing working solutions of different antibodies and enzyme substrate solution to utilize in ELISA.
5. How can be utilize cell extract or conditioned media in ELISA technique?