MEDICAL LABORATORY TECHNOLOGY

SUBJECT CODE – U3MB24ST

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UNIT – 3

UNIT III: COAGULATION MECHANISM & COUNTING OF BLOOD CELLS (08)

Haemoglobin: Composition, estimation. Coagulation mechanism- Coagulation factors, bleeding time and clotting time. PCV & ESR.

1. ESTIMATION OF HEMOGLOBIN BY SAHLI's METHOD (HEMATIN METHOD)

INTRODUCTION TO HEMOGLOBIN

Hemoglobin (Hb or Hgb) is a red color pigment present in red blood cells (RBCs) comprises Fe^{2+} and Globin protein. It is Hemoglobin in RBCs that carries the oxygen from the lungs to the tissues and CO_2 from body tissues to the lungs for excretion.

Hemoglobin (Hb or Hgb) is responsible for the appearance of Red color RBCs and blood. Hemoglobin is a chromoprotein consisting of Globin molecule attached to 4 red colored Heme molecules. Hemoglobin synthesis requires the coordinated production of Heme and Globin. Heme is a prosthetic group that medicates reversible binding of oxygen by hemoglobin. Globin is the protein that surrounds and protects the Heme molecule.

The Estimation of hemoglobin in the blood is commonly prescribed in various physiological and pathological conditions and as both diagnostic and prognostic test especially in case of suspected Anemia which can be caused by various factors.

Nowadays in many laboratories, the Hemoglobin estimation is done by using Automatic Hematology Analyzers but still in many other labs the following method is Commonly used to determine the Hemoglobin concentration in patient's blood.

- Sahli's Method a.k.a. Acid Hematin Method
- Cyanmethemoglobin Method (CMG) a.k.a Drabkin's Method

ESTIMATION OF HEMOGLOBIN BY SAHLI's METHOD

PRINCIPLE OF SAHLI's METHOD / ACID HEMATIN METHOD

The principle of Sahli's Method or Acid hematin method is quite easy that when the blood is added to N/10 Hydrochloric acid (HCl), the hemoglobin present in RBCs is converted to acid hematin which is a dark brown colored compound. The color of the formed acid hematin complex corresponds to the Hemoglobin concentration in the blood and is matched with the standard which is a reference brown glass given in the Sahli's apparatus by diluting with N/10 hydrochloric acid or distilled water until the color of acid hematin complex match with the color of the standard.

REAGENTS REQUIRED FOR SAHLI's METHOD / ACID HEMATIN METHOD

- N/10 hydrochloric acid (It is prepared by diluting concentrated hydrochloric acid 0.98 ml in distilled water and volume is made up 100 ml).
- Distilled water

APPARATUS & EQUIPMENTS REQUIRED FOR SAHLI's METHOD / ACID HEMATIN METHOD

- Sahli's Apparatus
    - Hemoglobin pipette (0.02 ml or 20 µl capacity)
    - Sahli's graduated Hemoglobin tube
    - Thin glass rod Stirrer for Hemoglobin Tube
    - Sahli's Comparator box with brown glass standard

- Spirit swab
- Blood Lancet
- Dry cotton swab
- Pasteur pipette

PROCEDURE OF SAHLI's METHOD / ACID HEMATIN METHOD

⇒ N/10 Hydrochloric acid is taken in Hemoglobin tube (has two graduations – one side gm/dl, and other side shows the Hb %age), up to the mark 20 – the lowest marking (yellow marking).

⇒ Venous or Capillary blood is drawn up to 20µl mark of hemoglobin pipette exactly.

⇒ For capillary blood draw, boldly prick the tip of the middle or ring finger with the help of Blood lancet or pricking needle. Wipe out the first drop of blood and suck the blood from the second drop in Hb pipette up to the mark of 20 µl. Fill the Hb pipette by capillary action.

⇒ Wipe out the surface of the pipette with the help of tissue paper/ cotton so that excess blood may not be added to the Hb tube.

⇒ Dispense the blood into N/10 hydrochloric acid taken in the hemoglobin tube, rinse the pipette with the same solution and mix properly with the help of stirrer.

⇒ Place the tube at room temperature for 10 minutes for complete conversion of hemoglobin into acid hematin.

⇒ After the reaction completes, place the Hb tube in the column in Sahli's Comparator box and start diluting the dark brown coloured compound (Acid Hematin) formed in the Hb tube using the N/10 HCl or distilled water by adding drop by drop of it into the solution and mix with the help of stirrer after each addition.

⇒ This process is done until the endpoint comes matching the color of standard with the color of the test.

⇒ Once the color is matched with the standard brown glass, lift the stirrer up and note down the reading in Sahli's Hb tube by taking the lower meniscus in consideration.

Note: Usually in colored solution, the upper meniscus is considered for taking the reading but in this case, it is a transparent color solution and lower meniscus can be recorded in order to give the exact reading.

⇒ Now add one more drop of distilled water and mix it properly with the help of stirrer. If color is still matching with the standard add another drop till it matches with the standard and note down the reading and, if it gets lighter after adding the first extra drop, it shows reading taken before dilution was correct. Note down that reading as the final result.

⇒ Reading of this method is expressed in Hemoglobin gm/dl (gram/100 ml) of blood.

PRECAUTIONS TO BE TAKEN WHILE PERFORMING ESTIMATION OF HEMOGLOBIN BY SAHLI's METHOD / ACID HEMATIN METHOD:

⇒ Sahli's apparatus especially the Hemoglobin pipette and Sahli's Hemoglobin tube should be clean and dry before use.

⇒ Suck the blood exactly up to the mark of 20 µl (0.02 ml) and air bubbles should not be present in the pipette with blood.

⇒ Mix well the acid and blood and wait for at least 10 minutes after adding the blood in acid.

⇒ Add distilled water drop by drop and mix well after each dilution. Avoid over dilution of the content.

