PATHOLOGY AND MICRO BIOLOGY LAB MANUAL

B.TECH DEGREE PROGRAMM

Department of Biomedical Engineering

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PATHOLOGY AND MICRO BIOLOGY LAB MANUAL (17BMCC83)

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Dr. J. Madhusudhanan
Subject in charge

1. STERILIZATION TECHNIQUES

Exp:
Date:

Aim:

To sterilize the materials used for culturing microorganisms

Background:

It is the process to destroy all micro organisms without damaging or altering the substance being sterilized.

***Types*:**

***Dry heat*:**

Dry heat done using hot air ovens used to sterilize glassware, metal instruments and the materials that cannot be penetrated by steam and it was done at 180^{o} C for 2-3 hrs.

Moist heat:

Moist heat is done using autoclaves Steam is bactericidal and kills microbes by denaturing proteins present in the cell wall. Moist heat is used in sterilization of culture media and other liquids used.

Gaseous chemicals:

Highly effective for sterilization

Filter sterilization:

This type of sterilization used to sterilize solutions that are thermo labile. Function by entrapping micro organisms within the porous structure of the filter matrix. Ex: antibiotics, sugars, amino acids, vitamins.

Radiation:

Working area routinely sterilized using UV radiation and these lamps are called UV lamps, which emit radiation chiefly at wavelength at 2537 A.

Chemical disinfectants:

They are used to kill vegetative bacteria, fungi, viruses and occasionally spores. Example: phenolic compounds and hypo chlorides.

Procedure:

- Keep the glassware in the inoculation chamber and switch on UV light and let it glow for 30 minutes.
- Switch off the UV lamp.

- Check the sterility of working area by swabbing with sterile cotton plugs. The plates were filled with suitable agar and inoculated with swabs.
- Plates were incubated at 37^{o}C.

Observation:

2. PREPARATION OF CULTURE MEDIA FOR MICRO ORGANISMS (liquid media)

Exp:
Date:

Aim:

To learn the principles, and techniques of preparing nutrient broth for the microbial culture.

Background:

A common medium (Nutrient broth) used for growing bacteria (non fastidious organism). It contains beef extract, peptone and NaCl as ingredients. This liquid medium is nutrient broth and can be supplemented with other substances like sugars, and other inorganic salts to meet the requirements of any particular organisms. These media are always kept sterile until they are used.

Requirements:

Beef extract, peptone, NaCl, agar, conical flask, cotton wool, measuring cylinder, pH meter etc.

Composition of Nutrient broth:

Beef extract - 1g
Peptone - 1g
NaCl - 0.5 g
Distilled water - 100 ml

Procedure:

- Hundred milliliter of distilled water is taken in a conical flask.
- Weigh the above ingredients and dissolve in distilled water.
- The pH of the medium is adjusted to 7.2 with the help of 0.1N sodium hydroxide.
- Distribute equal quantity of the nutrient broth in test tubes and plug the tubes with cotton.
- Sterilize the medium at 121^{o}C for 15 minutes in an autoclave.
- After 15 minutes remove the test tube and keep in a clean place.

Observation:

3. ABO BLOOD GROUPING

Exp:
Date:

Aim:

To identify the ABO blood grouping of the given blood sample.

Introduction:

Agglutination reactions occur between high molecular weight antigens and multivalent antibodies. Agglutination occurs when multiple antigens on the surface of multiple cells are cross-linked. When this cross-linking of blood cells occurs it is called hemagglutination. Hemagglutination reactions are used to determine blood-type. The presence of the carbohydrate antigens A and B are determined by specific antibodies that are typically isolated from rabbits. When anti-A antibodies are mixed with type A blood the blood will agglutinate, as will type AB blood, which carries both antigens. Type O blood on the other hand lacks both the A and B antigen and will not agglutinate. The Rh blood typing scheme is similar in principle to the AB-typing scheme. The Rh type is actually a number of several different antigens. The Rh blood group refers to the "positive" (+) or "negative" (-) that is appended to the AB-typing scheme.

Some blood types contain antibodies that trigger immune reactions against other blood types. In general:

- If you have type A blood, you should only receive types A or O blood.
- If you have type B blood, you should only receive types B or O blood.
- If you have type AB blood, you can receive types A, B, AB, or O blood.
- If you have type O blood, you should only receive type O blood.

If you have type AB blood, you're known as a "universal recipient," and can receive any ABO category of donor blood. If you have type O blood, you're known as a "universal donor," as anyone can receive type O blood. Type O blood is often used in emergencies when there isn't enough time to perform blood typing tests.

Materials:

Microscope slide, Anti-A, anti-B, anti-Rh antisera, Sterile Lancet, Alcohol wipe, Sterile toothpicks, Sterile cotton ball & bandage and other routine materials.

Procedure:

1. Towards the bottom of a microscope slide write A, B and Rh.
2. Clean the middle finger of your non-dominate hand with the alcohol wipe.
3. Pierce the disinfected finger with the lancet. This is easiest if you squeeze the finger and **quickly** pierce it.
4. Place a drop of blood above each of the labels on the slide.
5. Add a drop of anti-A antisera to the drop of blood above the A label and mix with a sterile toothpick.
6. Repeat with the other two antisera using a new toothpick each time.
7. Examine the slide for agglutination.

Observation:

4. CROSS MATCHING OF BLOOD

Exp:
Date:

Introduction:

Blood typing is the first step. This test finds out if you have blood type A, AB, B, or O. Your blood is also tested to find out if your Rh type is negative or positive. It's important for your healthcare provider to know your blood type in order to select a donor blood that's compatible before doing the cross match. An intermediate step between blood typing and cross matching is called a recipient antibody screen. This test checks for unexpected antibodies in your blood. If unexpected antibodies are found, this can delay the selection of compatible donor blood.

Cross matching is a way for your healthcare provider to test your blood against a donor's blood to make sure they are fully compatible. It's essentially a trial transfusion done in test tubes to see exactly how your blood will react with potential donor blood. It's important for donor blood to match your own as closely as possible. Otherwise, your immune system might create antibodies against the donor blood cells. In this case, your immune system correctly views the donor cells as foreign, but incorrectly views them harmful. This can lead to a dangerous and possibly fatal reaction. Blood typing reveals what type of blood you have. This depends on the presence of certain antigens on your red blood cells (RBCs). Antigens are proteins that trigger your immune system to produce antibodies. There are four main types of blood:

- type A, which contains type-A antigens
- type B, which contains type-B antigens
- type AB, which contains type-A and type-B antigens
- type O, which contains neither type-A nor type-B antigens

Blood will also be classified as Rh positive (+) or Rh negative (-), based on the presence or absence of a particular protein on your RBCs, known as rhesus factor. Cross matching is a test used to check for harmful interactions between your blood and specific donor blood or organs.

Principle:

Cross matching is based on the principle of serological detection of any clinically significant irregular/unexpected antibodies in either donor or recipient's blood. There are two types of cross matches:

Major Cross Match: It involves testing the donor's red cells with recipient's serum to determine the presence of any antibody which may cause hemolysis or agglutination of donor red cells. This is more important than minor cross match.

Minor Cross Match: It involves testing of donor's plasma with recipient's red cells to determine the presence of any antibody which may cause hemolysis or agglutination of recipient's red cells.

