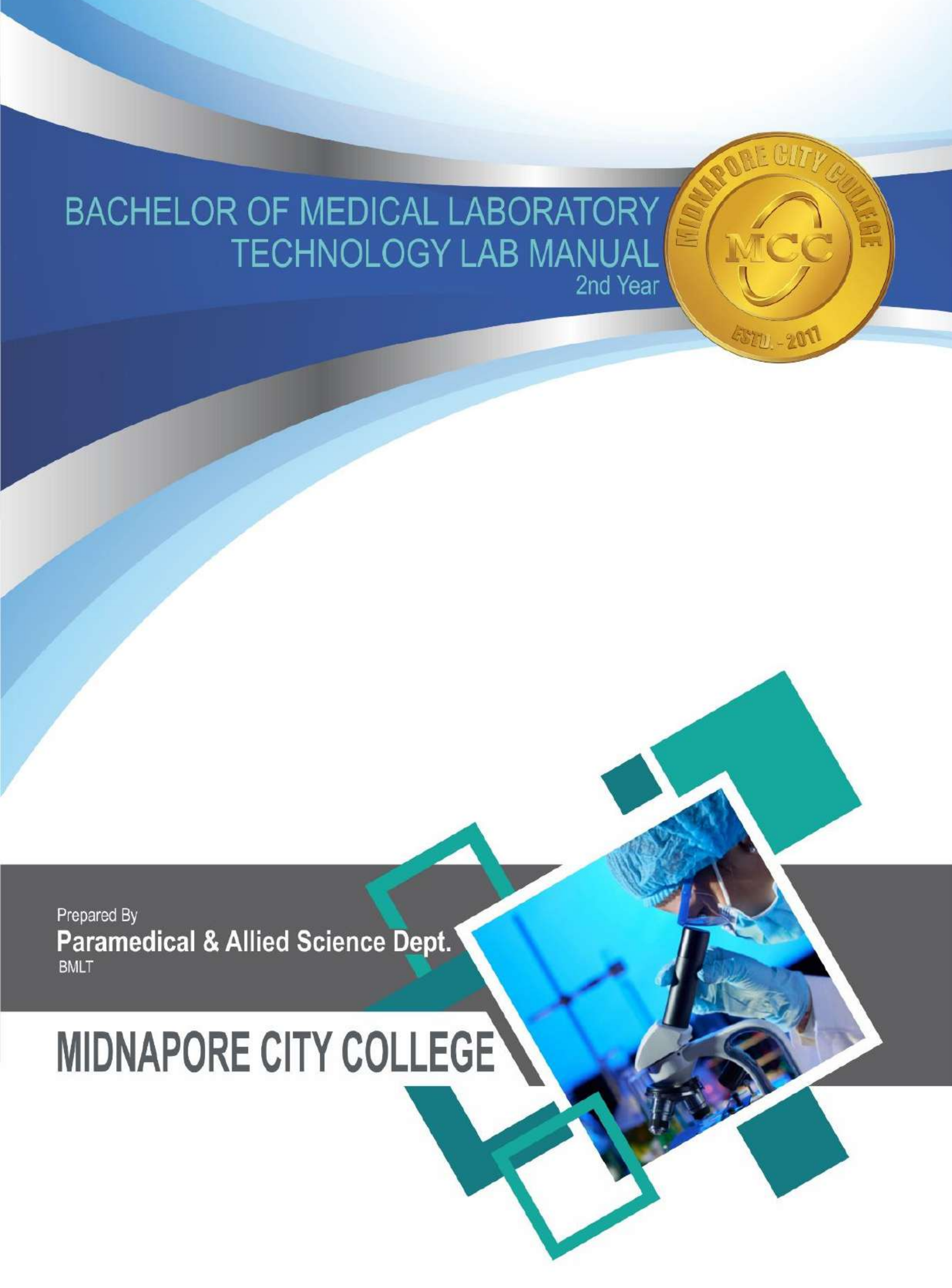


BACHELOR OF MEDICAL LABORATORY TECHNOLOGY LAB MANUAL

2nd Year

Prepared By
Paramedical & Allied Science Dept.
BMLT

MIDNAPORE CITY COLLEGE

BMLT (WBUHS) 2nd SEMESTER

Paper-201

EMBEDDING

Embedding is the process in which the tissues or the specimens are enclosed in a mass of the embedding medium using a mould. Since the tissue blocks are very thin in thickness, they need a supporting medium in which the tissue blocks are embedded. This supporting medium is called embedding medium. Various embedding substances are paraffin wax, celloidin, synthetic resins, gelatine, etc.

The choice of embedding media depends upon

1.Type of microscope 2. Type of microtome 3. Type of tissue eg. hard tissue like bone or soft tissue like liver biopsy

Paraffin wax with a higher melting point (56 to 62oC) is used for embedding. The molten wax is filtered inside the oven through a course filter paper into another container. This will protect the knife edge.

OTHER TYPES OF EMBEDDING MEDIA

1.Carbowax: It is a water-soluble wax. Therefore, tissues are directly transferred to water soluble wax after fixation and washing.

Methacrylate: It is easily miscible with alcohol and gives a clear and hard block when polymerised Polymerization takes place in the presence of a catalyst. Any trace of water causes uneven polymerization and formation of bubbles in the block around the tissue.

2.Epoxy Resin (Araldite): Epoxy polymers of araldite is used in higher resolution work and to see greater details. Epoxy resins are used for electron microscopy. Epoxy polymers of araldite differ from methcrylate in that they are crosslinked causing the cured solid block of araldite to be insoluble in any solvent. Longer filtration is required because the viscosity of resin is greater than methacrylate. For electron microscopy araldite is obtained as casting resinCY212, ahardner DDSA and an amine accelerator, DMP (ditrimethylamino methyl phenol) Blocks are suitably cured before sectioning for 48 to 60 hours at 60oC.

3.Agar embedding: It is mainly used in double embedding. Multiple fragments and friable tissue may be impregnated in one block when sectioning on the cryostat. Another use of agar embedding is for FNAC specimens.

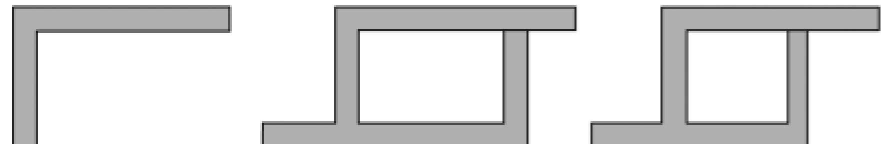
4.Celloidin media: Celloidin is a purified form of nitrocellulose. It is used for cutting hard tissues.

5.Gelatin: Its melting point is less than the melting point of agar. Gelatin may be used when frozen sections are required on friable and necrotic tissues.

Preparation of blocks

TYPES OF MOULDS

A variety of moulds are used for embedding. These may be LEUCKHARD embedding moulds (L mould) paper blocks, plastic moulds. Most of the laboratories use L moulds. L moulds are made up of metal, easy to procure, reusable and may be adjusted to make different size of blocks. One limb of the"L" is longer than the other. The two "Ls" are jointed to form a side of the rectangular box that act as a cast to make the mould.



Plastic moulds: Most of the laboratories use plastic embedding rings now. These are relatively inexpensive, convenient and support the block during sectioning and are designed to fit it on the microtome. This eliminates the step of mounting or attaching the block on a holder (metal or wooden holder).

Tissue-Tek System1 or Mark1 system : In this system plastic embedding rings with stainless steel moulds allow rapid embedding and cutting of tissues. In this system the blocks are stored with the plastic rings; the angle does not change for further requirement of sections.

The disadvantage of this method is that the space required for storing is more.

Tissue-Tek system 2or Mark 2 system : The Mark 2 system has provided a cassette to hold tissue during processing and has a stainless-steel lid on the plastic cassette. The cassette has a rough surface on one side of it with a slope where the accession number or the marking is done using a permanent marker.

Tissue embedding machine

All the blocking steps can be performed with the help of tissue embedding machine. The embedding machine contains the following parts –

Mould warmer, cassette bath, working surface warmer with a nozzle for pouring the wax, forceps well and cold plate.

The cold plate is of high efficiency refrigeration system having temperature control ranging from different freezing points to 4- or 5degree C. It can occupy about 50-60 blocks.

Large 3-5 litre capacity paraffin reservoir with adjustable temperature of 45- 75-degree C.

Optional vacuum lids, which allows for vacuum infiltration of tissues.

It has a forceps warmer convenient drain for excess wax.

The embedding machines are available with many other features.

Method of Embedding

1. Open the tissue cassette, check requisition form entry to ensure the correct number of tissue pieces is present.

2. Select the mould; there should be sufficient room for the tissue with allowance for at least a 2 mm surrounding margin of wax. Leuckhart mould method-This is the traditional embedding method. The “L moulds are adjusted according to the shape and size of the tissue. Glycerine may be applied to the L pieces and also to the metal or glass plate on which the moulds are placed for embedding. Simple glossed wall or floor tiles may also be used in place of glass plate.

3. Fill the mould with paraffin wax.

4. Using warm forceps select the tissue, taking care that it does not cool in the air; at the same time.

5. Place the tissue in the mould according to the side to be sectioned. This side should be facing down against the mould. A small amount of pressure may be used in order to have more even embedding.

6. Chill the mould on the cold plate, orienting the tissue and firming it into the wax with warmed forceps. This ensures that the correct orientation is maintained and the tissue surface to be sectioned is kept flat.

7. Insert the identifying label or place the labelled embedding ring or cassette base onto the mould

8. Add more paraffin into the mould to fill the cassette and mould.

9. Cool the block on the cold plate.

10. Remove the block from the mould.

11. Cross check block, label and requisition form.

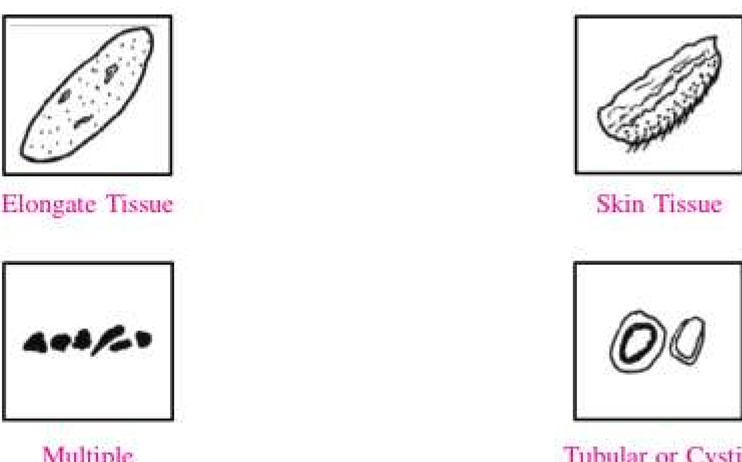
Some general considerations are as follows:

Elongate tissues are placed diagonally across the block.

Tubular and walled specimens such as vas deferens, cysts and gastrointestinal tissues are embedded so as to provide transverse sections showing all tissue layers.

Tissues with an epithelial surface such as skin, are embedded to provide sections in a plane at right angles to the surface (hairy or keratinized epithelia are oriented to face the knife diagonally).