⇒ The matching of color should be done against the natural source of light or electrical tube light (white light) to avoid any visual errors.

⇒ Blood sample and N/10 HCl acid should be taken in an accurate and precise amount in the Hb tube.

⇒ The Hb pipette should be wiped off properly in order to avoid the excess addition of blood in the Acid.

ADVANTAGES OF SAHLI's METHOD / ACID HEMATIN METHOD

⇒ It is the simple and easy method and may be done at any place because apparatus can be picked up anywhere.

DISADVANTAGES OF SAHLI's METHOD / ACID HEMATIN METHOD

⇒ Visual intensity may be different for different individuals by this method, we are not able to measure the inactive hemoglobin.

⇒ This method estimates only oxy Hemoglobin. Carboxyhemoglobin and methemoglobin cannot be estimated.

⇒ The endpoint disappears soon so it is difficult to know the actual endpoint and also the Proper stable standard is not available

⇒The resulting solution is not a clear solution but a suspension due to the action of hydrochloric acid on the proteins and lipids.

NORMAL VALUES OF HEMOGLOBIN

- Adult Male: 14-16 gm/dl
- Adult Female: 13-15 gm/dl
- Newborn: 16-18 gm/dl

CLINICAL SIGNIFICANCE OF HEMOGLOBIN ESTIMATION

Hemoglobin estimation gives a brief idea of the pathological conditions to the physician so that your physician can easily understand the cause of pathology and prescribe an effective treatment for it.

Raised Hemoglobin Content

- Polycythemia Vera
- Associated with Hypoxia
- Cyanotic Congenital Heart disease
- High Altitudes
- Heavy smoking
- Methemoglobinemia
- Elevated erythropoietin levels
    - Tumors of Kidney, Liver, CNS, Ovary etc.

    - Renal Diseases (Hydronephrosis & Vascular impairment)
- Adrenal hypercorticism
- Therapeutic androgens
- Relative causes of high hemoglobin content
    - Dehydration – Water deprivation, Vomiting, Diarrhea
    - Plasma loss – Burns, Enteropathy

Reduced Hemoglobin Content

Low Hemoglobin value means anemia caused by the following conditions

- Leukemia
- Tuberculosis
- Iron deficiency anemia
- Parasitic infections severely in hookworm infection
- Sickle cell anemia
- Thalassemia
- Aplastic anemia
- Hemolytic anemia
- Loss of blood

FACTS ABOUT HEMOGLOBIN

- Each gram of hemoglobin carries 1.34 ml oxygen
- Each gram of Hb contains 3.33 mg of iron
- Total Hb content in a healthy body is approximately 600 gms.

2.Erythrocyte Sedimentation Rate (ESR) :

The **erythrocyte sedimentation rate (ESR)** is a common hematological test for nonspecific detection of inflammation that may be caused by infection, some cancers and certain autoimmune diseases. It can be defined as the rate at which Red Blood Cells (RBCs) **sediment in a period of one hour.**

Principle of ESR

When anticoagulated blood is allowed to stand in a narrow vertical glass tube, undisturbed for a period of time, the RBCs – under the influence of gravity- settle out from the plasma. The rate at which they settle is measured as the number of millimeters of clear plasma present at the top of the column after one hour(mm/hr). This mechanism involves three stages:

- **Stage of aggregation** : It is the initial stage in which piling up of RBCstakes place. The phenomenon is known as Rouleaux formation. It occurs in the first 10-15 minutes.
- **Stage of sedimentation** : It is the stage of actual falling of RBCs in which sedimentation occurs at constant rate. This occurs in 30-40 minutes out of 1 hour, depending upon the length of the tube used.

- **Stage of packing** : This is the final stage and is also known as stationary phase. In this, there is a slower rate of falling during which packing of sedimented RBCs in column occurs due to overcrowding. It occurs in final 10 minutes in 1 hour.