Procedure:

- Draw a sample of blood using needle from one of your veins, usually on the inside of your elbow.
- Start by disinfecting the area with an antiseptic. An elastic band will be placed around the upper part of the arm, causing the vein to swell up with blood.
- A needle that gently be inserted into the vein and collect the blood sample in a tube.
- Once collected enough blood, the needle can be removed and unwrap the band from the arm.
- The puncture site will be cleaned and, if needed, bandaged.
- The blood sample will then be labelled and tested.
- Mix some of the blood with commercially available anti-A and anti-B antibodies.
- If the blood cells agglutinate**,** or clump together, it means the sample has reacted with one of the antibodies. This is called forward typing.
- Next, will perform reverse typing. This calls for some of the serum to be mixed with type A and type B cells.
- The sample then is checked for signs of reaction. Following this we have to perform Rh typing.
- This is when they mix some of the blood with antibodies against Rh factor. Signs of any reaction will be noted.

Observation:

NOTE:

Depending on the results of the blood typing, the blood will be classified as type A, B, AB, or O. It will also be classified as Rh+ or Rh-. There is no "normal" or "abnormal" blood type. The results of the cross matching test will help to assess if it's safe for the patients to receive specific donor blood or organs.

Back typing:

If the serum causes clumping only when mixed with:

- type B cells, have type A blood
- type A cells, have type B blood
- type A and B cells, have type O blood

If the serum doesn't cause clumping when mixed with either type A or B cells, the blood sample has type AB blood.

Rh typing:

If the blood cells clump when mixed with anti-Rh antibodies, the blood sample has Rh+ blood. If they don't clump, then the blood sample has Rh- blood.

Crossmatching:

If the blood cells clump when mixed with a donor sample, the donor blood or organ is incompatible with the blood.

5. HEMOGLOBIN ESTIMATION

Exp:
Date:

Aim:

To estimate the hemoglobin present in the given blood sample.

Introduction:

Hemoglobin is a conjugated protein that comprises of 90% of dry weight of RBC. Hemoglobin is tetramer containing two pairs of similar polypeptide chains called globin chains. To each of the four chains is attached heme which is complex of iron in ferrous form and protoporphyrin.

Principle:

N/10 HCl converts hemoglobin into soluble unstable acid hematin. The colour intensity of the acid hematin after dilution is compared with standard brown glass in the comparator.

***Materials required*:**

Sahli's hemoglobinometer, Hemoglobin pipette, Hemoglobin tube, Comparator, Stirrer, N/10 HCl, Concentrated HCl, Distilled water.

Procedure:

Collection of blood:

- Finger prick (in children and adults)
- From veins (venous blood)
- Blood collected in EDTA (1.0 to 1.2mg/ml) or double oxalate (2mg/1ml) in appropriate proportion (Double oxalate is mixture of 3 parts of ammonium oxalate and 2 parts of potassium oxalate)
- Heal prick (in infants)
  - Add N/10 HCl to the hemoglobinometer tube up to its lowest mark i.e 2g% (If N/10 HCl is taken above the mark the color of undiluted solution is lighter than standard and if N/10 HCl is taken less then all the hemoglobin is not converted).

- Take blood up to 20 cu mm mark on the pipette and transfer it to the hemoglobinometer tube containing N/10 HCl.
- Leave the solution for 10 minutes (for the conversion of hemoglobin to acid hematin)
- After 10 minutes add distilled water drop by drop and mix it by stirrer until the color matches with the color of comparator. While matching the color glass rod should be removed from the solution.
- The lower meniscus of the solution should be taken as the result which expresses hemoglobin content as g%
- If the hemoglobin is too low (less than 3g/dl) then 40µl blood is added to HCl upto 20 marks in the tube.
- Color is matched and result is halved.

Observation **:**

6. BLEEDING TIME AND CLOTTING TIME

Exp:
Date:

Aim:

The Bleeding and Clotting time test refers to a test that is performed on a sample of blood to measure the time taken for it to clot or coagulate. This test is also known as the BT CT test.

Principle:

In a bleeding time test, it is assessed what the rapidness with which the blood can clot and it can stop bleeding is. In this test, a small puncture is made in the skin of the person. By performing this test, it can be easily determined the way in which the platelets work together to form clots.

Clotting time is the time taken for blood to clot in a person. Clotting factors determine the clotting time in a person. Thus blood clotting factors play an important role in bleeding and clotting.

Platelets are found in the blood. Their function is to accumulate near the site of injury or puncture to seal the wound and reduce or stop the amount of blood that is flowing away from the body.

Procedure:

The trained person has to perform this procedure.

- The site of puncture is cleaned with an antiseptic to ensure that there is a minimal infection.
- A pressure cuff is placed around the arm, and it is inflated
- The pressure cuff is placed on the upper arm.
- Now two cuts that are small in size are made on the lower arm.
- These cuts cause a little bleeding. These are extremely shallow cuts.
- Then the cuff is removed from the arm.

- The bleeding time and clot time is checked using a timer.
- Every 30 seconds the blood from the cuts is blotted with blotting paper till the bleeding stops.
- Once the procedure is completed the cuts are bandaged.

Observation:

Reference range:

Reference range	Interpretation
2-7 minutes (Bleeding Time)	Normal
8-15 minutes (Clotting Time)	Normal

7. Study and Handling of Light Microscope

Exp:
Date:

Aim:

To study the parts, types and handling of light microscope.

Principle:

The light microscope is an instrument for visualizing fine detail of an object. It does this by creating a magnified image through the use of a series of glass lenses, which first focus a beam of light onto or through an object, and convex objective lenses to enlarge the image formed.

Light microscopes visualize an image by using a glass lens and magnification is determined by, the lens's ability to bend light and focus it on the specimen, which forms an image. When a ray of light passes through one medium into another, the ray bends at the interface causing refraction**.** The bending of light is determined by the refractive index**,** which is a measure of how great a substance slows the speed of light. The direction and magnitude of the bending of the light are determined by the refractive indexes of the two mediums that form the interface.

A medium with a lower refractive index such as glass to air, it normally speeds up the light penetration and making light bend away from the normal and when light is passed through a medium with a greater refractive index such as air to glass, it normally slows down and bends towards the normal, perpendicularly to the surface.

If an object is put between these two mediums i.e between water and air, in this case, a prism, the prism will bend the light at an angle. This is how the microscopic lenses work, they bend the light at an angle. The lens (convex) on receiving the light rays, it focuses the rays at a specific point known as the focal point (F-point)**.** The measure of distance from the center of the lens and the focal point is known as the focal length.

A microscope uses lenses whose strength is predetermined, in that, the strength of a lens is directly related to the focal length i.e short focal length magnifies objects more than lenses with a long focal length.

Microscopy works strictly with a factor of resolution whereby resolution being the ability of a lens to be able to differentiate small objects that are closely packed together. The resolution of a light microscope is determined by a numerical aperture of its lens system and by the wavelength of the light it employs; a numerical aperture a definition of the light wavelengths produced when the specimen is illuminated.