Multiple tissue pieces are aligned across the long axis of the mould, and not placed at random. Incorrect placement of tissues may result in diagnostically important tissue elements being missed or damaged during microtomy. In circumstances where precise orientation is essential, tissue should be marked or agar double embedded. Usually, tissues are embedded with the surface to be cut facing down in the mould.



MICROTOME AND SECTION CUTTING

A microtome (from the Greek mikros, meaning "small", and temnein, meaning "to cut") is a tool used to cut extremely thin slices of material, known as sections.

Various types of microtomes are available. Most commonly used microtome for routine histopathology is rotary microtome. The most common applications of microtomes are:

Traditional Histology Technique: Tissues are hardened by replacing water with paraffin. The tissue is then cut in the microtome at thicknesses varying from 2 to 50 µm. From there the tissue can be mounted on a microscope slide, stained with appropriate aqueous dye(s) after prior removal of the paraffin, and examined using a light microscope.

Cryosectioning Technique: Water-rich tissues are hardened by freezing and cut in the frozen state with a freezing microtome or microtome-cryostat; sections are stained and examined with a light microscope. This technique is much faster than traditional histology (15 minutes vs 16 hours) and is used in conjunction with medical procedures to achieve a quick diagnosis. Cryosections can also be used in immuno histochemistry as freezing tissue stops degradation of tissue faster than using a fixative and does not alter or mask its chemical composition as much.

Electron Microscopy Technique: After embedding tissues in epoxy resin, a microtome equipped with a glass or gem grade diamond knife is used to cut very thin sections (typically 60 to 100 nanometer). Sections are stained with an aqueous solution of an appropriate heavy metal salt and examined with a transmission electron microscope (TEM). This instrument is often called an ultramicrotome. The ultramicrotome is also used with its glass knife or an industrial grade diamond knife to cut survey sections prior to thin sectioning. These sections are of 0.5 to 1 µm thickness and are mounted on a glass slide and stained to locate areas of interest under a light microscope prior to thin sectioning for the TEM. Thin sectioning for the TEM is often done with a gem quality diamond knife. z Botanical Microtomy Technique:

Hard materials like wood, bone and leather require a sledge microtome. These microtomes have heavier blades and cannot cut as thin as a regular microtome.

Rotary Mictrotome - It is most commonly used microtome. This device operates with a staged rotary action such that the actual cutting is part of the rotary motion. In a rotary microtome, the knife is typically fixed in a horizontal position. A rotary action of the hand wheel actuate the cutting movement. Here the advantage over the rocking type is that it is heavier and there by more stable. Hard tissues can be cut without vibration. Serial sections or ribbons of sections can easily be obtained. The block holder or block (depends upon the type of cassette) is mounted on the steel carriage that moves up and down and is advanced by a micrometer screw. Auto-cut microtome has built in motor drive with foot and hand control. With suitable accessories the machine can cut thin sections of paraffin wax blocks and 0.5 to 2.0 micrometer thin resin sections.

Fig. 9.1: Rotary Microtome

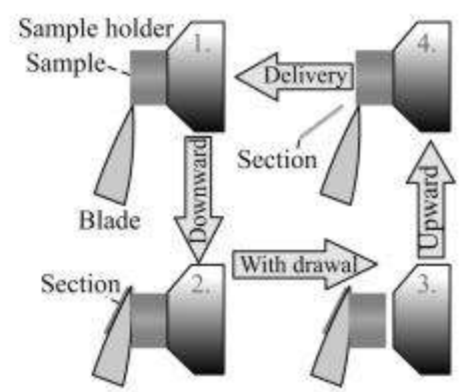


Fig. 9.2: Principle of sample movement for making a cut on a rotary microtome

In the figure to the left, the principle of the cut is explained. Through the motion of the sample holder, the sample is cut by the knife position 1 to position 2), at which point the fresh section remains on the knife. At the highest point of the rotary motion, the sample holder is advanced by the same thickness as the section that is to be made, allowing for the next section to be made. The flywheel in microtomes can be operated by hand. This has the advantage that a clean cut can be made, as the relatively large mass of the flywheel prevents the sample from being stopped during the sample cut. The flywheel in newer models is often integrated inside the microtome casing. The typical cut thickness for a rotary microtome is between 1 and 60 µm. For hard materials, such as a sample embedded in a synthetic resin, this design of microtome can allow for good “Semi-thin” sections with a thickness of as low as 0.5 µm.

PROPERTIES OF HEMATOXYLIN

1. Hematoxylin has no staining property.

2. Hematin with mordant such as ammonium or potassium alum forms lake which functions as cationic dye and stains anionic tissue components.

3. Hematin in an aqueous solution can be acidic or an alkaline dye depending on pH.

4. Hematin has affinity for several tissues with an appropriate mordant.

Progessive staining - When tissue is left in the stain just long enough to reach the proper end point. The slides have to be examined at different interval to find out when the staining is optimum.

Regressive staining - In this method the tissue is overstained and then destained (differentiate) until the proper endpoint is reached. Harris hematoxylin is a regressive stain; the overstaining is removed by acid - alcohol. The removal of this excess dye is called differentiation. The hematoxylin alum gives a reddish hue to the tissues because of acidic pH. To convert this colour to the final blue, alkaline pH is required. This process is called “blueing”. It is done either by tap water or by ammonium hydroxide.

Preparation of Harris’s hematoxylin

Ingredients:

Hematoxylin 5gm

Absolute alcohol 50ml

Ammonium alum 100gm

Distilled water 1000ml

Mercuric oxide 2.5gm

Glacial acetic acid 40ml

Method - Dissolve the hematoxylin in absolute alcohol and ammonium alum in hot water. Mix the two solutions and heat to boiling. Remove from flame, and add mercuric oxide and cool rapidly. Glacial acetic acid if added gives brisk nuclear staining, but life of the solution is reduced. Hence if acetic acid is to be added, it should be added in working solution.

Preparation of Mayer’s hematoxylin

Ingredients :

Hematoxylin 1.0gm

Distilled water 1000ml

Ammonium alum 50gm

Sodium iodate 0.2gm

Citric acid (reduces pH) 1.0gm

Chloral hydrate (preservative) 50gm

Method - Hematoxylin is dissolved in distilled water using gentle heat. Then alum is added and dissolved. Then sodium iodate, citric acid and chloral hydrate are added respectively.

EOSIN

Eosin is used as the counterstain that stains the cytoplasm rose coloured. The intensity of the eosin is individual choice. The most widely used eosin is "eosin Y". The "Y" stands for yellowish. It is available in either water soluble or alcohol soluble form. Most laboratories use the water-soluble form of eosin Y in an alcohol-water solution which is described here.

Eosin Y (water soluble) 1.0gm

Distilled water 80ml

95% alcohol 320ml

Glacial acetic acid 0.4ml

METHOD OF STAINING

1. Deparaffinize sections in xylene, 10-20 minutes. Filter Hematoxylin.

2. Rehydrate sections: 100% alcohol for 1-2 minutes 95% alcohol for 1-2 minutes

3. Rinse in tap water 4. Rinse in distilled water 5. Stain with Hematoxylin for 3-5 minutes

6. Wash in tap water

7. Differentiate section with 1% HCl in 70% alcohol 1-2 dips and check under microscope. If necessary, return slides to HCl for further differentiation.

8. Wash slides in running tape water for 15 minutes

9. Stain slides in Eosin for 1-4 minutes

10. Dehydration and Differentiation: 95% alcohol 5-6 dips 100% alcohol 5-6 dips

11. Clear slides in xylene 2 times

12. Mount slides with mounting media (Permount or DPX).

PERIODIC ACID – SCHIFF (PAS) STAIN

Periodic acid causes oxidation of 1:2 glycol groups in the tissues to di-aldehydes. The di-aldehyde reacts with fuchsin – sulfurous acid solution (Schiff's) to form a magenta-colored compound.

Aim: To demonstrate glycogen, epithelial mucin, fungi, amoeba and basement membrane.

Control: Liver and intestine

Reagents

Periodic acid 1%

Distilled water 100 mL

Schiff's reagent

Basic fuchsin 1 gm

Distilled water 200 ml

1N hydrochloric acid 20 ml

Sodium or Potassium metabitesulfite 1gm

Activated charcoal 2gm

1.Dissolve basic fuchsin in boiling distilled water

2.Shake for 5 minutes and cool to 50°C z Filter and add 1N solution.

3.Cool further and add sodium or potassium metabisulfite.

4.Keep for 18 hours in dark.

5.Add activated charcoal, shake well, filter and store the solution at 40°C.

Procedure

Bring the sections to water. z Dip the slide in Periodic acid solution for 5-10 minutes.

Wash in tap water and rinse in distilled water. Z

Put Schiff's reagent on the section for 20 minutes.

Wash thoroughly in running water.

Counerstain with Hematoxylin, dehydrate, clear and mount in DPX.

Result

Glycogen (except non-sulfated acid mucopolysaccharide), basement membrane, fungi, parasites and other positive substances – magenta z Nucleus – blue or violet.

STAIN FOR RETICULIN FIBRES

Aim: To identify reticulin fibers in sections.

Principle: Reticulin fibers are treated with potassium permagnate to produce sensitized sites for silver deposition. Silver is in a form readily able to precipitate as metallic silver. Formalin, a reducing agent causes deposition of metallic silver at pH 9.0.Excess silver is removed by sodium thiosulphate solution .Treatment with gold chloride produces permanent precipitate.

Control: Normal liver.

Reagents

Acidified potassium permagnate

0.5% potassium permagnate 95ml

3% sulfuric acid 5ml

Solution should be made fresh.

Silver nitrate solution

To 5ml of 10% aqueous silver nitrate,

add strong ammonia drop by drop until the precipitate which has formed initially is dissolved. Add 5ml of 3% sodium hydroxide. Again add strong ammonia drop by drop till the precipitate is completely dissolved. Add distilled water to make it 50ml and keep it in a jar.

2% Oxalic acid

Oxalic acid 2gm

Distilled water 100ml

4% aqueous iron alum

Ferric ammonium sulphate

Distilled water 100ml

10% Formalin

Formaldehyde 10ml

Distilled water 90ml

0.2% Gold chloride

Gold chloride 0.2%

Distilled water 100ml

Store in refrigerator

2% Sodium thiosulphate

Sodiun thiosulphate 2gm

Distilled water 100ml

Neutral red (acidified)

Neutral red 1gm

Distilled water 100ml

Glacial acitic acid 1ml

Dissolve the dye in distilled water. Add the acid, mix, filter and store.

Procedure 1. Deparaffinize and bring the sections to water. 2. Oxidize in acidified potassium permagnate for 3 minutes. 3. Rinse in distilled water. 4. Decolorize with 2% oxalic acid for 1 minute. 5. Rinse in distilled water. 6. Put iron alum for 10 minutes. 7. Rinse in distilled water. 8. Put ammonical silver solution for 10 seconds. 9. Rinse in distilled water. 10. Immediately reduce with formalin for 2 minutes. 11. Wash in running tap water for 2 minutes. 12. Tone in 0.2% gold chloride for 2 minutes. 13. Rinse in distilled water. 14. Fix in 2% thiosulphate for 2 minutes. 15. Wash in water for 2 minutes. 16. Counter-stain with neutral red for 2 minutes. 17. Dehydrate, clear in xylene and mount in DPX.

Result

Reticulin fibres -black

Nuclei – red

VERHEOFF STAIN FOR COLLAGEN

Aim: To identify collagen and elastic tissue in the same section.