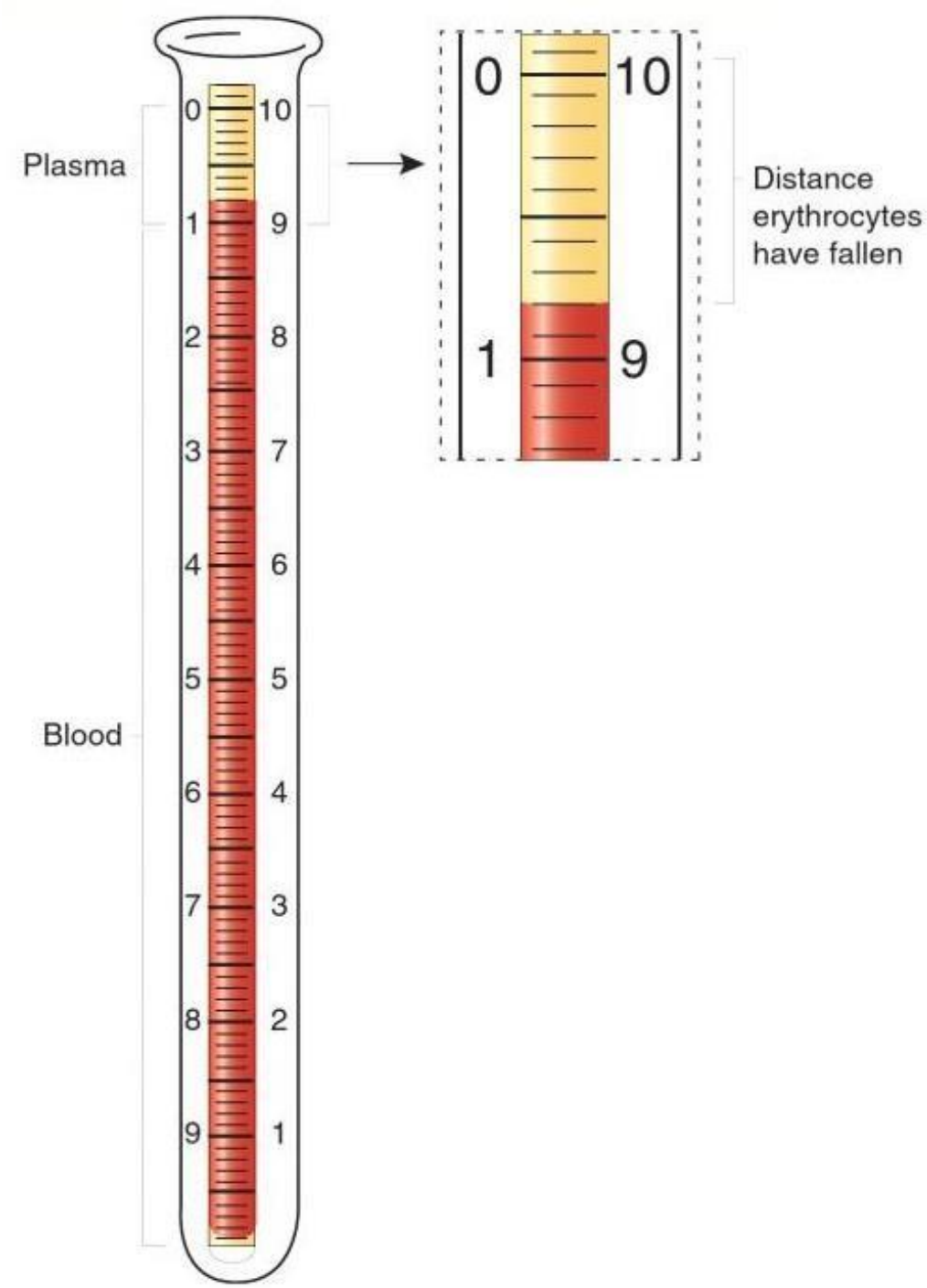
Methods of ESR determination

There are two main methods to determine ESR :

- Wintrobe's method
- Westergren's method

Each method produces slightly different results. Mosely and Bull (1991) concluded that Wintrobe's method is more sensitive when the ESR is low, whereas, when the ESR is high, the Westergren's method is preferably an indication of patient's clinical state.

Wintrobe's method



This method uses Wintrobe's tube, a narrow glass tube closed at the lower end only. The Wintrobe's tube has a **length of 11 cm** and **internal diameter of 2.5 mm**.

It contains 0.7-1 ml of blood. The lower 10 cm are in cm and mm. The marking is 0 at the top and 10 at the bottom for ESR.

This tube can also be used for PCV. The marking is 10 at the top and 0 at the bottom for PCV.

Requirements :

- Anticoagulated blood (EDTA, double oxalate)
- Pasteur pipette
- Timer
- Wintrobe's tube
- Wintrobe's stand

Procedure:

1. Mix the anticoagulated blood thoroughly.
2. By using Pasteur pipette, fill the Wintrobe's tube upto '0' mark. There should be no bubbles in the blood.
3. Place the tube vertically in ESR stand and leave undisturbed for 1 hour.
4. At the end of 1 hour, read the result.

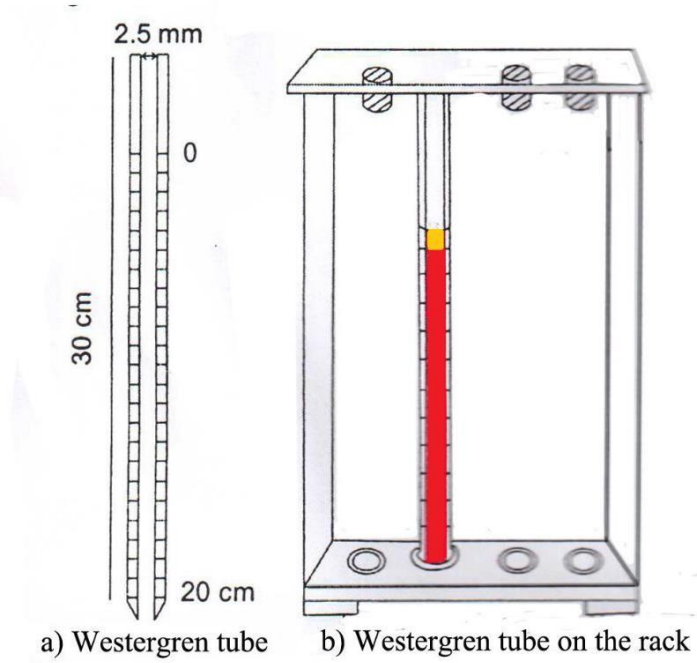
Normal values :

For Male :0-9 mm/hr

Female : 0-20 mm/hr

Westergren's method

It is better method than Wintrobe's method. The reading obtain is magnified as the column is lengtheir. The Westregren tube is open at both ends. It is **30 cm in length** and **2.5 mm in diameter**. The lower 20 cm are marked with 0 at the top and 200 at the buttom. It contains about 2 ml of blood.



a) Westergren tube b) Westergren tube on the rack

Requirements :

- Anticoagulated blood (0.4 ml of 3.13% trisodium citrate solution + 1.6 ml blood)
- Westergren tube
- Westergren stand
- Rubber bulb (sucker)

Procedure :

1. Mix the anticoagulated blood thoroughly.
2. Draw the blood into the tube upto 0 mark with the help of rubber bulb.
3. Wipe out blood from bottom of the tube with cotton.
4. Set the tube upright in stand. Make sure the pipette fits snugly to eliminate possible leakage and that the pipette is in vertical position.
5. Leave the tube undisturbed for 1 hour.
6. At the end of 1 hour, read the result.

Normal values Male :0-

10 mm/hr Female : 0-15

mm/hr

Clinical Significance of ESR

The erythrocyte sedimentation rate (ESR) is a non-specific test. It is raised in a wide range of infectious, inflammatory, degenerative, and malignant conditions associated with changes in plasma proteins, particularly increases in fibrinogen, immunoglobulins, and C-reactive protein.
The ESR is also affected by many other factors including anaemia, pregnancy, haemoglobinopathies, haemoconcentration and treatment with anti-inflammatory drugs.