A minimum distance (d) between two objects that distinguishes then to be two separate entities, determined by the wavelengths of the light can be calculated by an Abbe equation using the wavelength of the light that illuminated the specimen (Lambda, λ) and the numerical aperture (NA, n sin Θ) i.e. **$d=0.5\ \lambda/n \sin \Theta$**

Introduction:

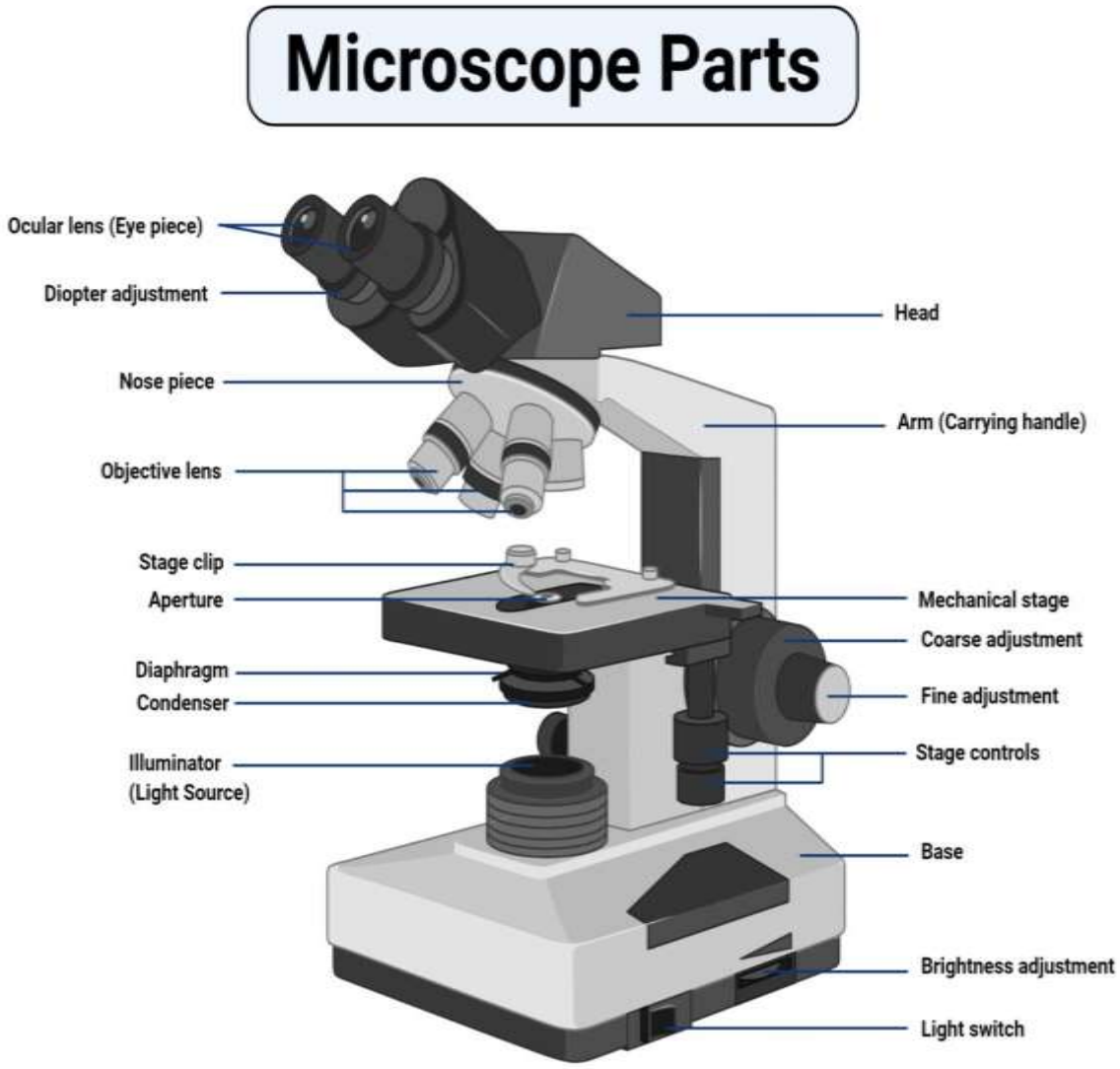
With the evolved field of Microbiology, the microscopes used to view specimens are both simple and compound light microscopes, all using lenses. The difference is simple light microscopes use a single lens for magnification while compound lenses use two or more lenses for magnifications. This means, that a series of lenses are placed in an order such that, one lens magnifies the image further than the initial lens.

The modern types of Light Microscopes include:

1. Bright field Light Microscope
2. Phase Contrast Light Microscope
3. Dark-Field Light Microscope
4. Fluorescence Light Microscope

Bright-field Light Microscope (Compound Light Microscope):

- This is the most basic optical Microscope used in microbiology laboratories which produces a dark image against a bright background. Made up of two lenses, it is widely used to view plant and animal cell organelles including some parasites such as *Paramecium* after staining with basic stains.
- Its functionality is based on being able to provide a high-resolution image, which highly depends on the proper use of the microscope. This means that an adequate amount of light will enable sufficient focusing of the image, to produce a quality image. It is also known as a compound light microscope.



It is composed of:

- Two lenses which include the objective lens and the eyepiece or ocular lens.
- Objective lens is made up of six or more glasses, which make the image clear from the object
- The condenser is mounted below the stage which focuses a beam of light onto the specimen. It can be fixed or movable, to adjust the quality of light, but this entirely depends on the microscope.
- They are held together by a sturdy metallic curved back used as an arm and a stand at the bottom, known as the base, of the microscope. The arm and the base hold all the parts of the microscope.

- The stage where the specimen is placed, allowing movement of the specimen around for better viewing with the flexible knobs and it is where the light is focused on.

- Two focusing knobs i.e the fine adjustment knob and the coarse adjustment knob, found on the microscopes' arm, which can move the stage or the nosepiece to focus on the image. the sharpen the image clarity.

- It has a light illuminator or a mirror found at the base or on the microbes of the nosepiece.

- The nosepiece has about three to five objective lenses with different magnifying power. It can move round to any position depending on the objective lens to focus on the image.

- An aperture diaphragm also is known as the contrast, which controls the diameter of the beam of light that passes through the condenser, in that, when the condenser is almost closed, the light comes through to the center of the condenser creating high contrast. But when the condenser is widely open, the image is very bright with very low contrast.

Magnification by Bright field Microscope (Compound light microscope):

During visualization, the objective lens remains parfocal which means, when the objective lens is changed, the image still remains in focus. The objective lens plays a major role in focusing the image on the condenser forming an enlarged clear image within the microscope, which is then further magnified by the eyepiece to a primary image.

What is seen in the microscope as an enlarged clear image of the specimen is known as the virtual image. To calculate the magnification, multiply the objective and eyepiece objective magnification together. The magnification is standard, i.e not too high nor too low, and therefore depending on the magnification power of the lenses, it will range between 40X and 100X.

Calculation of magnification = Magnification of objective lens / magnification of the eyepiece lens

The objective lens plays a vital role in not only enlarging the image but also making it clear for viewing, a feature known as resolution. Resolution according to Prescott, is the ability of a lens to separate or distinguish between small objects closely linked together. Whereas the eyepiece magnifies the image at the end of the viewing, its magnification range is lower than that of the objective lens at 8X-12X (10X standard) and that of the objective lens at 40X-100X, magnification, and resolution of the microscope is highly dependent on the objective lens.

Applications of the Bright Field Light Microscope (Compound light microscope):

Vastly used in Microbiology, this microscope is used to view fixed and live specimens, that have been stained with basic stains. This gives contrast for easy visibility under the microscope. Therefore it can be used to identify basic bacteria cells and parasitic protozoans such as *Paramecium.*

Phase contrast Microscope:

- This is a type of optical microscope whereby small light deviations know as phase shifts occur during light penetration into the unstained specimen. These phase shifts are converted into the image to mean, when light passes through the opaque specimen, the phase shifts brighten the specimen forming an illuminated (bright) image in the background.

- The phase-contrast microscope produces high contrast images when using a transparent specimen more so those of microbial cultures, thin tissue fragments, cell tissues, and subcellular particles.