Principle: In the presence of ferric salts (oxidizers) elastic fibers stain with hematoxylin, along with the nuclei.

Control: skin

Reagents

1. Verhoeff's solution: Freshly prepared solution gives best result.

Solution A
Hematoxylin 5gm
Absolute alcohol 100ml
Dissolve hematoxylin with the aid of heat, cool and filter.
Solution B
Ferric chloride 10gm
Distilled water 100ml
Solution C
Iodine 2gm
Potassium iodide 4gm
Distilled water 100ml
Add 8ml of solution B into 20ml of solution A and then add 8ml of solution C.

2. 2% Ferric chloride solution
3. 1% aqueous solution of acid fuchsin
4. Saturated aqueous solution of picric acid
5. Van Gieson's stain

Acid Fuchsin 1% (aqueous) 5ml
Saturated aqueous solution of picric acid 100ml

6. Sodium thiosulphate, 5% (aqueous solution)

Procedure

1. Deparaffinize and take the section to water.
2. Stain in Verhoeff solution until the section is black.
3. Wash in distilled water.
4. Differentiate in 2% Ferric chloride with agitation for few minutes. Check differentiation by rinsing in distilled water. Under the microscope the elastic fibers and nuclei should stain black and rest of the tissue should be light grey.
5. Put in 5% sodium thiosulphate for 1 minute.
6. Wash in tap water for 5minutes.
7. Counter-stain with Van Gieson's stain for 1-2 minutes.
8. Differentiate in 95% alcohol.
9. Dehydrate in absolute alcohol two times.
10. Clear in xylene and mount in DPX.

Result

Elastic fibres- black
Nuclei - black
Collagen- red
Other tissues- yellow

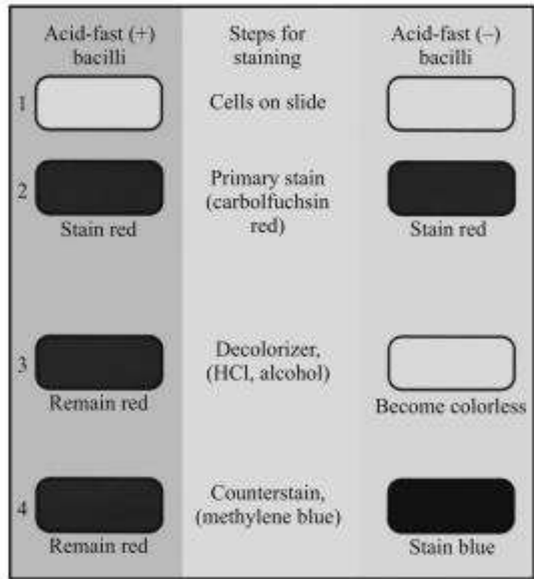
ACID-FAST STAINING

The Ziehl–Neelsen stain, also known as the acid-fast stain, widely used differential staining procedure. The Ziehl – Neelsen stain was first described by two German doctors; Franz Ziehl (1859 to 1926), a bacteriologist and Friedrich Neelsen (1854 to 1894) a pathologist. In this type some bacteria resist decolourization by both acid and alcohol and hence they are referred as acidfast organisms. This staining technique divides bacteria into two groups namely acid-fast and non acid-fast. This procedure is extensively used in the diagnosis of tuberculosis and leprosy. Mycobacterium tuberculosis is the most important of this group, as it is responsible for the disease called tuberculosis (TB) along with some others of this genus Principle Mycobacterial cell walls contain a waxy substance composed of mycolic acids. These are β-hydroxy carboxylic acids with chain lengths of up to 90 carbon atoms. The property of acid fastness is related to the carbon chain length of the mycolic acid found in any particular species

Ziehl- Neelsen Procedure

1. Make a smear. Air Dry. Heat Fix.
2. Flood smear with Carbol Fuchsin stain. Carbol Fuchsin is a lipid soluble, phenolic compound, which is able to penetrate the cell wall
3. Cover flooded smear with filter paper
4. Steam for 10 minutes. Add more Carbol Fuchsin stain as needed
5. Cool slide
6. Rinse with DI water
7. Flood slide with acid alcohol (leave 15 seconds). The acid alcohol contains 3% HCl and 95% ethanol, or you can declorase with 20% H_2SO_4
8. Tilt slide 45 degrees over the sink and add acid alcohol drop wise (drop by drop) until the red color stops streaming from the smear

9. Rinse with DI water.
10. Add Loeffler's Methylene Blue stain (counter stain). This stain adds blue color to non-acid fast cells. Leave Loeffler's Blue stain on smear for 1 minute
11. Rinse slide. Blot dry.
12. Use oil immersion objective to view.



MAY-GRÜNWALD GIEMSA STAIN

This is one of the common Romanwsky stains used in cytology. It is useful for studying cell morphology in air-dried smears. It is superior to Papanicolaou to study the cytoplasm, granules, vacuoles, basement membrane material etc. For nuclear staining Papanicolaou is superior. Contents of the staining reagents:

May-Grünwald solution 0.2%
Methanol 99 %
May-Grünwald´s eosin-methylene blue 0.2 %
Contains: Eosin G, Methylene blue
Giemsa solution
Methanol 73 %
Glycerol 26 %
Giemsa´s Azur-Eosin-Methylene blue 0.6 %
Contains: Azur I, Eosin G, Methylene blue Phosphate buffer Potassium dihydrogen phosphate/ disodium hydrogen phosphate x $2H_2O$ 67.0 mmol/l
Storage Giemsa solution,
May-Grünwald solution: protected from light at 2-25°C. Unopened reagents may be used until the expiry date on the label. Phosphate buffer: at 2-8°C. Unopened reagents may be used until the expiry date on the label.
Preparation of working solutions

1. Buffered water: Dilute phosphate buffer with deionised or distilled water 1:20, e.g. 30 ml phosphate buffer + 570 ml deionised or distilled water.
2. Giemsa working solution : Mix 84 ml of Giemsa solution into 516 ml of buffered water.
3. May-Grünwald working solution: Mix 360 ml of May-Grünwald solution into 240 ml of buffered water.

Staining method

1. Fix the air-dried smear specimen in methanol for 10 -20 minutes
2. Stain with May-Grünwald working solution for 5 minutes
3. Stain with Giemsa working solution for 12 minutes
4. Wash with clean buffered water for 2, 5 and 2 minutes
5. Dry the slides in upright position at room temperature
6. Mount the slides with a coverslip using DPX Any modifications to the staining procedure/working solutions may affect the staining result, and are subject to precise method validation.

Papanicolaou stain

Papanicolaou formula

1. Harris' hematoxylin Hematoxylin 5g Ethanol 50ml Potassium alum 100g Distilled water (50°C) 1000ml Mercuric oxide 2-5g Glacial acetic acid 40ml
2. Orange G 6 Orange G (10% aqueous) 50ml Alcohol 950ml Phosphotungstic acid 0-15g
3. EA 50 0.04 M light green SF 10ml 0.3M eosin Y 20ml Phosphotungstic acid 2g Alcohol 750ml Methanol 250ml Glacial acetic acid 20ml Filter all stains before use.

Original Papanicolaou staining method:

1. 96% ethyl alcohol 15 seconds
2. 70% ethyl alcohol 15 seconds
3. 50% ethyl alcohol 15 seconds
4. Distilled water 15 seconds
5. Harris hematoxylin 6 minutes
6. Distilled water 10 dips
7. Hydrochloric acid 0.5% solution, 1-2 quick dips
8. Distilled water 15 seconds
9. Few dips in 0.1% ammoniated water. The smear turns to blue.
10. 50% ethyl alcohol 15 seconds
11. 70% ethyl alcohol 15 seconds
12. 96% ethyl alcohol 15 seconds
13. OG-6 (orange) 2 minutes
14. 96% ethyl alcohol 10 dips
15. 96% ethyl alcohol 10 dips
16. EA 50 eosin yellowish 3 minutes
17. 96% ethyl alcohol (10 dips)
18. 100% ethyl alcohol (10 dips) 19. Xylene (10 dips)
20. Mount: in DPX using coverslip

Results: The nuclei should appear blue/black The cytoplasm (non-keratinising squamous cells) – blue/green Keratinising cells- pink/orange.

Physical examination of cerebrospinal fluid

1. Color

Under physiological condition the CSF is a clear colorless liquid. Any presence of colored substances in CSF is pathological. A color is most often caused by presence of hemoglobin, methemoglobin or bilirubin. Admixture of blood results in slight rose to red color, denoted as erythrochromic (sanguinolent) appearance of CSF. The blood can contaminate the CSF sample artificially due to damaged local blood vessels during the lumbar puncture. In this case, if the CSF is taken subsequently to three test tubes, the color tends to weaken from one tube to another. Also, the blood color can be removed by centrifugation of the CSF sample. In contrast, in intracranial bleeding the color of CSF should be the same in all test tubes, and should resist the centrifugation, although in a very fresh bleeding the supernatant can still be colorless. A yellow xanthochromic color of CSF is due to presence of bilirubin originating from catabolism of hemoglobin (older bleeding). The xanthochromy can persist up to 3 – 4 weeks since the bleeding episode (see below spectrophotometry of CSF). Presence of methemoglobin manifests as ochre yellow to brown color.

2. Turbidity

The CSF sample is cloudy usually due to presence of leukocytes, which appear in the CSF in purulent neuroinfections. Intensity of the turbidity is proportional to amount of leukocytes. Presence of erythrocytes can also manifest as turbidity.

5. Chemical examination of cerebrospinal fluid

1. Total protein in CSF The CSF contains about 200-fold less protein than blood plasma. About 80 % of the CSF proteins come from plasma. The entry of proteins to the CSF is affected by their molecular weight, charge, plasmatic concentration and condition of the blood-brain barrier. Larger molecules (e.g. IgM) travel more slowly than smaller ones (e.g. IgG, albumin). The remaining 20 % of proteins is produced intrathecally1 (e.g. part of immunoglobulins, β2-microglobulin). Some plasmatic proteins are modified in the CSF areas (e.g. transferrin, prealbumin). In minimal amounts also structural proteins are found in the CSF. From a clinical point of view, an increase in total protein in CSF, called hyperproteinorachia, is significant. It can be caused by the following mechanisms: • If the blood-CSF barrier is impaired, passage of proteins to the CSF is pathologically high. An obstruction of CSF pathways results in a severe damage to the blood-CSF barrier downstream of the blockade and proteins from plasma enter the CSF (albumin as well as highmolecular-weight fibrinogen). • Intrathecal synthesis of immunoglobulins in activation of immune system. • Abnormal spectrum of serum proteins will project also to the CSF, for instance monoclonal gammapathy results in presence of the same immunoglobulins in CSF. • Elevation of structural proteins due to injury of CNS tissues. • Tumor infiltration of meninges Estimation of total protein in CSF is useful mainly as a quickly accessible test providing basic information on the condition of blood-CSF barrier. One of the recommended assays for quantitative estimation of total protein in CSF is the reaction with pyrogallol red (see the measurement of protein in urine). A preliminary qualitative information on whether the CSF protein is increased can be provided by the Pandy's reaction, in which globulins and in part also albumin are denatured by the aqueous solution of phenol. Physiological values: Sp2 - Total protein (proteinorachia) 0.20 − 0.45 g/l Pandy's reaction: negative (protein < 0.2 g/l) 5.2. Albumin in CSF The entire albumin found in CSF has to come from blood, because albumin is not formed in the CNS. Rather, it is synthesized in the liver and enters the CSF via

passage through blood-CSF barrier. Albumin represents about 57 % of total CSF protein. An increased concentration of albumin in CSF always means a disorder in blood-CSF barrier. For a more detailed evaluation of 1 Intrathecal synthesis means synthesis within CNS and structures filled with CSF. 2 Sp – cerebrospinal fluid Cerebrospinal fluid 5 the blood-CSF barrier condition an albumin quotient Qalb is used.
It takes into account concentration of albumin in CSF (AlbCSF) and in serum (Albserum).

$$Q_{alb} = \frac{Alb_{CSF}}{Alb_{serum}}$$

The albumin quotient is used for: • assessment of blood-CSF barrier impairment, • calculation of intrathecal synthesis of immunoglobulins. Pathological increased values of albumin quotient are found due to impaired blood-brain barrier, which may occur in inflammatory diseases of CNS (meningitis of various etiology), multiple sclerosis or an obstruction in the CSF pathways. Albumin in the CSF is measured by sensitive immunochemical assays (immunoturbidimetry, immunonephelometry, ELISA).