Causes of a significantly raised ESR :

- All types of anemias except sickle cell anemia
- African trypanosomiasis (rises rapidly)
- Visceral leishmaniasis

Acute and chronic inflammatory conditions and infections including:

- HIV disease
- Tuberculosis
- Acute viral hepatitis
- Arthritis
- Bacterial endocarditis
- Pelvic inflammatory disease
- Ruptured ectopic pregnancy
- Systemic lupus erythematosus

- Myelomatosis, lymphoma, Hodgkins disease, some tumours
- Drugs, including oral contraceptives

Causes of Reduced ESR :

- Polycythaemia
- Poikilocytosis
- Newborn infants
- Dehydration
- Dengue haemorrhagic fever
- Other conditions associated with haemoconcentration

3.MICRO METHOD FOR ESTIMATION OF PACKED CELL VOLUME (PCV) OR HEMATOCRIT

Principle

Anticoagulated whole blood is centrifuged in a capillary tube of uniform bore to pack the red cells. Centrifugation is done in a special microhematocrit centrifuge till packing of red cells is as complete as possible. The reading (length of packed red cells and total length of the column) is taken using a microhematocrit reader, a ruler, or arithmetic graph paper.

Equipment

1. Microhematocrit centrifuge: It should provide relative centrifugal force of 12000 g for 5 minutes.
2. Capillary hematocrit tubes: These are disposable glass tubes 75 mm in length and 1 mm in internal diameter. They are of two types: plain (containing no anticoagulant) and heparinised (coated with a dried film of 2 units of heparin). For plain tubes, anticoagulated venous blood is needed. Heparinised tubes are used for blood obtained from skin puncture.
3. Tube sealant like plastic sealant or modeling clay; if not available, a spirit lamp for heat sealing.
4. Microhematocrit reader; if not available, a ruler or arithmetic graph paper.

Specimen

Venous blood collected in EDTA (dipotassium salt) for plain tubes or blood from skin puncture collected directly in heparinised tubes. Venous blood should be collected with minimal stasis to avoid hemoconcentration and false rise in PCV.

Method

1. Fill the capillary tube by applying its tip to the blood (either from skin puncture or anticoagulated venous blood, depending on the type of tube used). About 2/3rds to 3/4ths length of the capillary tube should be filled with blood.
2. Seal the other end of the capillary tube (which was not in contact with blood) with a plastic sealant. If it is not available, heatseal the tube using a spirit lamp.
3. The filled tubes are placed in the radial grooves of the centrifuge with the sealed ends toward the outer rim gasket. Counterbalance by placing the tubes in the grooves opposite to each other.

4. Centrifuge at relative centrifu-gal force 12000 g for 5 minutes to completely pack the red cells.
5. Immediately remove the tubes from the centrifuge and stand them upright. The tube will show three layers from top to bottom: column of plasma, thin layer of buffy coat, and column of red cells.
6. With the microhematocrit reader, hematocrit is directly read from the scale. If hematocrit reader is not available, the tube is held against a ruler and the hematocrit is obtained by the following formula:

$$\frac{\textbf{Length of red cell column in mm}}{\textbf{Length of total column in mm}}$$

To obtain PCV, the above result is multiplied by 100.

GENERAL NOTES

1. Prolonged application of tourniquet during venes puncture causes hemoconcentration and rise in hematocrit.
2. Excess squeezing of the finger during skin puncture dilutes the sample with tissue fluid and lowers the hematocrit.
3. Correct proportion of blood with anticoagulant should be used. Excess EDTA causes shrinkage of red cells and falsely lowers the hematocrit.
4. Inadequate mixing of blood with anticoagulant, and inadequate mixing of blood before testing can cause false results.
5. Low hematocrit can result if there are clots in the sample.
6. Centrifugation at lower speed and for less time falsely increases PCV.
7. A small amount of plasma is trapped in the lower part of the red cell column which is usually insignificant. Increased amount of plasma is trapped in microcytosis, macrocytosis, spherocytosis, and sickle cell anemia, which cause an artifactual rise in hematocrit. Larger volume of plasma is trapped in Wintrobe tube than in capillary tube.
8. As PCV requires whole blood sample, it is affected by plasma volume (e.g. PCV is higher in dehydration, and lower in fluid overload).
9. Expression of PCV: Occasionally, PCV is expressed as a percentage. In SI units, PCV is expressed as a volume fraction. Conversion factor from conventional to SI units is 0.1 and from SI to conventional units is 100.
10. Rules of 3 and 9: These rules of thumb are commonly used to check the accuracy of results and are applicable only if red cells are of normal size and shape.
    - ***Hemoglobin (gm/dl) × 3 = PCV***
    - ***Red cell count (million/cmm) × 9 = PCV***
11. Automated hematocrit: In automated hematology analyzers, hematocrit is obtained by multiplying red cell count (in millions/cmm) by mean cell volume (in femtoliters).

REFERENCE RANGES

- Adult males: 40-50%
- Adult females (nonpregnant): 38 45%
- Adult females (pregnant): 36-42%

- Children 6 to 12 years: 37-46%
- Children 6 months to 6 years: 36 42%
- Infants 2 to 6 months: 32-42%
- Newborns: 44-60%

CRITICAL VALUES

- Packed cell volume: < 20% or > 60%

4.BLEEDING TIME (IVY Method) Principle

A blood pressure cuff is placed on the patient's arm above the elbow and inflated to 40 mmHg to create venostasis. A standardized incision is made on the volar surface of the forearm utilizing a disposable bleeding time device. A stopwatch is started and at 30-second intervals the blood is blotted away using filter paper. The bleeding time is the length of time required for bleeding to cease.[1,2]

Quality Control

Quality control is accomplished through the standardization of the procedure. The wound should be a standard length and depth and a constant pressure of 40 mmHg should be maintained throughout the procedure.