- The principle behind the working of the phase-contrast microscope is the use of an optical method to transform a specimen into an amplitude image, that's viewed by the eyepiece of the microscope.

- The PCM can be used to view unstained cells also known as the phase objects, which means that the morphology of the cell is maintained and the cells can be observed in their natural state, in high contrast and efficient clarity. This is because if the

specimens are stained and fixed, they kill most cells, a characteristic that is uniquely undone by the brightfield light microscope.

- The shifts that occur during light penetration, become converted to changes in amplitude which causes the image contrast.

- Coupled with contrast-enhancing elements such as fluorescence, they produce better visuals of the specimens' image.

Parts of the Phase Contrast Microscope:

The instrumentation of the Phase Contrast Microscope is based on its light pathways from receiving the source of light to the visualization of the image.
Therefore its sequentially made up of: Light source (Mercury arc lamp), Collective lens, Aperture, Condenser, Condenser annular, Specimen, Objective, Phase plate, Deflected light, Phase ring

The functioning of the Phase Contrast microscope

- The change caused by the deviated scattered (Deflected) light and the undeviated light that reaches the specimen which is absorbed, create at a certain wavelength, producing color. The difference created by the scattered light and that of the absorbed light is known as amplitude variations. These amplitude variations are sensitive to allowing visualization by photographic equipment like the Phase Contrast Microscope, hence seen by the human eye.

- The Condenser of the phase-contrast microscope has an opaque disk that is known as an annular ring, with a transparent ring that produces a cone of light, that passes through a specimen. Due to light variations some light bend at the specimen, caused by variations in light density, forming an image at the objective lens. The undeviated light will strike the phase ring on the phase plate and the deviated light will miss the phase ring passing through the phase plate directly, this forms an image.

- The Phase-Contrast Microscope is designed with objective lenses that have the ability to perform multiple functions when combined with contrast-enhancing techniques, for example, fluorescence. The objective lenses are located in the internal phase plate with variation in the light absorption and phase displacement i.e undiffraction, creating a wide spectrum for contrasting the specimen and forming a strong contrast in the background.

Applications of Phase-Contrast Microscope:

- Determine morphologies of living cells such as plant and animal cells
- Studying microbial motility and structures of locomotion
- To detect certain microbial elements such as the bacterial endospores

Dark-field Light Microscope:

This is a specialized type of bright field light microscope which has several similarities to the Phase-Contrast Microscope. To make a dark field Microscope, place a darkfield stop underneath and a condenser lens which produces a hollow cone beam of light that enters the objective only, from the specimen. This technique is used to visualize living unstained cells.

This is effected by the way illumination is done on the specimen in that, when a hollow cone beam of light is transmitted to the specimen, deviated light (unreflected/unrefracted) rays do not pass through the objectives but the undeviated (reflected/refracted) light passes through the objectives to the specimen forming an image. This makes the surrounding field of the specimen appear black while the specimen will appear illuminated. This is enabled by the dark background this the name, dark-field Microscopy.

Applications of the Dark Field Microscope:

- It is used to visualize the internal organs of larger cells such as the eukaryotic cells

- Identification of bacterial cells with distinctive shapes such as *Treponema pallidum*, a causative agent of syphilis.

Fluorescent Microscope:

The above-discussed microscopes will normally produce images after a light has been transmitted and passed through the specimen.

In the case of the fluorescent Microscope, the specimen emits light. By adding a dye molecule to the specimen. This dye molecule will normally become excited when it absorbs light energy, hence it releases any trapped energy as light. The light energy that is released by the excited molecule has a long wavelength compared to its radiating light. The dye molecule is normally a fluorochrome, that fluoresces when exposed to the light of a certain specific wavelength. The image formed is a fluorochrome-labeled image from the emitted light

The **principle** behind this working mechanism is that the fluorescent microscope will expose the specimen to ultra or violet or blue light, which forms an image of the specimen that is emanated by the fluorescent light. They have a mercury vapor arc lamp that produces an intense beam of light that passes through an exciter filter. The exciter filter functions to transmit a specific wavelength to the fluorochrome stained specimen, producing the fluorochrome-labeled image, at the objective.

After the objective, there is a barrier filter that functions primarily to remove any ultraviolet radiation that may be harmful to the viewer's light, thus reducing the contrast of the image.

Applications of the Fluorescent Microscope:

- Used in the visualization of bacterial agents such as *Mycobacterium tuberculosis.*
- Used to identify specific antibodies produced against bacterial antigens/pathogens in immunofluorescence techniques by labeling the antibodies with fluorochromes.

- Used in ecological studies to identify and observe microorganisms labeled by the fluorochromes
- It can also be used to differentiate between dead and live bacteria by the color they emit when treated with special stains

Besides the above-discussed microscopes, there is one not commonly used microscope known as the Differential Interference Contrast Microscopy. It is very similar to the phase-contrast microscope whereby the images are formed from the variations in the light either deviated and or undeviated. The difference is, here two beams of light are emitted to the specimen and focused by a prism. One beam passes through the prism to the specimen while another passes through the glass slide clear area without the specimen. The two beams then combine and interfere with each other to form an image. It can be used to view cell structures such as endospores, bacterial cell walls, nuclei and granules for unstained specimens.

8. Total RBC Count

Exp:
Date:

Aim:

To count the total RBC present in the blood.

Principle:

The test is important because RBCs contain hemoglobin, which carries oxygen to the body's tissues. The number of RBCs you have can affect how much oxygen your tissues receive. The tissues need oxygen to function. The hematocrit is the volume of red blood cells in the body. A hematocrit test measures the ratio of RBCs in the blood. Platelets are small cells that circulate in the blood and form blood clots that allow wounds to heal and prevent excessive bleeding.

Materials required:

Hayme's solution consists of :

Na Cl = 1 G (Isotonic solution).

Na_2SO_4 = 5 gram. It will prevent rouleux formation.

$HgCl_2$ = 0.5 G acts as antiseptic.

D.H_2O = 200 mL

Gower's solution consists of:

Na Cl for an isotonic solution.

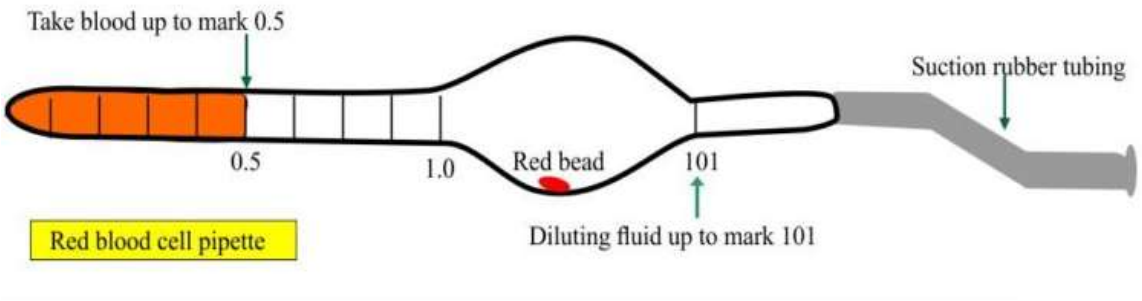
Na2SO4 = 12.5 G

Glacial acetic acid = 33.3 G

D.H2O = 200 mL

Procedure:

- RBCs counting solution is Hayem's or Gowers isotonic saline.
- Make a dilution of 1:200 with a diluting solution. Fill the red bulb pipette up to 0.5 marks.