Physiological values:
Sp-albumin: 120 − 300 mg/l Albumin quotient
Qalb depends on age: up to 15 years of age ≤ 5 × 10-3
up to 40 years of age ≤ 6.5 × 10-3
up to 60 years of age ≤ 8 × 10-3

TEST: SEMEN ANALYSIS PRINCIPLE:

For a successful pregnancy to occur there are several variables to consider when analyzing semen or cryopreserving human spermatozoa for artificial insemination. Among these variables are volumes of semen per ejaculate, sperm count, motility and morphology.

SPECIMEN REQUIREMENTS: A period of 48 to 72 hours of abstaining from ejaculation (including masturbation) is required to obtain accurate sperm concentrations. Period of less than 48 hours can result in decreased sperm concentrations, especially in older men. Intervals that exceed 4 days result in increased sperm concentrations with decrease in motile and viable forms. A sample should be obtained at the laboratory or physician's office where the analysis will be performed. If this is not possible, the specimen must be brought to the laboratory within 2 hours of collection and maintained at body temperature during transport. This can be accomplished by carrying the sample in an inside pocket or holding it against the body with undergarments. Samples exposed to extreme heat or cold may exhibit increased viscosity and/or coagulation of the seminal fluid, resulting in a decrease in motility.
REFERENCES: WHO Laboratory Manual for the Examination and Processing of Human Semen, 5th edition, World Health Organization 2010.

Normal Range: Normal semen should have the following parameters:
1. Volume: > 1.5 ml
2. Count: > 15 x 106/ml

3. Motility: > 40% motile NOTE:
Quality of Motility: 1 + = Sperm moving but no forward progression 2 + = Sperm moving aimlessly with slow forward progression 3 + = Sperm moving at moderate speed with forward progression 4 + = Sperm moving at high speed with straight-line forward progression
4. Morphology: See test result
5. Viscosity: Moderate.
Turnaround Time: 24 hours

2ND YEAR BMLT MICROBIOLOGY PRACTICAL (202)

Cultivation of Fungi

Principle: Because the structural components of molds are very delicate, even simple handling with an inoculating loop may result in mechanical disruption of their components.

The following slide culture technique is used to avoid such disruption. A deep concave slide containing a suitable nutrient medium with an acidic pH, such as Potato dextrose agar (PDA), is covered by a removable coverslip. Mold spores are deposited in the surface of the agar and incubated in a moist chamber at room temperature. Direct microscopic observation is then possible without fear of disruption or damage to anatomical components. Molds can be identified as to spore type and shape, type of sporangia, and type of mycelium.

Materials: Cultures, Potato dextrose agar (PDA), Petridish, Lactophenol cotton blue, slide, cover slip.

Procedure:

1. Following aseptic technique and with the help of a sterile inoculating loop, inoculated PDA plates with the supplied samples.

2. Incubate all plates at room temperature, 30°C, for 2 to 5 days. Note: Do not invert the plates.

3. 1. Examine each mold plate under the low and high power of a dissecting microscope by following LCB mount.

Result:

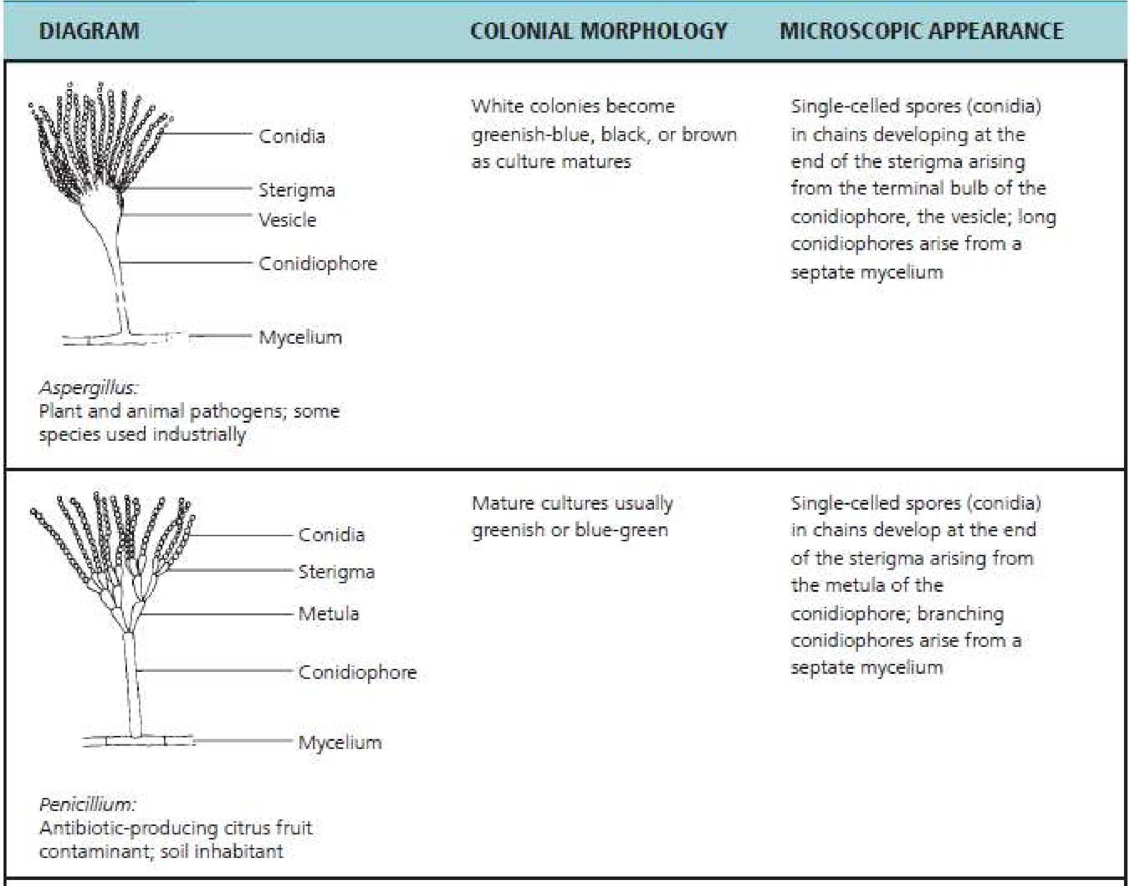


DIAGRAM	COLONIAL MORPHOLOGY	MICROSCOPIC APPEARANCE
Conidia; Sterigma; Vesicle; Conidiophore; Mycelium *Aspergillus:* Plant and animal pathogens; some species used industrially	White colonies become greenish-blue, black, or brown as culture matures	Single-celled spores (conidia) in chains developing at the end of the sterigma arising from the terminal bulb of the conidiophore, the vesicle; long conidiophores arise from a septate mycelium
Conidia; Sterigma; Metula; Conidiophore; Mycelium *Penicillium:* Antibiotic-producing citrus fruit contaminant; soil inhabitant	Mature cultures usually greenish or blue-green	Single-celled spores (conidia) in chains develop at the end of the sterigma arising from the metula of the conidiophore; branching conidiophores arise from a septate mycelium

<u>KOH Mount</u>

Principle: A KOH test is a simple, non-invasive procedure for diagnosing fungal infections of the skin or nails. KOH is a strong alkali. When specimen such as skin, hair, nails or sputum is mixed with 10% w/v KOH, it softens, digests and clears the tissues (e.g., keratin present in skins) surrounding the fungi so that the hyphae and conidia (spores) of fungi can be seen under a microscope. Microscopic examination of KOH preparation reveals the presence of fungal structure and aids in diagnosing mycoses.

Materials: Microscope slide and cover glass, 20% potassium hydroxide (KOH), and Microscope.

Procedure:

➢ The affected skin or nail is gently scraped with a small scalpel or the edge of a glass slide.

➢ The scrapings from the skin are placed on a microscope slide and a few drops of a potassium hydroxide (KOH) solution are added.

➢ The slide is heated for a short time and then examined under the microscope using lactophenol cotton blue.

Results and Interpretation: Potassium hydroxide (KOH) solution is alkaline and has the ability to dissolve keratin that is scraped from the outer layer of the skin. As the KOH dissolves the material binding the skin cells together, any fungus present is released. This allows for the identification of organisms such as dermatophytes.

Fig.- Fungal hyphae in a (KOH) preparation of skin scales as seen with the 10x objective.

<u>**LCB Mount**</u>

Principle: The lactophenol cotton blue (LCB) wet mount preparation is the most widely used method of staining and observing fungi. It has the following constituents: 1) Phenol kills fungus; 2) Lactic acid acts as a clearing agent and helps preserve the fungal structures, 3) Cotton blue is an aniline dye that stains the chitin in the fungal cell walls which adds colour to the fungal preparation thereby enhancing and contrasting the structures; and 4) Glycerol is a viscous substance that prevents drying of the prepared slide specimen.

Materials: Microscope slide and cover glass, and Microscope.

Procedure:

1) After grease free of the glass slide, a drop of LCB was placed on the slide.
2) Aseptically add the supplied sample mixed to the dye and placed a cover glass on it.
3) Observed under microscope.

Results and Interpretation: Lactophenol cotton blue solution is a mounting medium and staining agent used in the preparation of slides for microscopic examination of fungi. Fungal spores, hyphae, and fruiting structures stain blue while the background stains pale blue.

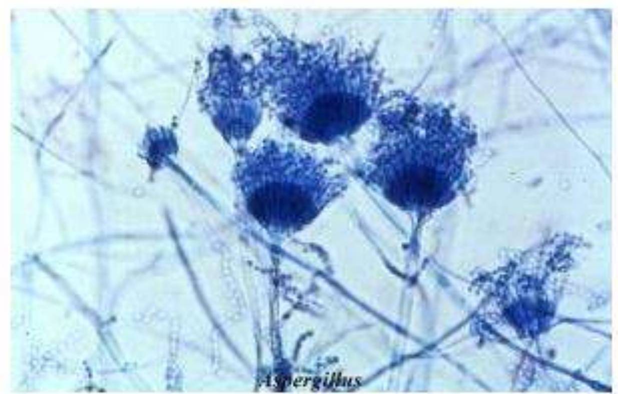


Fig: *Aspergillus* spp in LPCB Mount

Germ Tube Test

Principle:

Formation of germ tube is associated with increased synthesis of protein and ribonucleic acid. Germ Tube solutions contains tryptic soy broth and fetal bovine serum, essential nutrients for protein synthesis. It is lyophilized for stability. Germ tube is one of the virulence factors of *Candida albicans*. This is a rapid test for the presumptive identification of *C. albicans*.