Reagents and Equipment

1. Blood pressure cuff
2. Sterile disposable bleeding time device
3. Stopwatch
4. Whatman No. 1 filter paper
5. Alcohol swabs
6. Butterfly bandage

Procedure

1. Select the site for the puncture. This site should be located on the volar surface of the forearm approximately 5 cm below the antecubital crease.
2. Prepare the site by cleansing with an alcohol swab. Allow alcohol to dry completely.
3. Place a blood pressure cuff around the upper arm and inflate to 40 mm Hg.
4. With a disposable bleeding time device, make a standard incision and start a stopwatch.
5. At 30-second intervals, blot the blood from the puncture site using filter paper. Care must be taken to avoid touching the wound at any time.
6. When blood is no longer drawn to the filter paper, stop the timer. Record the length of time required for bleeding to stop.
7. Release the blood pressure cuff and apply a butterfly bandage over the puncture site.

Reference Interval

Each laboratory should establish its own reference interval. The general reference interval is 1-9 minutes.

Comments

1. A platelet count should be performed prior to the bleeding time. A platelet count less than 100.0 x 10^9/L will result in a prolonged bleeding time.
2. The direction of the incision should be consistent. A horizontal incision gives a longer bleeding time than a vertical incision.
3. The sensitivity of the procedure is determined by venostasis and the direction of the incision. The most sensitive technique is a horizontal incision with venostasis.
4. If the bleeding has not ceased at 15 minutes, stop the timer and apply pressure to the incision site. The bleeding time should be reported as greater than 15 minutes.
5. Disposable bleeding time devices are also available for newborns. These devices make a standard incision that is shorter and not as deep as the adult devices. In performing a bleeding time on a newborn, the pressure applied by the blood pressure cuff must be adjusted to the weight of the newborn.
6. Since the ingestion of aspirin and many aspirin-like drugs results in a prolonged bleeding time, a true bleeding time is obtained by requiring the patient to remain aspirin-free for seven days.
7. The bleeding time is prolonged in thrombocytopenia, hereditary and acquired platelet dysfunctions, von Willebrand's disease, afibrinogenemia, severe hypofibrinogenemia, and some vascular bleeding disorders.

5..Clotting Time (C T)

Indications

1. Clotting Time is advised to find bleeding disorder, most likely due to clotting factors deficiency.
2. To Diagnose hemophilia.

Sample

- **Clotting Time** is done on a fresh blood sample, and the patient needed to be in the lab.

Precaution

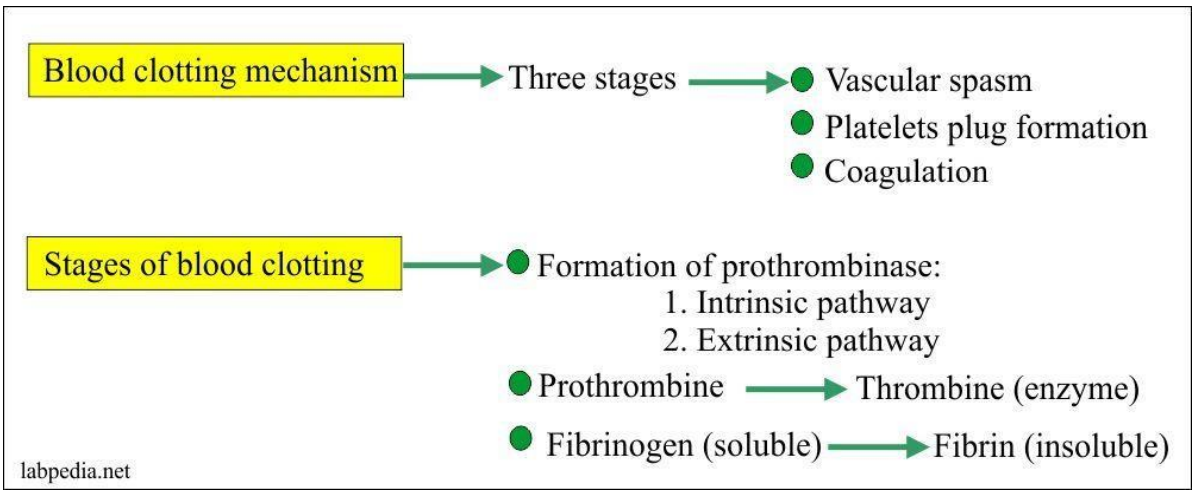
1. It does not diagnose mild coagulation disorders.
2. Blood should be taken in the least traumatic manner.
3. The premature activation of the clotting process must be avoided to ensure an accurate result.
4. Avoid hemolysis of the sample.
5. It is important to get the history of the patient:
    1. Note physical appearance, site, the severity of the disease, and frequency of the bleeding episodes.

2. Get an accurate history of the drugs.
3. A patient and the family history in detail needed.
4. Also, consider other contributing or underlying diseases.

Pathophysiology

1. For the clot formation, prothrombin is converted into thrombin.
2. Thrombin converts soluble fibrinogen into insoluble fibrin.
3. For this process, clotting factors are needed, along with calcium.
4. Also assisted by the factors produced by platelets and damaged tissue.

5. So clotting time is the time needed for the generation of thrombin from the complex system of clotting.



UNIT IV: STAINING METHODS

Haemotology Staining

There are various types of Blood cells present in our body, having different shapes and sizes, which take up the stains as per their structures.

Some of the Components of the blood cells are 1.Basophilic i.e. they have a

great affinity for acidic dyes

2. Acidophilic i.e. they have a high affinity for Basic Dyes

3. Neutral and has the high affinity for neutral stains.

Therefore, the stains used in Hematology laboratory are the combination of these three types of stains.