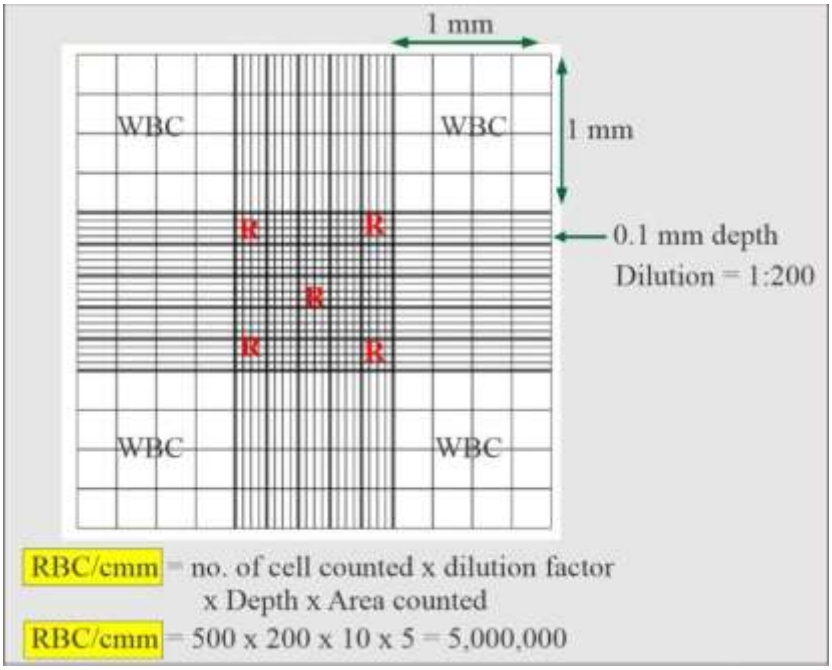
- Draw the solution to mark 101 of the RBC pipette
- Mix the blood thoroughly in the pipette.
- Discard the first few drops (4 to 5) and then fill the Neubauer chamber.

- Make sure that the chamber is free of air bubbles.
- The distribution of the cells should be uniform over the ruled area.
- Allow for 2 minutes to settle the cells.
- Now count RBCs in the Neubauer chamber.
- Use 40 X to count the RBCs.
- For RBCs, use the center square, which has 25 smaller squares.
- Count the corner 4 squares and one central square.
- Count only the RBCs which fall on the left and top border of these squares.
- Repeat the count twice and divide by 2 to get the average.

The formula for RBCs count is:

Multiply factor = 10 x 200 / 0.2 = 10,000

Multiply RBCs count with 10,000 = RBCs million/cmm.



Observation:

NOTE:

- The normal RBC range for men is 4.7 to 6.1 million cells per microliter (mcL).
- The normal RBC range for women who aren't pregnant is 4.2 to 5.4 million mcL.
- The normal RBC range for children is 4.0 to 5.5 million mcL.

9. Peripheral smear study (i) Morphology (ii) WBC Differential count

Exp:
Date:

Introduction:

Peripheral blood is the fluid that travels through your heart, arteries, capillaries, and veins. Its most important function is to transport oxygen and other nutrients to your body's cells and tissues and to remove carbon dioxide and other waste products from the body. Peripheral blood also plays an essential role in the immune system, delivery of hormones, and temperature regulation.

Taking a blood sample, also known as venipuncture, is a minimally invasive way to gather information about the types of cells circulating throughout the body. The components of the blood may be examined along with the levels of red blood cells, white blood cells, and platelets. The examination of the blood is useful in the diagnosis of many conditions, including leukemia, lymphoma, and myelo-proliferative disorders.

A fresh, well-made, peripheral blood smear is crucial for accurate cell morphology assessment. The components contributing towards the final result are fourfold: The quality of the smear, the quality of the stain, the quality of the microscope and the skill and experience of the microscopist.

A white blood cell (WBC) count measures the number of white blood cells in your blood, and a WBC differential determines the percentage of each type of white blood cell present in your blood. A differential can also detect immature white blood cells and abnormalities, both of which are signs of potential issues.

A WBC count can also be called a leukocyte count, and a WBC differential can also be called a leukocyte differential count.

- neutrophils
- lymphocytes
- monocytes
- eosinophils
- basophils

The three basic types of blood cells

Measuring changes in your blood cell levels can help your doctor evaluate your overall health and detect disorders. The test measures the three basic types of blood cells.

Red blood cells

Red blood cells carry oxygen throughout your body and remove carbon dioxide. A CBC measures two components of your red blood cells:

- hemoglobin: oxygen-carrying protein
- hematocrit: percentage of red blood cells in your blood

Low levels of hemoglobin and hematocrit are often signs of anemia, a condition that occurs when blood is deficient in iron.

White blood cells

White blood cells help your body fight infection. A CBC measures the number and types of white blood cells in your body. Any abnormal increases or decreases in the number or types of white blood cells could be a sign of infection, inflammation, or cancer.

Platelets

Platelets help your blood clot and control bleeding. When a cut stops bleeding, it's because platelets are doing their job. Any changes in platelet levels can put you at risk for excessive bleeding and can be a sign of a serious medical condition.

Materials required:

Gloves, 70%alcohol, glass slide, gauze,

Procedure:

1. Label pre-cleaned slides (preferably frosted-end) with patient's name (or other identifier), date and time of collection.
2. Wear gloves.
3. Clean slides with 70 to 90% alcohol and allow to dry. Do not touch the surface of the slide where the blood smear will be made.
4. Select the finger to puncture, usually the middle or ring finger. In infants, puncture the heel.
5. Clean the area to be punctured with 70% alcohol; allow to dry.
6. Puncture the ball of the finger, or in infants puncture the heel.
7. Wipe away the first drop of blood with clean gauze.
8. Touch the next drop of blood with a clean slide. Repeat with several slides (at least two thick and two thin smears should be made). If blood does not well up, gently squeeze the finger.

Observation:

Reference:

Blood component	Normal levels
red blood cell	In men: 4.32-5.72 million cells/mcL In women: 3.90-5.03 million cells/mcL
hemoglobin	In men: 135-175 grams/L In women: 120-155 grams/L
hematocrit	In men: 38.8-50.0 percent In women: 34.9-44.5 percent
white blood cell count	3,500 to 10,500 cells/mcL
platelet count	150,000 to 450,000/mcL

Test	Adult normal cell count	Adult normal range (differential)	Low levels (leukopenia and neutropenia)	High levels (leukocytosis and neutrophilia)
white blood cells (WBC)	4,300-10,000 (4.3-10.0) white blood cells/mcL	1% of total blood volume	<4,000 white blood cells/mcL	>12,000 white blood cells/mcL
neutrophils (ANC)	1,500-8,000 (1.5-8.0) neutrophils/mcL	45-75% of total white blood cells	**mild:** 1,000-1,500 neutrophils/mcL **moderate:** 500-1,000 neutrophils/mcL **severe:** <500 neutrophils/mcL	>8,000 neutrophils/mcL

10. Manual paraffin tissue processing and section cutting (demonstration)

Exp:
Date:

Introduction:

Embedding tissue into paraffin blocks supports the tissue structure and enables very thin sections to be cut and mounted onto microscope slides for analysis.