Materials: Test tubes, Cover slip, Conical flask, Incubator, Centrifuge, Microscope, Microscope slide and cover glass.

Procedure:

- Prepare tryptic soy broth and sterilize, in which put 0.5 ml of human blood serum into a test tube.
- Using a Pasteur pipette, touch a colony of yeast and gently emulsify it in the serum. Note: Too large of an inoculum will inhibit germ tube formation.
- Incubated the tube at 37°C for 2 to 4 hours.
- Transfer a drop of the serum to a slide for examination.
- Coverslip and examine microscopically under low and high-power objectives.

Results and Interpretation:

Positive Test: A short hyphal (filamentous) extension arising laterally from a yeast cell, with no constriction at the point of origin. Germ tube is half the width and 3 to 4 times the length of the yeast cell and there is no presence of nucleus. Examples: *Candida albicans* and *Candida dubliniensis*

Negative Test: No hyphal (filamentous) extension arising from a yeast cell or a short hyphal extension constricted at the point of origin. Examples: *C. tropicalis, C. glabrata* and other yeasts.

Fig.-Germ tube formation in *Candida albicans*

<u>Identification of Common Medically Important Fungi:</u>

Candida albicans

Division: Deuteromycotina

Class: Blastomycetes

Order: Cryptococcales

Family: Cryptococcaceae

Genus: *Candida*

Species: *albicans*

(i) *Candida albicans* is small, oval, and yeast like unicellular fungus.
(ii) It shows pseudomycelial growth and multilateral budding. The cell elongates and develops into pseudomycelium.
(iii) The genus comprises of imperfect forms of ascomycetous and basidiomycetous yeasts of various genera.
(iv) *C. albicans* is the imperfect state of Syringospora, which is a member of basidiomycotina.
(v) They form chlamydospores and produce spherical clusters of blastospores.
(vi) The colonies are moderate in size, smooth and pasty. The older colonies have honey comb like appearance in the centre and develop radical furrows.
(vii) The possess a capsular form of polysaccharide that shows pyrogenic activity.
(viii) Examples are *C. albicans*, *C. utilis*, etc.

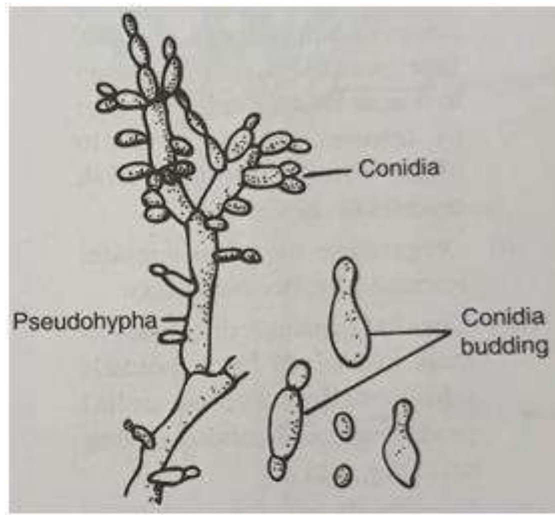


Fig.-Structure of *Candida* sp.

Aspergillus

Class: Deuteromycetes

Order: Moniliales

Family: Moniliaceae

Genus: *Aspergillus*

(i) Colonies on Czapek Dox agar are white (*A. versicolor*) on white at first and becoming yellowish (*A. flavipes*), blue green (*A. sydowi*), lime green (*A. flavus*),

cinnamon to deeper brown shades with age (*A. terreus*), blackish brown to black with slight yellowish mycelia (*A. niger*).

(ii) Vegetative mycelim septate branched hyphae colourless.

(iii) Conidial apparatus developed as stalk and heads from footcells (thick-walled hyphal cells) producing conidiophores at long axis.

(iv) Conidiophores septate or un-septate, broadening into elliptical, hemispherical or globose fertile vesicles.

(v) Vesicles bear phialides in one series (uniseriate), or two series (biseriate).

(vi) Phialides clustered in terminal groups or radiating from entire surface.

(vii) Conidia (conidia bearing cells) elliptical, globose, smooth walled, rough or spinulose walls produced in chains.

(viii) Some species produce cleistothecia e.g. *A. versicolor*, *A. ruber*, some strains produce sclerotia e.g. *A. niger*; some species produce irregularly globose, ovoid or elongated heavily walled abundant hulls cells e.g. *A. granulosis*.

(ix) They play a significant role in production of amylase (a. niger), diastase (*A. flavipes*, *A. parasiticus*), otomycosis in humans (*A. niger*), etc.

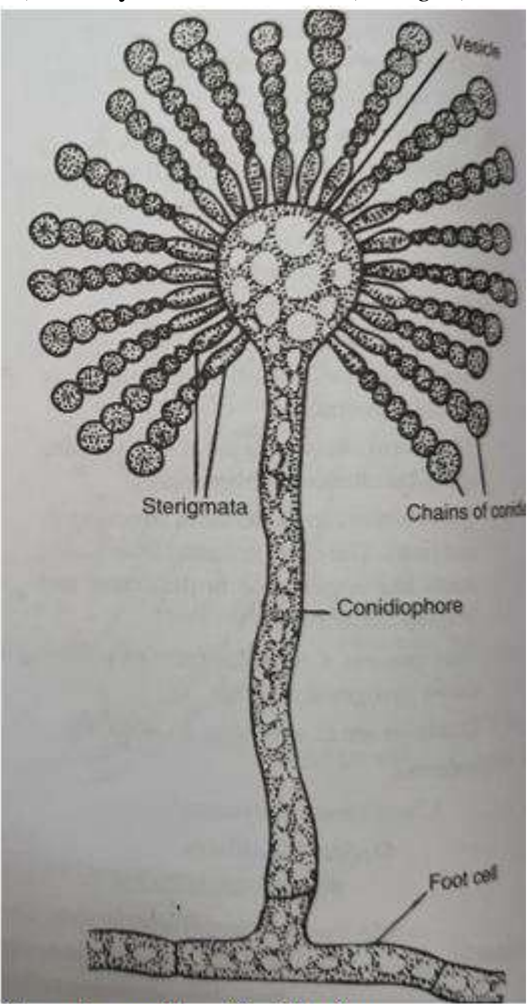


Fig.-*Aspergillus:* Conidiophore and chains of conidia

Penicillium

Class: Deuteromycetes

Order: Moniliales

Family: Moniliaceae

Genus: *Penicillium*

(i) Colonies are of various colors (i.e. green, bluish green, greyish green), central area raised or smooth, may be zonate with age, radially furrowed.
(ii) Vegetative hyphae creeping, wooly or cottony, floccose to semifloccose, slow or fast growing.
(iii) Hyphae branched and septate producing branched or unbranched conidiophores each with one, two more verticil of phialides (conidia bearing cells) and metulae (verticils of secondary and sometimes primary branchlets).
(iv) Conidia borne in chains typically forming brush like head, not enclosed in slime, well differentiated footcells not present.
(v) Conidia globose, ovate or elliptical with smooth or rough surface.
(vi) Some species produce cleistothecia which ripe early or late.
(vii) The species that produce penicillins are *P. notatum, P. chrysogenum*, etc.
(viii) The other important species are: *P. citrinum, P. granulatum, P. ochraceum, P. javancium, P. lividium, P. citreoviride, P. restrictum, P. waksmani, P. regulosum, P. lilacinum* (destructive parasite of nematodes causing diseases in plants), etc.

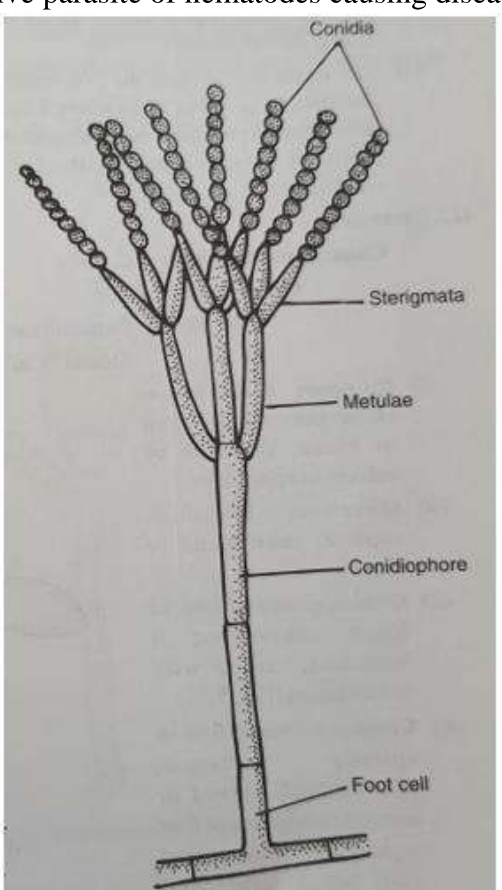
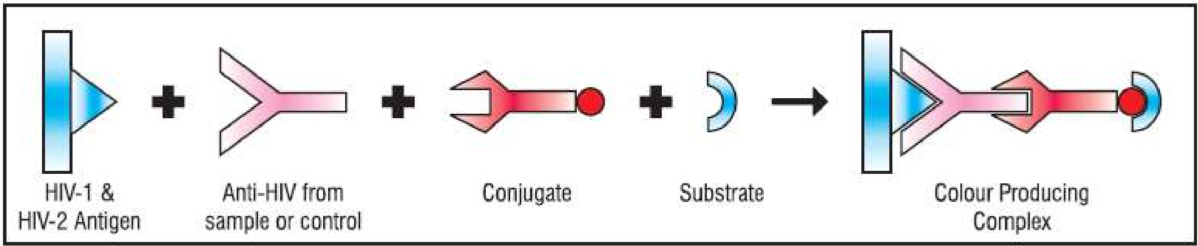


Fig.-Structure of *Penicillum*

Serological Test Related to Viral Diagnosis

HIV-ELISA [Microwell ELISA Test for the Detection of Antibodies to HIV-1 and HIV-2 in Human Serum/ Plasma]

Principle: HIV envelope proteins proteins gp41, C terminus of gp 120 for HIV-1, and gp 36 for HIV-2 representing immunodominant epitopes are coated onto microtiter wells. Specimens and controls are added to the microtiter wells and incubated. Antibodies to HIV-1 and HIV-2 if present in the specimen, will bind to the specific antigens absorbed onto the surface of the wells. The plate is then washed to remove unbound material. Horseradish peroxidase (HRP) conjugated antihuman IgG is added to each well. This conjugate will bind to HIV antigen- antibody complex present. Finally, substrate solution containing chromogen and hydrogen peroxide is added to the wells and incubated. A blue colour will develop in proportion to the amount of HIV-1 and / or HIV-2 antibodies present in the specimen. The colour reaction is stopped by a stop solution. The enzyme substrate reaction is read by EIA reader for absorbance at a wavelength of 450 nm. If the sample does not contain HIV-1 or HIV-2 antibodies then enzyme conjugate will not bind and the solution in the wells will be either colourless or only a faint background colour develops.