Besides the dyes, a buffer is added to the stain which acts as the mordant and enhances the staining reaction, results in the better morphology of the blood cells under the microscope.

Romanowsky stains are such types of stains that are universally employed for the staining of blood cells.

Almost all the Romanowsky group of stains has two essential components i.e. Methylene blue & Eosin or Azure dye.

Methylene blue is a basic dye that has a high affinity for the acidic components of the cell i.e. Nucleus and Eosin/ Azure is the Acidic dye which has the high affinity for the basic components of the cells i.e. the cytoplasm and Granules in some cells.

Most of the Romanowsky stains are prepared with Methyl alcohol (Methanol) so that they act as

a fixative as well as the cellular stain. There are 4 different types of Romanowsky stains commonly used in Hematology laboratory for staining the blood cells –

- Wright stain
- Leishman Stain
- Giemsa Stain
- Field's Stain
- Wright's Stain
- Jaswanth Singh – Bhattacharji stain
- Peroxidase stain.

1. Wright's Stain

- Commercially prepared Wright's stains are available and make the staining procedure relatively simple.
- **Staining Solution**
- 1. Wright's stain: Dissolve 0.1 g Wright's stain into 60 mL absolute methanol. Allow the solution to stand in a tightly sealed brown bottle for 1–2 weeks. Filter the solution before using.
- 2. Wright's buffer: Dissolve 3.80 g Na_2HPO_4 (dibasic sodium phosphate) and 5.47 g KH_2PO_4 (monobasic potassium phosphate) into 500 mL distilled water. Bring the total volume to 1000 mL with distilled water.
- **Staining Procedure**
- 1. Flood an air-dried film with Wright's stain and allow to stand for 1–3 minutes.
- 2. Add an equal amount of Wright's buffer and mix by gently blowing on the slide until a metallic green sheen forms on the surface. Allow it to stand for 2–6 minutes (the exact time must be determined for each batch of stain).
- 3. Gently rinse the stain from the slide using tap water or distilled water and a wash bottle or beaker.
- 4. Prop up the slide and allow it to air-dry.
- **Staining Results**
- A satisfactorily stained blood film will reveal erythrocytes with a yellowish red cytoplasm. The erythrocytes of lower vertebrates and leukocytes will have dark violet nuclei. Heterophils of lower vertebrates will exhibit red-orange, rod-shaped cytoplasmic granules. Eosinophils will exhibit a pale blue cytoplasm and redorange round granules in many species. Basophils will exhibit dark purple cytoplasmic granules. The cytoplasm of thrombocytes of lower vertebrates and the platelets of mammals will stain colorless to light blue with red granules
- **Wright–Giemsa Stain**
- Commercially prepared Wright–Giemsa stains are available and make the staining procedure relatively simple.
- **Staining Solution**
- 1. Dissolve 300 mg powdered Wright's stain and 30 g powdered Giemsa stain into 100 mL absolute methanol. Allow the solution to stand for 1–2 days in a tightly sealed brown bottle.
- 2. Filter the solution and use as indicated for theWright's staining procedure.
- **Staining Results**
- The results are similar to Wright's stain alone except the cell nuclei become reddish purple instead of violet.

2. Leishman stain

Leishman stain is a mixture of Methylene blue, and Eosin dye, prepared in Alcohol medium and diluted with buffer or distilled water during staining procedure. Leishman stain is a differential stain that is used to variably stain the various components of the cells and it can be used to study the adherence of pathogenic bacteria to the human cells. It differentially stains the human and bacterial cells and appeared as purple and pink colored bodies respectively. The Leishman stain is one

of the best stains for routine blood stain to stain the Peripheral blood smear for the examinations of blood film under the microscope and is satisfactory for malaria and other blood parasites. Giemsa stain gives better results in parasitic studies.

Principle of Leishman staining

Leishman Stain is a neutral stain for blood smears which was devised by the British surgeon W. B. Leishman (1865–1926). It consists of a mixture of eosin (an acidic stain), and Methylene blue (a basic stain) in Methyl alcohol and is usually diluted and buffered during the staining procedure. It stains the different components of blood in a range of shades between red and blue.

It is based on a methanolic mixture of "polychromed" Methylene blue and eosin. The methanolic stock solution is stable and also serves the purpose of directly fixing the smear eliminating a prefixing step.

Leishman stain is commonly used when there is need to examine the Blood smear for the Various blood cells, Differential Leucocyte count, Type of Anemia, Toxic Granules & Platelet count etc. and also used to differentiate nuclear and cytoplasmic morphology of the various cells of the blood like Platelets, RBCs, WBCs as well as for the parasites. This stain is the most dependable stain for Peripheral blood film examination.

The working principle of the Leishman stain is same as described above. As it is a type of Romanowsky stains, it contains both the Acidic and Basic dyes which have the affinity for Basic and Acidic components of the Blood cells respectively.

The acidic dye, Eosin, variably stains the Basic components of thecells
i.e. the cytoplasm, Granules etc.