Materials and Reagents:

Waterbath, Container with ice, Glass microscope slides, Microtome and blade, Oven

Procedure:

Sectioning:

- Chill paraffin-embedded tissue blocks on ice before sectioning. Cold wax allows thinner sections to be obtained by providing support for harder elements within the tissue specimen. The small amount of moisture that penetrates the block from the melting ice will also make the tissue easier to cut.
- Fill a water bath with ultrapure water and heat to 40-45^{o}C.
- Place the blade in the holder, ensure it is secure and set the clearance angle. The clearance angle prevents contact between the knife facet and the face of the block. Follow the microtome manufacturer's instructions for guidance on setting the clearance angle. For Leica blades this is normally between 1^{o} and 5^{o}
- Insert the paraffin block and orientate so the blade will cut straight across the block.
- Carefully, approach the block with the blade and cut a few thin sections to ensure the positioning is correct. Adjust if necessary.
- Trim the block to expose the tissue surface to a level where a representative section can be cut. Trimming is normally done at a thickness of 10-30 µm.
- Cut sections at a thickness of about 4-5 µm (you will probably need to discard the first few sections as they are likely to contain holes caused by trimming).
- Using tweezers, pick up the ribbons of sections and float them on the surface of the water in the water bath so they flatten out. Use the tweezers to separate the sections.
- Use microscope slides to pick the sections out of the water bath and store upright in a slide rack.

- Place the slide rack into an oven and allow sections to dry overnight at 37^{o}C.

Observation:

11. Cryo processing of tissue and cryosectioning (demonstration)

Exp:
Date:

Introduction:

The cryosection procedure is also known as the frozen section procedure. It is a laboratory procedure used to perform microscopic analysis of a specimen. This procedure is most commonly used in oncological surgery. A pathologist is necessary for the intraoperative consultation. The pathologist evaluates and examines the specimen. The pathologist then reports if the specimen is benign or malignant. They are also responsible for informing if the resection margin is clear of cancer. The cryosectioning procedure practiced today is based on the technique described by Dr. Louis B. Wilson in 1905.

Cryosections are a good way to visualize the fine details of the cell. Although less stable than resin and paraffin-embedded sections, cryosections are typically more superior for the preservations and detection of antigens through microscopy. Cryosection preparation can usually be done in a day. The rapid freezing helps decrease the formation of ice crystals and minimizes morphological damage. Cryosections can be used in various procedures such as in situ hybridization, immunohistochemistry, and enzymatic degradation.

Materials required:

Fixative (such as formaldehyde), Staining solution (such as toluidine blue, hematoxylin, eosin, or others), Fresh tissue sample, Brush, Container for storage of tissue sample, Cryostat with metal grids, Microscope slides, Plastic or metal tissue mold, Moistened tissues and other routine lab wares.

Procedure:

The procedure for cryosectioning can be done quickly as it is relatively simple.

A) Cryostat preparation

i) Use proper cleaning agents and clean the cryostat.

ii) Insert a new sterile blade for the cryostat.

iii) When it comes to frozen embedded block or fresh frozen specimens, use some frozen embedding media to adhere the sample to the mount in the proper cutting position. Ensure that the cutting surface is parallel to the blade.

iv) Allow the specimen to equilibrate reaching cryostat temperature. This takes approximately 20 minutes.

B) Cryosection preparation

i) Freeze a tissue sample up to 2.0 cm in diameter in OCT using a suitable tissue mold. Freeze the OCT with tissue onto the metal grids fitting the cryostat. At room temperature, OCT is viscous but freezes at -20^0C. Depending on the type of tissue, optimal freezing temperature may differ. For example, brain tissues are optimally frozen at -3^0C in M-1 medium.

ii) Cut sections that are approximately 5 to 15μm in the cryostat at a temperature of -20^0C. the temperature of the cutting chamber can be adjusted based on the tissue under study. These sections can be moved with the help of toothpicks and brushes if necessary. A camel hair brush can be helpful in guiding the merging section over the blade.

iii) Remove the folds and wrinkles present on the cut sections.

iv) Within 1 minute of cutting the tissue section, transfer it to a room temperature slide by touching the slide to the tissue. This allows the cut section to adhere to the slide. Using your gloved finger, rub the underside of the slide to help transfer heat as this helps with the adhesion. All this should be accomplished within 1 minute of cutting the section. This avoids the freeze drying of the specimen. Using silanized or poly-L-lysine coated slides improve the adherence of the section.

v) An optional step is to use ultraviolet treatment of the slide to increase the adherence and with sterilization. This can be done by incubating the slide for 15 to 20 minutes under the ultraviolet light. It works best if an ultraviolet sterilization hood is utilized. The light breaks down the membrane slightly helping with adhesion and sterilization. It is important to not incubate it longer than 30 minutes as it risks damaging the membrane.

vi) To evaluate the preservation and orientation of the tissue, the first slide of each set can be stained using toluidine blue, eosin, hematoxylin, or various aqueous stain.

vii) Immerse the slide immediately into a fixative. Some researchers air-dry the section onto the slide at air temperature to maximize adherence before fixation. However, this technique has a disadvantage where the surface tension forces distortion of the cells resulting in the loss of high-resolution detail. It can also lead to changes in the results of immunostaining.

viii) Any unused tissue should be covered using a layer of OCT to avoid freeze drying and storing leftover samples at -70^{0}C. For long-term storage, adding moistened tissue to the container helps prevent desiccation especially in a frost-free freezer.

Observation:

12. Urine physical and chemical examination

Exp:
Date:

Aim:
To examine the given urine sample by physical and chemical method.

Introduction:
In order to get valid results from urine analysis, an appropriate and correct technique of urine sample collection is essential. For majority of urine examinations the first early morning urine is the most suitable. It is recommended the urine collection is preceded by at least eight hours of lying position. The early morning urine is more concentrated and acidic compared to the later samples, and suitable especially for chemical examination. The urine taken later during the day is more affected by liquid intake, food and physical activity. Urine is typically obtained by a spontaneous micturition. The actual urine collection is to be performed after thorough washing and wiping of the external urethral orifice. A perfectly clean, dry, and capped vessel should be used; in particular, it must be free from detergents and disinfectants that distort chemical analyses. The middle flow of urine is best for analysis. The initial flow is always contaminated with cells and bacteria from around the external urethral orifice. Therefore, the patient should dispose the first flow or urine into closet, and pick up the following portion of urine into the collection vessel. In certain situations it might be necessary to obtain the urine sample by percutaneous suprapubic puncture of urinary bladder, or by urinary bladder catheterisation. In women the examination of urine is avoided shortly before, and shortly after the menstruation. For majority of qualitative and semiquantitative chemical analyses with urine test strips, no stabilising additives into the urine sample are necessary. If, however, it is not possible to examine the urine sample within two hours since its collection, the urine should be kept refrigerated or chemically conserved.

Chemical examination of urine:
The routine chemical examination of urine involves qualitative tests for protein, glucose, hemoglobin, ketone bodies and bile pigments. These components are mostly present in urine from healthy individuals as well, but in tiny amounts undetectable by the routine tests. Various pathological conditions increase their concentration in urine.

1. Proteins

a) Sulfosalicylic acid test

Place about 1 ml of the sample being tested into a test tube and add 5-10 drops of 20% sulfosalicylic acid. A white precipitate is produced if protein is present. This is a very sensitive test.

b) Heller's test

Place carefully about 1 ml of concentrated nitric acid (warning: corrosive !) in a test tube. Incline the tube and slowly pour down the side of the tube in a manner to produce a stratification (two separated layers) about 1 ml of the sample being tested. A white ring appears between the two layers if the test is positive.

c) Heat coagulation

Place about 1 ml of the sample being tested into a test tube, add about 0.2 ml of acetate buffer (pH=4.6) and heat to boiling. If protein is present, white turbidity appears.