Procedure:

A. Preparation of Reagents: Prepare the following reagents before or during assay procedures. Reagents and samples should be at room temperature (20-30ºC) before beginning the assay and can remain at room temperature during testing. Return reagents to 2-8ºC after use. All containers used for preparation of reagents must be cleaned thoroughly and rinsed with distilled or deionized water. Prewarm the incubator at 37ºC.

Microlisa-HIV Strip: Bring foil pack to room temperature (20-30ºC) before opening to prevent condensation on the microwell strips.

a. Break-off the required number of strips needed for the assay and place in the well holder. Take the strip holder with the required number of strips, considering that two negative & three positive controls should be included in the run while opening the fresh kit. However, for one or two strips, one negative and two positive controls and for more strips at least two negative and three positive controls should be included in each subsequent run.

b. Unused wells should be stored at 2-8ºC, with desiccant in an aluminium pouch with clamp & rod. Microwells are stable for 30 days at 2-8ºC from the date of opening of sealed pouch, when stored with desiccant along with clamp & rod.

Caution: Handle microwell strip with care. Do not touch the bottom exterior surface of the wells.

B. Sample Preparation:

I. Microwell Dilution:

a) Pipette 100 µl of sample diluent in to the microwell.

b) Add 10 µl of serum sample to be tested.

c) Ensure thorough mixing of the sample to be tested.

II. Preparation of Wash Buffer:

a) Check the buffer concentrate for the presence of salt crystals. If crystals are present in the solution, resolubilize by warming at 37ºC until all crystals dissolve.

b) Prepare at least 50 ml. (2ml. concentrated buffer with 48 ml. water) of buffer for each microlisa strip used. Mix well before use.

c) Mix 20 ml. 25 X wash buffer concentrate with 480 ml. of distilled or deionized water.

Working Wash Buffer is stable for 2 months when stored at 2-8ºC.

III. Preparation of Working Conjugate: Dilute conjugate concentrates 1:100 in conjugate diluent. Do not store working conjugate. Prepare a fresh dilution for each assay in a clean glass vessel. Determine the quantity of working conjugate solution to be prepared from table given below. Mix solution thoroughly before use.

IV. Preparation of working substrate solution: Mix TMB substrate and TMB Diluent in 1:1 ratio to prepare working substrate. Do not store working substrate. Prepare a fresh dilution for each assay in a clean plastic/glass vessel. Determine the quantity of working substrate solution to be prepared from table. Mix solution thoroughly before use.

V. Wash Procedure:

1. Incomplete washing will adversely affect the test outcome.

2. Aspirate the well contents completely into a waste container. Then fill the wells completely with wash buffer avoiding overflow of buffer from one well to another and allow to soak (approx. 30 seconds). Aspirate completely and repeat the wash and soak procedure 4 additional times for a total of 5 washes.

3. Automated washer if used should be well adjusted to fill each well completely without over filling

4. Tap upside down on absorbent sheet till no droplets appear on the sheet, taking care not to dislodge the wells.

VI. Test Procedure: Once the assay has started, complete the procedure without interruption. All the reagents should be dispensed in the centre of the well and the tip of the pipette should not touch the wall of the microwell. Fit the strip-holder with the required number of Microlisa-HIV strips. The sequence of the procedure must be carefully followed. Arrange the assay control wells so that well A-1 is the reagent blank. From well A-1 arrange all controls in a horizontal or vertical configuration. Configuration is dependent upon reader software.

1. Add 100 µl sample diluent to A-1 well as blank.

2. Add 100µl Negative Control in each well no. B-1 & C-1 respectively. Negative Control is ready to use and hence no dilution is required.

3. Add 100µl Positive Control in D-1, E-1 & F-1 wells. Positive Control is ready to use and hence no dilution is required.

4. Add 100 µl sample diluent in each well star ting from G-1 followed by addition of 10µl sample. (Refer Microwell Dilution)

5. Apply cover seal.

6. Incubate at 37ºC + 2ºC for 30 min. + 2 min.

7. While the samples are incubating, prepare Working Wash Solution and Working Conjugate as specified in Preparation of Reagents.

8. Take out the plate form the incubator after the incubation time is over and, wash the wells 5 times with Working Wash solution according to the wash procedure given in the previous section (wash procedure).

9. Add 100 µl of Working Conjugate Solution in each well including A-1.

10. Apply cover seal.

11. Incubate at 37ºC + 2ºC for 30 min. + 2 min.

12. Aspirate and wash as described in step no. 8.

13. Add 100 µl of working substrate solution in each well including A-1.

14. Incubate at room temperature (20 - 30ºC) for 30 min. in dark.

15. Add 100 µl of stop solution.

16. Read absorbance at 450 nm within 30 minutes in ELISA READER after blanking A-1 well. (Bichromatic absorbance measurement with a reference wavelength 600 - 650 nm is recommended when available).

Result: Calculation

Abbreviations

NC -	Absorbance of the Negative Control
$NC\bar{x}$ -	Mean Negative Control
PC -	Absorbance of the Positive Control
$PC\bar{x}$ -	Mean Positive Control

TEST VALIDITY:

Blank acceptance Criteria

Blank must be <0.100 in case of differential filter being used. In case differential filter is not available in the reader the blank value may go higher.

Negative Control Acceptance Criteria:

NC must be ≤ 0.150. If it is not so, the run is invalid and must be repeated.

Positive Control Acceptance Criteria:

1. PC must be ≥ 0.50
2. Determine the mean ($PC\bar{x}$) value If one of three positive control values is outside of these limits, recalculate $PC\bar{x}$ based upon the two acceptable positive control values.
3. If two of the three positive control values are outside the limits, the assay is invalid and the test must be repeated.

CUT OFF VALUE

Absorbance (O.D.)

NC	-	0.042 B1 Well	PC	-	1.412	D1 Well
	-	0.040 C1 Well		-	1.392	E1 Well
Total:		0.082 2 Wells		-	1.407	F1 Well
			Total :		4.211	3 Wells

$NC\bar{x} = 0.082/2 = 0.041$ $PC\bar{x} = 4.211/3 = 1.403$

The cut off value is calculated by adding Mean Negative Control ($NC\bar{x}$) and Mean Positive Control ($PC\bar{x}$) as calculated above and the sum is divided by 6.

$$\text{Cut off Value} = \frac{NC\bar{x} + PC\bar{x}}{6}$$

e.g. $NC\bar{x} = 0.041$, $PC\bar{x} = 1.403$

$$\text{Cut off Value} = \frac{0.041 + 1.403}{6} = \frac{1.444}{6} = 0.240$$

Interpretation:

1. Test specimens with absorbance value less than the cut off value are non-reactive and may be considered as negative for anti-HIV.

2. Test specimens with absorbance value greater than or equal to the cut off value are reactive for anti-HIV by Microlisa-HIV.

Note: Test specimens with absorbance value within 10% below the cut off should be considered suspect for the presence of antibodies and should be retested in duplicate.

3. Specimens with absorbance value equal to or greater than the cut off value are considered initially reactive by the criteria of Microlisa-HIV. Original specimen should be retested in duplicate.

4. If both duplicate retest sample absorbance value is less than cut off value, the specimen is considered non-reactive.

5. If any one of the duplicates retest sample absorbance value is equal to or greater than the cut off or both duplicate retest value are equal to or greater than the cut off, the specimen is considered reactive by the criteria of Microlisa HIV. Further confirmation by other EIA assays or confirmation assays including Western Blot or PCR is recommended.

GAC- ELISA Test for the Detection of Dengue IgG Antibodies in Human Serum/Plasma

Principle: Antibodies to Dengue if present in the specimen, will bind to the Anti-human IgG antibodies adsorbed onto the surface of the wells. The plate is then washed to remove unbound material. Horseradish peroxidase (HRPO) conjugated dengue antigen (DEN1-4) is added to each well. This dengue antigen conjugate will bind to Dengue specific IgG antibodies which is complexed with anti- human IgG antibodies. Finally, substrate solution containing chromogen and hydrogen peroxide is added to the wells and incubated. A blue colour will develop in proportion to the amount of Dengue antibodies present in the specimen. The colour reaction is stopped by a stop solution. The enzyme substrate reaction is read by EIA reader for absorbance at a wavelength of 450 nm. If the sample does not contain Dengue IgG antibodies then enzyme conjugate will not bind and the solution in the wells will be either colourless or only a faint background colour develops.

Preparation of Reagents:

Prepare the following reagents before or during assay procedures. Reagents and samples should be at room temperature (20-30ºC) before beginning the assay and can remain at room temperature during testing. Return reagents to 2-8ºC after use. All containers used for preparation of reagents must be cleaned thoroughly and rinsed with distilled or deionized water. Prewarm the incubator to 37ºC.

i) Anti human IgG antibodies coated strips: Bring foil pack to room temperature (20-30ºC) before opening to prevent condensation on the microwell strips.

a. Break-off the required number of strips needed for the assay and place in the well holder. Take the strip holder with the required number of strips, taking into account that one each of negative & positive control & three calibrators should be included in the run while opening the fresh kit. However, for one or two strips, one each of negative, positive control & two calibrator and for more strips one each of negative and positive control & three calibrator should be included in each subsequent runs.

b. Unused wells should be stored at 2-8oC, with dessicant in aluminium pouch with clamp & rod. Microwells are stable for 30 days at 2-8ºC from the date of opening of sealed pouch, when stored with desiccant along with clamp & rod.

Caution: Handle microwell strip with care. Do not touch the bottom exterior surface of the

wells.

ii) Sample Preparation:

TUBE DILUTION: Mark the tubes carefully for the proper identification of the samples. Dilute the serum samples to be tested, with sample diluent 1:100 in separate tubes (1 ml. sample diluent + 10 µl serum samples). Use a separate tip for each sample and then discard as biohazardous waste.

iii) Preparation of Working Wash Buffer:

a) Check the buffer concentrate for the presence of salt crystals. If crystals are present in the solution, resolubilize by warming at 37°C until all crystals dissolve.

b) Prepare at least 25ml. (1ml. concentrated buffer with 24 ml. water) of buffer for each strip used. Mix well before use.

c) Mix 20 ml. of 25X wash buffer concentrate with 480 ml. of distilled or deionized water. Working wash buffer is stable for 2 months when stored at 2-8°C.

iv) Preparation of working substrate solution: Mix TMB substrate and TMB Diluent in 1:1 to prepare working substrate.

Do not store working substrate. Prepare a fresh dilution for each assay in a clean plastic/glass vessel. Determine the quantity of working substrate solution to be prepared from table. Mix solution thoroughly before use. Discard unused solution. A deep blue color present in the substrate solution indicates that the solution has been contaminated and must be discarded.

Test Procedure:

Once the assay has started, complete the procedure without interruption. All the reagents should be dispensed in the centre of the well and the tip of the pipette should not touch the wall of the microwell.

Fit the strip-holder with the required number of Anti-human IgM coated strips. The sequence of the procedure must be carefully followed. Arrange the assay control wells in a horizontal or vertical configuration. Configuration is dependent upon reader software.