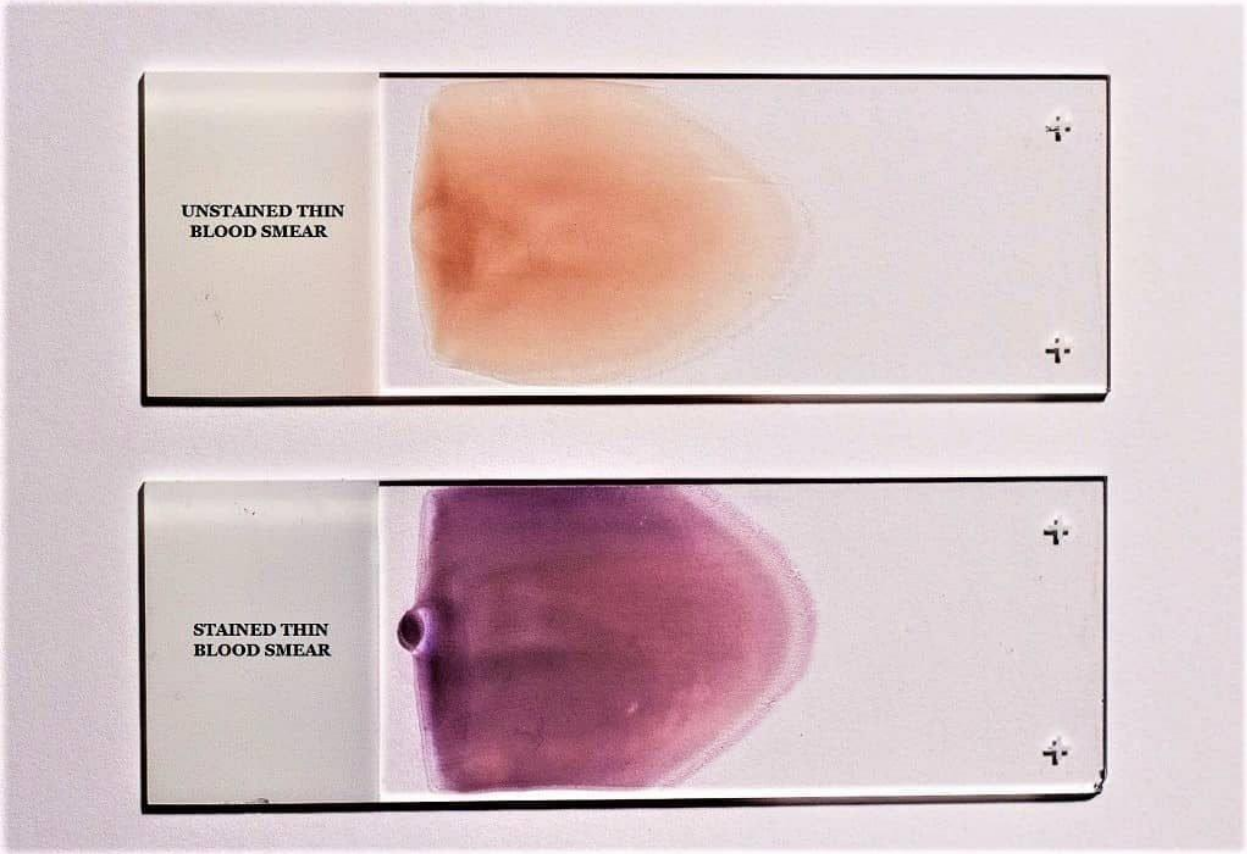
And the Basic dye, Methylene blue stains the Acidic components, especially the Nucleus of the cell. The stain must be diluted for use with Phosphate buffer to pH 6.8 or 7.2, depending on the specific technique used. The pH 6.8 is preferred when the morphology of blood cells is to be examined and pH 7.2 is good for parasitic studies.

MATERIALS REQUIRED FOR THE LEISHMAN STAIN

- Leishman Stain (Stock Solution)
- Microscopic Glass Slide
- Phosphate buffer (pH 6.8)
- Graduated pipettes
- Measuring cylinder
- Distilled Water
- Pasteur pipette
- Coplin Jar
- Blood Specimen – The specimen used usually consists of fresh whole blood collected by finger puncture (capillary puncture) or the EDTA anticoagulated whole blood, collected by venipuncture and it should be less than 1-hour old for better results.

PROCEDURE OF LEISHMAN STAINING

The Procedure of Leishman staining may vary as per the purpose of staining that means whether the staining is done for the examination of Blood cells Morphology, Toxic Granules in Leucocytes, Type of Anemia etc. and also, the protocol may vary as per the Standard Operating Procedures (SOPs) of the Laboratory.



STAINED & UNSTAINED THIN BLOOD SMEAR

The thick smears are commonly stained either with Giemsa stain or Field's Stain which gives better results and contrast for the Parasitic studies and especially useful in identification of malaria parasite in the blood.

The Leishman staining can be done in 2 ways either by immersing the Blood smear slides in the reagent filled Coplin jars or by covering the smear with Leishman stain which is placed horizontally on Staining Rack.

Covering method

Prepare a thin blood smear on a clean and dry microscopic glass slide and air dry it.

The preparation of thin blood Smear

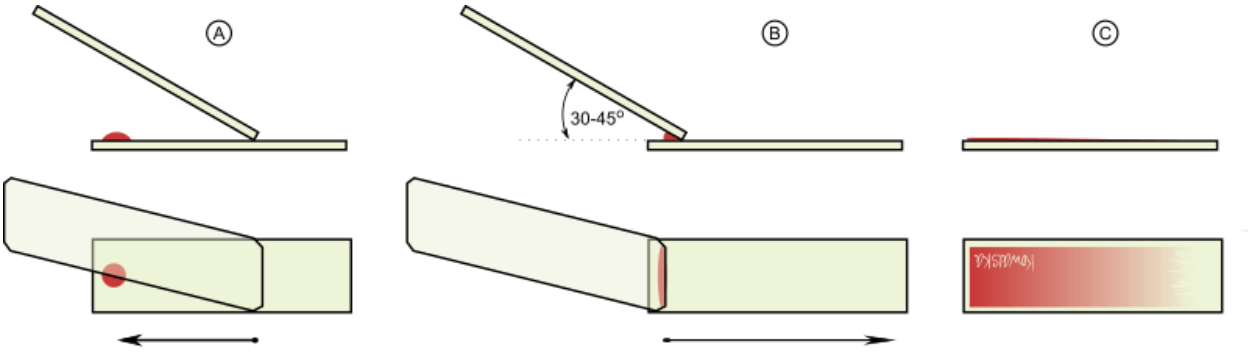
the blood sample is obtained by venipuncture, use a capillary tube to transfer a drop of blood from the tube onto the clean grease-free microscopic glass slide. If a finger puncture or heel puncture is made, discard the first drop of blood and then dispense a drop of blood from the puncture site onto the clean microscopic glass slide. Place the drop of blood in the center on one side of the glass slide leaving about 1 cm margins.