2. Glucose

a) Fehlings's test

Take a clean test tube and prepare Fehlings's reagent by mixing equal volumes (about 1 ml) of Fehling I (copper(II) sulfate) and Fehling II (NaOH, NaK – tartarate). The reagent prepared is dark blue in colour, without any precipitate inside. Take another test tube and put there about 1 ml of the sample being tested. Add equal volume of Fehlings's reagent prepared in the previous step. Heat the content of the test tube to boiling. If the test is positive, reddish brown (orange, olive-green) precipitate is formed.

b) Benedict's test

Place about 1 ml of Benedict's reagent in a test tube. Add 4-5 drops of the sample being tested and heat the content of the test tube to boiling. If the test is positive, reddish brown (orange, olive-green) precipitate is formed. *(In principle, Benedict's reagent is only a modification of Fehlings's reagent.)*

c) Nylander's test

Place about 1 ml of the sample being tested into a test tube, add about 1 ml of Nylander's reagent and heat the content of the test tube to boiling. If the test is positive, the solution turns grayish-yellow due to formation of black precipitate of metallic bismuth.

3. Ketone bodies

a) Lestradet's test

Take a small round filter paper and place it unfolded on the white tile. Use a little spoon (it is inside the plastic box with the reagent) to put Lestradet's reagent onto the center of filter paper. Moisten the reagent on the filter paper with a drop of the sample being tested. If purple colour develops within 1 minute, the test is positive.

b) Legal's test

Take a clean test tube and disolve few grains of solid sodium nitroprusside in about 1 ml of water. Take another test tube and put there about 5 ml of the sample being tested, add 5 drops of sodium nitroprusside solution prepared in the previous step and 5 drops of 10% NaOH. Red colour appears due to the presence of creatinine. Add few drops of concentrated acetic acid. If ketone bodies are present, the coloration turns to deeper colour.

4. Blood and hemoglobin

Heitz - Boyer's test

In a test tube combine about 1 ml of the sample being tested with equal volume of the Heitz-Boyer reagent. Carefully overlay with hydrogen peroxide. In the presence of hemoglobin (blood) a red-violet ring appears at the interface of two layers.

5. Bilirubin

a) Naumann's test

In a test tube, mix about 5 ml of the sample being tested with talc powder. Prepare what you need for filtration (little funnel, filtrate paper) and filter the mixture to separate talc with bilirubin adsorbed. After the filtration, put a drop of Fouchet's reagent (a solution of FeCl3 and trichloroacetic acid) on the talc on filtration paper. A blue colour indicates that bilirubin is present. This test is more sensitive than the other tests.

b) Hamarsten's test

Place carefully about 0.5 ml of the mixture of acids (HCl and HNO3 - warning: corrosive !) in a test tube. Add about 2 ml of ethanol and few drops of the sample being tested. In the presence of bilirubin, green coloration appears.

c) Gmelin's test

Place carefully about 1 ml of concentrated nitric acid (warning: corrosive !) in a test tube. Incline the tube and slowly pour down the side of the tube in a manner to produce a stratification (two separated layers) about 1 ml of the sample being tested. A green ring appears between the two layers if the test is positive. *(Very same procedure as described for Heller's test; protein – white ring, bilirubin – green ring.)*

d) Rosin's test

Place about 1 ml of the sample being tested into a test tube. Carefully overlay with alcoholic solution of iodine. A green ring at the interface of two layers indicates the presence of bilirubin.

6. Urobilinogen

Ehrlich's aldehyde test

Place about 1 ml of the sample being tested into a test tube. Add few drops of Ehrlich's aldehyde reagent. A red colour suggests that "Ehrlich positive substances" are present. This is a very sensitive test.

Note:

- Dark yellow urine may be an indication of dehydration.
- Bright yellow urine is often caused by multivitamin supplements.
- Red or pink urine may be a sign of bleeding or simply a result of having eaten beets.
- Brown or greenish-brown urine may be a sign of hepatitis or other liver problems.
- Green urine is sometimes seen in people who have been on the sedative Diprivan (propofol) for a long period of time.

The 10 most commonly performed reagent tests are:

- Bilirubin, a yellowish pigment associated liver problems
- Erythrocytes (red blood cells), a sign of bleeding
- Glucose, elevations that suggest diabetes
- Ketones, elevations that also suggest diabetes
- Leukocytes (white blood cells), a sign of infection

- Nitrites, suggestive of a bacterial infection
- pH, which measures how acidic the urine is
- Protein, elevations that suggest kidney impairment
- Specific gravity (SG), which measures urine concentration
- Urobilinogen, seen with hepatitis and liver disease

Vitamin C (ascorbic acid) reagent strips are sometimes used to see if any abnormality in the results is caused by disease or a vitamin supplement you may have taken.

13. SIMPLE STAINING

Exp:
Date:

Aim:

The aim of the simple stain is to determine cell shape, size and arrangements of bacteria.

Principle:

In simple staining, the bacterial smear is stained with a single reagent, which produces a distinctive contrast between the organism and its background. Basic stains with a positively charged chromogen are preferred because bacterial nucleic acids and certain cell wall components carry a negative charge that strongly attracts and binds to the cationic chromogen. The purpose of simple staining is to elucidate the morphology and arrangement of bacterial cells. The most commonly used basic stains are methylene blue, crystal violet, and carbol fuchsin.

Materials Required:

Methylene blue, crystal violet, and carbol fuchsin, Microincinerator or Bunsen burner, inoculating loop, staining tray, microscope, lens paper, bibulous (highly absorbent) paper, and glass slides.

Procedure:

- Place a slide on the staining tray and flood the smear with one of the indicated stains, using the appropriate exposure time for each: carbol fuchsin, 15 to 30 seconds; crystal violet, 20 to 60 seconds; methylene blue, 1 to 2 minutes.
- Gently wash the smear with tap water to remove excess stain. During this step, hold the slide parallel to the stream of water; in this way you can reduce the loss of organisms from the preparation.
- Using bibulous paper, blot dry, but do not wipe the slide.
- Examine all stained slides under oil immersion.

Observation:

Note:

Bacilli and diplobacilli: Rod-shaped bacteria, purple

Spirilla: spiral-shaped bacteria, purple

Cocci: spherical-shaped, bacteria, purple

14. GRAM'S STAINING

Exp:
Date:

Aim:

To study and identify whether the given bacterial culture is gram positive of gram negative.

Principle:

Gram's stain was introduced in 1880 by the Danish bacteriologist Christian Gram. Gram's staining is employed to visualize and differentiate between organisms. It is useful of presumptive identification of organisms before carry out the other serological and biochemical test.

Bacteria can be classified into two classes: gram positive and gram negative. The bacteria, which retain the crystal violet, are termed as gram positive and the bacteria which lose the crystal violet are termed as gram negative.

The cell walls of negative bacteria are thinner and has high liquid content during the alcohol treatment it extracts the lipid this increases the porosity of cell. So the gram negative bacteria are decolorized and take up the color of the counter stain.

Iodine is used as a mordant, saffranin is used as a counter stain.

Materials required:

Crystal violet, gram's iodine, ethanol and saffranin, glass rod, hot plate, glass slides, 24 hrs old culture, microscope, distilled water etc.