1. Add 100 µl Negative Control in A-1well.

2. Add 100 µl calibrator in B-1, C-1 & D-1 wells.

3. Add 100 µl Positive Control in E-1 well.

4. Add 100 µl of each sample diluted in sample diluent (1:100), in each well star ting from F-1-well. (Refer TUBE DILUTION).

5. Apply cover seal.

6. Incubate at 37ºC ± 1ºC for 60 min. ± 1min.

7. While the samples are incubating, prepare working Wash Solution as specified in preparation

of reagents.

8. Take out the plate from the incubator after the incubation time is over and, wash the wells

5 times with working Wash Solution.

9. Add 100 µl of Enzyme Conjugate Solution in each well.

10. Apply cover seal.

11. Incubate at 37ºC ± 1ºC for 60 min ± 1min.

12. Aspirate and wash as described in step no. 8.

13. Add 100 µl of working substrate solution in each well.

14. Incubate at room temperature (20-30ºC) for 30 min. in dark.

15. Add 50 µl of stop solution.

16. Read absorbance at 450 nm and 630 nm (reference filter) within 30 minutes in ELISA Reader.

Calculation of Results:

a. Cut off value = mean O.D. of calibrator × calibration factor

b. Calculation of sample O.D. ratio: Calculate sample O.D. ratio as follows:

Sample O.D.

Sample O.D. ratio $= \frac{Sample\ O.D.}{Cut\ off\ Value}$

c. Calculation of Dengue IgG units: Calculate by multiplying the sample O.D. ratio by 10.

Dengue IgG units = sample O.D. ratio × 10.

e.g.: Mean O.D. of calibrator = 0.75

Calibration factor = 0.7

Cut off value = 0.75 × 0.7 = 0.525

e.g.: sample absorbance (O.D.) = 0.925

Cut off value = 0.525

Sample O.D. ratio = 0.925 / 0.525 = 1.761

Dengue IgG units = 1.761 × 10 = 17.61

Interpretation of Results:

a. If the Dengue IgG Units is < 9 then interpret the sample as Negative for Dengue IgG antibodies.

b. If the Dengue IgG Units is between 9 - 11 then interpret the sample as Equivocal for Dengue IgG antibodies. Equivocal samples should be repeated in duplicate and calculate the average dengue units. Sample that remain equivocal after repeat testing should be repeated by an alternative method or another sample should be collected.

c. If the Dengue IgG Units is > 11 then interpret the sample as Positive for Dengue IgG antibodies.

BMLT WBUHS 2nd Year Lab Manual

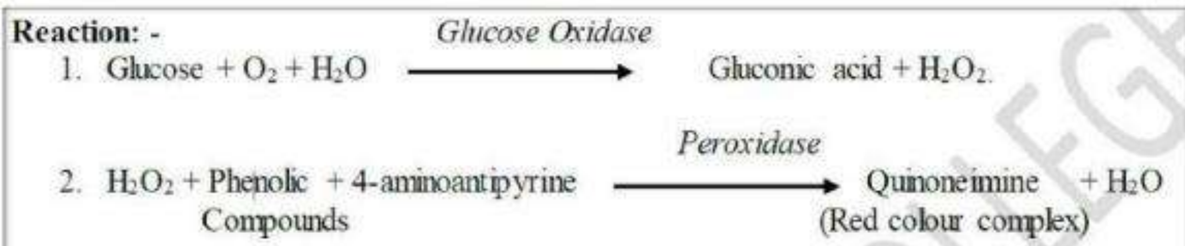
Paper -203

ESTIMATION OF PLASMA GLUCOSE BY GOD-POD METHOD

Principle:

Glucose oxidase (GOD) oxidizes the specific substrate β-D- glucose to gluconic acid and hydrogen peroxide (H2O2) is liberated. Peroxidase (POD) enzyme acts on hydrogen peroxide to liberate nascent oxygen (O2), then nascent oxygen couples with 4-amino antipyrine and phenol to form red quinoneimine dye. The intensity of the colour is directly proportional to the concentration of glucose present in plasma. The intensity of colour is measured by colorimeter at 530 nm or green filter and compared with that of a standard treated similarly. Final colour is stable for at least 2 hours if not exposed to direct sunlight.

Reaction:



Reagents:

1. Glucose colour reagent; it contains GOD, POD, 4- amino antipyrine, phenol & phosphate buffer (pH 7.5)

2. Glucose standard solution, Concentration = 100 mg/dl. 100 mg of anhydrous glucose is dissolved in 100 ml of distilled water

Procedure:

Pipette into clean, dry test tube labelled as Blank (B), Standard (S) and Test (T).

Then add the solution in each of test tubes separately as shown in table below

	BLANK	STANDARD	TEST
Glucose colour Reagent	1000 µl	1000 µl	1000 µl
Distilled Water	10 µl	-------	--------
Standard	-----	10 µl	--------
Plasma	-----		10 µl

Mix thoroughly and keep the tubes at 37˚C for 15 minutes

OD at 530 nm	0.02	0.45	0.58

Data: Plasma Glucose standard concentration is 100 gm/dl

CALCULATION

Concentration of Glucose = (OD of Test/OD of standard) × Concentration of standard

Result:

Plasma glucose concentration in given unknown blood sample = mg/dl.

Normal Range

	Fasting	After eating	2-3 hrs after eating
Normal	80 - 100	170 - 200	120 - 140
Pre Diabetic	101 - 125	190 - 230	140 - 160
Diabetic	126+	220 - 300	200+

Interpretation:

• Hyperglycemia:

• It is found in following conditions

I. Physiological:

• 1. Alimentary: After high carbohydrate diet

• 2. Emotional: Stress, anger, anxiety etc.

II. Pathological:

• 1. Diabetes mellitus

• 2. Hyperadrenalism

• 3. Hyperpituitarism

• Hypoglycemia:

• It is found in following conditions:

• **I. Physiological:**

– During starvation

– After Severe Exercise •

II. Pathological:

– Prolonged fasting

– Due to excess of insulin e.g.

• Excessive dose of insulin

• No food intake after insulin administration

• Tumours of pancreas (insulinoma)

– Glycogen storage disease

– Hypoactivity of adrenal and pituitary gland

UREA KIT

(Mod. Berthelot method)

For the determination of urea in serum, plasma and urine (or Invitro Diagnostic Use Only)

Summary

Urea is the end product of protein metabolism. It is synthesized in the liver from the ammonia produced by the catabolism of amino acids. It is transported by the blood to the kidneys from where it is excreted. Increased levels are found in renal diseases, urinary obstructions, shock, congestive heart failure and burns. Decreased levels are found in liver failure and pregnancy.

Principle

Urease hydrolyses urea to ammonia and CO_2. The ammonia further reacts with a phenolic chromogen and Hypochlorite to form a green coloured complex. Intensity of the colour formed is directly proportional to the amount of urea present in the sample.

Urea+ H_2O = Ammonia+CO_2

Ammonia + Phenolic chromogen = Green coloured complex + hypochlorite

Normal reference values

Serum/Plasma: 14- 40 mg/dl

Urine: Upto 20g/L

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	75 Assays	3x75Assays	2x150Assays
L1: Buffer Reagent	75 ml	3*75ml	2*150 ml
L2: Enzyme Reagent	7.5 ml	3*7.5ml	30 ml
L3: Chromogen Reagent	15 ml	2*22.5ml	2*30 ml
S: Urea Standard (40 mg/dl)	5 ml	5 ml	5 ml

Storage / stability

Contents are stable at 2-8°C till the expiry mentioned on Urea Nitrogen in mg/dl the tables.

Reagent Preparation: Reagents are ready to use for the given procedure.

Working Enzyme Reagent: For the flexibility and Convenience in performing large assay series, a working enzyme reagent may be made by pouring 1 bottle of L2.

Enzyme Reagent) into 1 bottle of L1 (Buffer Reagent). For smaller series combine 10 parts ofL1 (Buffer Reagent) and 1 part of L2 (Enzyme Reagent). Use 1 ml of the working reagent per assay instead of 1 ml of L1 and 0.1 ml of L2 as given in the procedure. The working enzyme reagent is stable for at least 4 weeks when stored at 2-8°C.

Working Chromogen Reagent: For larger volume cuvettes, dilute 1 part of L3 (Chromogen Reagent) wih 4 parts of fresh ammonia free distilled/ deionised water. Use 1 ml of working chrornogen instead of 0.2 ml in the assay. The working chromogen reagent is stable for at least 8 weeks when stored at 2-8°C in a tightly stoppered plastic bottle.

Sample material

Serum, plasma, Urine. Dilute urine 1+ 49 with distilled water before the assay (Results x 50). Urea is reported to be stable in the serum for 5 days when stored at 2-8°C.

Procedure

Wavelength/filter : 570 nm (Hg 578 nm)/ Yellow

Temperature : 37°C /R.T

Light path : 1 cm

Pipette into clean dry test tubes labelled as Blank (B), Standard (S), and Test (T)

Addition Sequence	B (ml)	S (ml)	T (ml)
Buffer Reagent (L1)	1.0	1.0	1.0
Enzyme Reagent (L2)	0.1	0.1	0.1
Distilled water	0.01	-	-
Urea Standard (S)	-	0.01	-
Sample	-	-	0.01

Mix well & incubate for 5 min. at 37°C or 10 min. at R.T. (25°C)

Chromogen Reagent (L3)	B (ml) 0.2	S (ml) 0.2	T (ml) 0.2

Mix well and incubate for 5 min. at 37°C or 10 min. at R.T (25°C). Measure the absorbance of the Standard (Abs.S), and Test Sample (Abs.T) against the blank, within 60 Min.

Calculations

Urea in mg/dl = (Abs.T/Abs.S) x 40

Urea Nitrogen in mg/dl = Urea in mg/dl x 0.467

Linearity

This procedure is linear upto 250 mg/dl. Using the working chromogen reagent (1 ml) the linearity is increased to 4000 mg/dl. If values exceed this limit, dilute the serum with normal saline (NaCl 0.9%) and repeat the assay. Calculate the value using the proper dilution factor.

Notes

Any contamination by ammonia or ammonium salts lead to erroneous results, hence plasma should not be collected with Fluoride or Heparin Ammonium salts.

The working enzyme reagent is not stable at elevated temperatures and should be stored at 2-8°C immediateley after use.

CREATININE KIT

(Mod. Jaffe's Kinetic method)

For the determination of Creatinine in serum and urine)

(For Invitro Diagnostic Use Only)

Summary

Creatinine is the catabolic product of creatinine phosphate which is used by the skeletal muscle. The daily production depends on muscular mass and it is excreted out of the body entirely by the kidneys. Elevated levels are found in renal dysfunction, reduced renal blood flow (shock, dehydration, congestive heart failure) diabetes acromegaly. Decreased levels are found in muscular dystrophy.

Principle:

Picric acid in an alkaline medium reacts with creatinine to form orange coloured complex with the alkaline picrate. Intensity of the colour formed during the fixed time is directly proportional to the amount of creatinine present in the sample.

Creatinine+ Alkaline Picrate → Orange Coloured Complex

Reference values:

Serum	Urine in 24hrs. collection
Males: 0.6-1.2 mg%	1.1-3.0 gm
Females: 0.5-1.1 mg%	1.0-1.8 gm

It is recommended that each laboratory establish its own normal range representing its patient population.