Procedure:

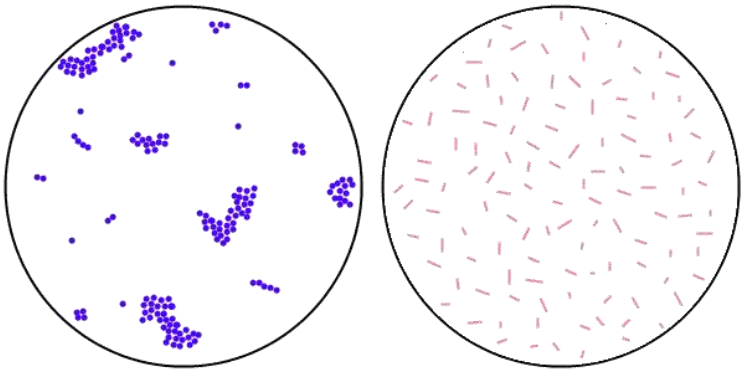
Preparation of a smear

- Using a sterilized inoculating loop, transfer loopful of liquid suspension containing bacteria to a slide (clean grease free microscopic slide) or transfer an isolated colony from a culture plate to a slide with a water drop.
- Disperse the bacteria on the loop in the drop of water on the slide and spread the drop over an area the size of a dime. It should be a thin, even smear.
- Allow the smear to dry thoroughly.

- Heat-fix the smear cautiously by passing the underside of the slide through the burner flame two or three times. It fixes the cell in the slide. Do not overheat the slide as it will distort the bacterial cells.

Staining

- Cover the smear with methylene blue and allow the dye to remain in the smear for approximately one minute (Staining time is not critical here; somewhere between 30 seconds to 2 minutes should give you an acceptable stain, the longer you leave the dye in it, the darker will be the stain).
- Using distilled water wash bottle, gently wash off the excess methylene blue from the slide by directing a gentle stream of water over the surface of the slide.
- Wash off any stain that got on the bottom of the slide as well.
- Saturate the smear again but this time with Iodine. Iodine will set the stain
- Wash of any excess iodine with gently running tap water. Rinse thoroughly. *(You may not get a mention of step 4 and 5 in some textbooks)*
- Wipe the back of the slide and blot the stained surface with bibulous paper or with a paper towel.
- Place the stained smear on the microscope stage smear side up and focus the smear using the 10X objective.
- Choose an area of the smear in which the cells are well spread in a monolayer. Center the area to be studied, apply immersion oil directly to the smear, and focus the smear under oil with the 100X objective.



Cocci in Cluster Bacilli

Observation:

The bacterial cells usually stain uniformly and the color of the cell depends on the type of dye used. If methyene blue is used, some granules in the interior of the cells of some bacteria may appear more deeply stained than the rest of the cell, which is due to presence of different chemical substances.

15. Widal slide test

Exp:
Date:

Introduction:

- Widal test is a serological test which is used for the diagnosis of enteric fever or typhoid fever. The test was developed by Greembaum and Widal in 1896. Typhoid or enteric fever is caused by a gram negative bacteria *Salmonella enterica* (*Salmonella* Typhi or *Salmonella* Paratyphi), found in the intestine of man. Salmonella paratyphi also causes Typhoid but of a milder form.
- *Salmonella* possess O antigen on their cell wall and h antigen on their flagella. On infection, these antigen stimulates the body to produce specific antibodies which are released in the blood. The Widal test is used to detect these specific antibodies in the serum sample of patients suffering from typhoid using antigen-antibody interactions. These specific antibodies can be detected in the patient's serum after 6 days of infection (fever).
- *Salmonella* Typhi possesses O antigen on the cell wall and H antigen on flagella. *Salmonella* Paratyphi A and *S.* Paratyphi B also possess O antigen on their cell wall and but have AH and BH antigen on their flagella respectively.
- The patient's serum is tested for O and H antibodies (agglutinins) against the following antigen suspensions (usually stained suspensions):
    - ***S***. Typhi 0 antigen suspension, 9, 12
    - ***S***. Typhi H antigen suspension, d
    - ***S***. Paratyphi A 0 antigen suspension, 1, 2, 12
    - ***S***. Paratyphi A H antigen suspension, a
    - ***S***. Paratyphi B 0 antigen suspension, 1, 4, 5, 12
    - ***S***. Paratyphi B H antigen suspension, b, phase 1
    - ***S***. Paratyphi C 0 antigen suspension, 6, 7
    - ***S.*** Paratyphi C H antigen suspension, c, phase 1
- *Salmonella* antibody starts appearing in serum at the end of first week and rise sharply during the 3rd week of endemic fever. In acute typhoid fever, O agglutinins can usually be detected 6–8 days after the onset of fever and H agglutinins after 10–12 days.

- It is preferable to test two specimens of sera at an interval of 7 to 10 days to demonstrate a rising antibody titre.
- *Salmonella* antigen suspensions can be used as slide and tube techniques.

Principle:

Bacterial suspension which carry antigen will agglutinate on exposure to antibodies to *Salmonella* organisms. Patients' suffering from enteric fever would possess antibodies in their sera which can react and agglutinate serial doubling dilutions of killed, coloured *Salmonella* antigens in a agglutination test.

The main principle of widal test is that if homologous antibody is present in patients serum, it will react with respective antigen in the reagent and gives visible clumping on the test card and agglutination in the tube. The antigens used in the test are "H" and "O" antigens of *Salmonella* Typhi and "H" antigen of *S.* Paratyphi. The paratyphoid "O" antigen are not employed as they cross react with typhoid "O" antigen due to the sharing of factor 12. "O" antigen is a somatic antigen and "H" antigen is flagellar antigen.

Materials required:

Fresh serum, stored at 2-8° Serum should not be heated or inactivated, Widal positive control, Widal test card or slide, Applicator stick and other routine lab glasswares.

The complete kit containing five vials containing stained Salmonella antigen

- *S.* Typhi————-O antigen
- *S.* Tyhhi————- H antigen
- *S.* Paratyphi ——AH antigen
- *S.* Paratyphi ——BH antigen

Procedure:

Preparation of Widal Antigens

- H suspension of bacteria is prepared by adding 0.1 per cent formalin to a 24 hours broth culture or saline suspension of an agar culture.
- For preparation of O suspensions of bacteria, the organisms were cultured on phenol agar (1:800) to inhibit flagella.
- Standard smooth strains of the organism are used; *S* Typhi 901, O and H strains are employed for this purpose.

- The growth is then emulsified in small volume of saline, mixed with 20 times its volume of alcohol, heated at 40° C to 50° C for 30 minutes and centrifuged.
- The antigens are treated with chloroform (preservative) and appropriate dyes are added for easy identification of antigens.

SLIDE TEST:

1. Place one drop of positive control on one reaction circles of the slide.
2. Pipette one drop of isotonic saline on the next reaction circle. (-ive Control).
3. Pipette one drop of the patient serum to be tested onto the remaining four reaction circles.
4. Add one drop of Widal TEST antigen suspension 'H' to the first two reaction circles. (PC & NC).
5. Add one drop each of 'O', 'H', 'AH' and 'BH' antigens to the remaining four reaction circles.
6. Mix contents of each circle uniformly over the entire circle with separate mixing sticks.
7. Rock the slide, gently back and forth and observe for agglutination macroscopically within one minute.

SEMI-QUANTITATIVE METHOD:

1. Pipette one drop of isotonic saline into the first reaction circle and then place 5, 10, 20, 40, 80 ul of the test sample on the remaining circles.
2. Add to each reaction circle, a drop of the antigen which showed agglutination with the test sample in the screening method.
3. Using separate mixing sticks, mix the contents of each circle uniformly over the reaction circles.
4. Rock the slide gently back and forth, observe for agglutination macroscopically within one minute.

Interpretation of Widal slide Test: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