Contents	2x 35 ml	2x75 ml	2x150 ml	2x500 ml

L1: Picric acid reagent	35 ml	75 ml	150 ml	2x250 ml
L2: Buffer Reagent	35 ml	75 ml	150 ml	2 x150 ml
S: Creatinine Standard (2 mg/dl)	5ml	2 x5 ml	15 ml	50 ml

Storage/stability

All reagents are stable at R.T. till the expiry mentioned on the label.

Reagent Preparation

Reagents are ready to use. Do not pipette with mouth. Working reagent for larger assay series a working reagent may be prepared by mixing equal volumes of Picric Acid Reagent and Buffer Reagent. The Working reagent is stable at R.T. (25-30 °C) for at least one week.

Sample material

Serum or Urine. References Creatinine is stable in serum for 1 day at 2-8°C Urine of 24 hours collection is preferred. Dilute the specimen 1:50 with distilled/ deionised water before the assay.

Procedure

Wavelength/filter

Temperature

Light path

Pipette into a clean dry test tube labelled Standard (S) or Test (T)

Addition Sequence	(S)/(T) 30°C / 37°C
Picric Acid Reagent (L1) Buffer reagent (L2)	0.5 ml 0.5 ml

Bring reagents to the assay temperature and add

Creatinine Standard (S)/ Sample / Diluted Urine	0.1 ml

Mix well and read the initial absorbance A, for the Standard and Test after exactly 30 seconds. Read another absorbance A, of the Standard and Test exactly 60 seconds later. Calculate the change in absorbance ΔA for both the Standard and Test.

For Standard ΔAS = A2S-A1S

For Test ΔAT = A2T – A1T

Calculations

Creatinine in mg/dl = (ΔAT/ ΔAS) X 2.0

Urine Creatinine in g/l = (ΔAT/ ΔAS) X 1.0

Urine Creatinine g/24Hrs = Urine Creatinine in g/L x Vol. of urine in 24 Hrs

Linearity

The procedure is linear upto 20 mg/dl of creatinine. If values exceed this limit, dilute the sample with distilled water and repeat the assay. Calculate the value using the proper dilution factor.

Wavelength/filter: 520 nm (Hg 548 nm)/Yellow Green

Temperature: 37°C

Light path: 1cm

URIC ACID KIT

(Uricase/PAP method)

For the determination of Uric Acid in serum or plasma

Summary

Uric acid is the end produced of purine metabolism. Uric acid is excreted to a large degree by the kidneys and to a smaller degree in the intestinal tract by microbial degradation. Increased levels are found in Gout, arthrits, impaired renal functions, and starvation, Decreased levels are found in Wilson's disease, Fanconis syndrome and yellow atrophy of the liver.

Principle

Uricase converts uric acid to allantoin and hydrogen peroxide. The hydrogen peroxide formed further reacts with a phenolic compound and 4 aminoantipyrine by the catalytic action of peroxidase to form a red coloured quinoneimine dye complex. Intensity of the colour formed is directly proportional to the amount of uric acid present in the sample.

Uric acid +H2O → (Uricase) → Allantoin +H2O2

O2+Aminoantieripynne+Phenolic compound = Red quinoneimine dye + H2O+ Phenolic compound

Nomal reference values:

Serum/Plasma (Males): 3.4 -7.0 mg/dl

(Females): 2.5-6.0 mg/dl

Contents	25 ml	75 ml	2x 75 ml	2x 150 ml
L1: Bufer Reagent	20ml	60ml	2 x60 ml	2 x120ml
L2: Enzyme Reagent	5ml	15 ml	2 x15 ml	2 x30 ml
S: Uric Acid Slandard (8mg/dl)	5ml	5ml	5ml	

Procedure:

Wavelength/filter: 520 nm (Hg 548 nm)/Yellow Green

Temperature: 37°C

Light path: 1 cm

Pipette into dean dry test tubes labelled as Blank (B), Standard (S), and Test (T):

Addition Sequence	**B (ml)**	**S (ml)**	**T(ml)**
Working reagent	1.0	1.0	1.0
Distillled water	0.02	-	-
Uric acid Standard (S)	-	0.02	-
Sample	-	-	0.02

Mix well and incubate at 37°C for 5 min. or at RT. (25°C) for 15 min. Measure the absorbance of the Standard (Abs.S) and test sample against the Blank, within 30 min.

Calculations

Uric acid in mg/dl = $(\Delta AT/ \Delta AS) \times 8.0$

Linearity

This procedure is linear upto 20 mg/dl. If values exceed this limit, dilute the serum with normal saline (Nacl 0.9%) and repeat the assay, Calculate the value using the proper dilution factor.

BILIRUBIN KIT

(Mod. Jendrassik & Grof's method)

For the determination of Direct & Total Bilirubin in serum.

(For Invitro Diagnostic Use Only)

Summary

Blirubin is mainly formed from the heme portion of aged or damaged RBCs. It then combines with albumin to form a complex which is not water soluble. This is referred to as indirect or unconjugated Bilirubin. In the liver this Bilirubin Complex is combined with glucuronic acid into a water soluble conjugate. This is referred to as conjugated or direct Blirubin. Elevated levels of bilirubin are found in liver diseases (Hepatitis, cirrhosis), excessive hemolysis/destruction of RBC (hemolytic jaundice) obstruction of the billary tract (obstrucive jaundice) and in drug induced reactions. The differentiation between the direct and indirect bilirubin is important in diagnosing the cause of hyperbilirubinemia.

Principle

Bilirubin reacts with diazotised sulphanilic acid to form a coloured azobilirubin compound. The unconjugated bilirubin couples with the sulphanilic acld in the presence of a cafein-benzoate accelerator. The intensity of the colour fomed is directy proportional to the amount of bilirubin present in the sample.

Bilirubin + Diazotized sulphanilic acid = Azobilirubin compound

Normal reference values

Serum (Direct): upto 0.2 mg/dl

(Total): upto 1.0 mg/dl

Procedure

Wavelength / filter: 546 nm/Yellow- Green

Temperature: R.T.

Light path: 1cm

Direct Bilirubin Assay

Pipette into clean dry test tubes labelled as Blank (B) and Test (T)

Addition Sequence	B(ml)	T (ml)
Direct Bilirubin Reagent (L1)	1.0	1.0
Direct Nitrite Reagent (L2)	-	0.05
Sample	0.1	0.1

Mix well and incubate at R.T. for exactly 5 min. Measure the absorbance of the Test Samples (Abs.T) immediately against their respective Blanks.

Total Bilirubin Assay

Pipette into clean dry test tubes labelled as Blank (B), and Test (T):

Addition Sequence	B(ml)	T (ml)
Total Bilirubin Reagent (L1)	1.0	1.0
Total Nitrite Reagent (L2)	-	0.05
Sample	0.1	0.1

Mix well and incubate at R.T. for 10 min. Measure the absorbance of the Test Samples (Abs.T) immediately against their respective Blanks.

Calculatlons

Total or Direct Biliubin in mg/dl = = Abs.T X 13

Linearity

This procedure is linear upto 20 mg/dl. If values exceed this limit, dilute the sample with distilled water and repeat the assay. Calculate the value using the proper dilution factor.

ALBUMIN KIT

Bromocresol green (BCG)

Quantitative determination of albumin

For In-Vitro and professional use only

Store at 2-8 °C

INTENDED USE

For the determination of Albumin concentration in human serum or plasma.

Principle

The method is based on the specific binding of bromocresol green (BCG), an anionic dye, and the protein at acid pH produce a color change of the indicator from yellow-green to green –blue with the resulting shift in the absorption wavelength of the complex. The intensity of the color formed is proportional to the concentration of albumin in the sample.

BCG + Albumin (pH-4.3) = BCG-albumin complex

Materials provided

1. Bromocresol green PH 4.2 (0.12mmol/L) reagent.

2. Albumin standard. Albumin aqueous primary standard 5g/dL.

Materials required but not provided

- Spectrophotometer or colorimeter capable of measuring absorbance at 630 nm.
- Matched cuvettes 1.0 cm light path.
- General laboratory equipment.

Samples

- Serum or EDTA plasma.
- Albumin in serum and plasma is stable for 2 weeks at 2-8 °C, and for up to 4 months at –20°C.

Procedure

Assay conditions:

Wavelength -630 nm (600-650)

Cuvette - 1 cm light path

Temperature -15-25°C

2. Adjust the instrument to zero with distilled water.

3. Pipette into a cuvette:

Tubes	Blank	Sample	Standard
Reagent	1.0 mL	1.0 mL	1.0 mL
Sample	-	5µL	-
Standard	-	-	5µL

4. Mix and incubate for 10 minutes at room temperature (15-25°C).

5. Read the absorbance (A) of the samples and standard, against the blank.

The colour is stable for 60 minutes at room temperature.

Calculations

[(A) Sample/ (A) Standard] X 5 (Standard conc) = g/dL albumin

In the sample

Conversion factor: g/dl X 144.9 = µmol/L

Reference values

3.5 to 5.0 g/dl

These values are for orientation purpose; each laboratory should establish its own reference range.

Clinical significance

One of the most important serum proteins produced in the liver is albumin. This molecule has an extraordinarily wide range of functions, including nutrition, maintenance of oncotic pressure and transport of Ca ++, bilirubin, free fatty acid, drugs and steroids.

Variation in albumin levels indicate liver diseases, malnutrition, skin lesions such as dermatitis and burns or dehydration.

Clinical diagnosis should not be made on a single test result; it should integrate clinical and other laboratory data.

TOTAL PROTEIN KIT

(Biuret method)

For the determination of Total Proteins in serum and plasma.

(For lnvltro Diagnostic Use Only)

Summary

Proteins are constituents of muscle. enzymes, hormones and several other key functional and structural entities in the body. They are involved in the maintenance of the normal distribution of water between blood and the tissues. Consisting mainly of albumin and globulin the fractions vary 1ndependently and widely in diseases. Increased levels are found mainly in dehydration. Decreased levels are found mainly in malnutrition, impaired synthesis, protein losses as in hemorrhage or excessive protein catabolism.

Principle

Proteins, in an alkaline medium, bind with the cupric ions present in the biuret reagent to form a blue-violet coloured complex. The intensity of the colour formed is directly proportional to the amount of Proteins present in the sample.

Proteins+cu^{2+} = Blue-Violet Coloured Complex

Normal reference values

Serum & Plasma: 6.0-8.0g/dl

It is recommended that each laboratory establish its own normal range representing its patient population.

Procedure

Wavelength/filter: 550nm (Hg546nm)/Yellow-Green

Temperature: R.T./37°C

Light path: 1 cm

Pipette into dean dry test tubes labelled as Blank (B), Standard (S). and Test (T) :

Addition Sequence	B (ml)	S (ml)	T (ml)
Biuret reagent (L1)	1.0	1.0	1.0
D1stilled water	0.02	-	-
Protein Standard (S)	-	0.02	-
Sample	-	-	0.02

Mix well and incubate at 37°C for 10 min. or at R.T. for 30 min. Measure the absorbance of the standard (Abs.S), and Test sample (Abs.T) against the blank, within 60 Min.

Calculations

Total Proteins in g/dl = (Abs.T/Abs.S) X 8

Linearity

This procedure is linear upto 15 g/dl. If values exceed this limit, dilute the sample with distilled water and repeat the assay. Calculate the value using the proper dilution factor.

- **Serum Globulin** = (Serum total protein – Serum albumin)g/dL