⇒ Place the specimen glass slide on a flat surface and hold it with the index finger and the thumb of the left hand (for Right-handed peoples). Now, Place a smooth, clean edge of the spreader slide on the specimen slide at an angle of about 30° – 45°.

Move the spreader slide toward the drop of blood until the contact is made with the drop of the blood at the specific angle. Then move the spreader slide smoothly and rapidly forward over the specimen slide, drawing the blood behind it into a thin film that should be tongue- shaped.

⇒ Allow the blood smear to air-dry completely. Do not blow air on the slide from any source in an effort to enhance drying, it may distort the smear.

⇒ Using a lead pencil or glass marking pencil, write the Name, Identification no. and the Date on the frosted end of the slide. Do not use a wax pencil or marker or any Pen as it dissolves and washed out during the staining process.



PREPARATION OF PERIPHERAL BLOOD SMEAR

⇒ Now, cover the well dried, thin blood smear with undiluted Leishman Stain solution by counting the drops of Leishman stain.

⇒ Let it stand for 2 minutes, the methanol present in the stain fixes the smear onto the glass slide.

⇒ After 2 minutes, add twice the amount of distilled water or Phosphate buffer solution and mix the content by swirling or by blowing gently. Incubate the slides for at least 10 min at 37 °C. This will stain the blood cells.

⇒ Rinse the slides thoroughly with Phosphate buffer solution up to 2 minutes or until it acquires a purple-pinkish tinge.

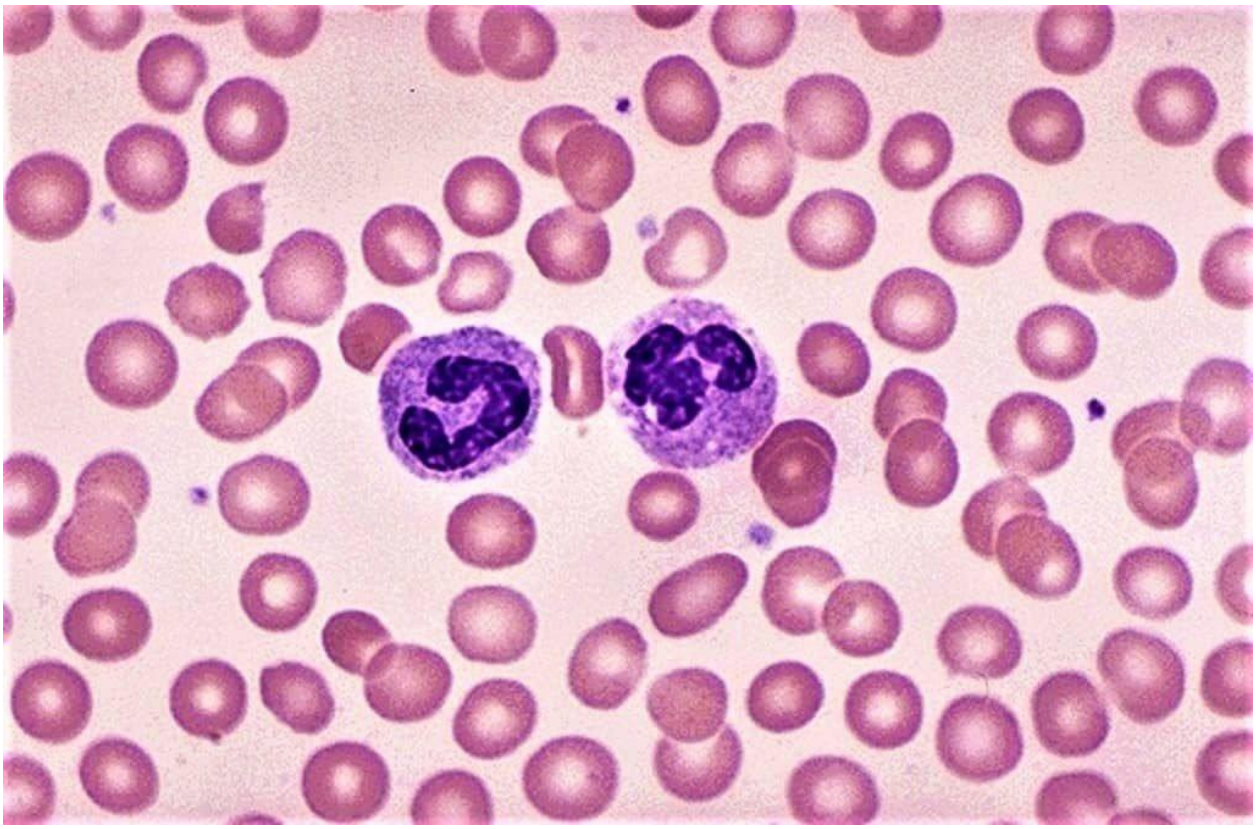
⇒ Air dry the slides in a tilted position so that the water easily remove out of the slides.

⇒ Now you can mount the smears with mounting media, e.g. Gurri's neutral mounting media or any other mounting medium which do not decolorizes the smear. Do not use Canada balsam as it may decolorize the smear.

⇒ Let it dry in air for few hours and then observe the slides under oil immersion objective lens of the microscope.

RESULTS UNDER THE MICROSCOPE

The various blood cells will be observed under the microscope as follows:



NORMAL BLOOD SMEAR STAINED WITH LEISHMAN STAIN UNDER THE MICROSCOPE

The color of Nuclei by Leishman Stain

Chromatin	Purple
Nucleoli	Light blue

The color of Cytoplasm by Leishman Stain

Erythrocytes	Pink
Reticulocytes	Dark blue
Lymphocytes	Blue
Monocytes	Grey blue

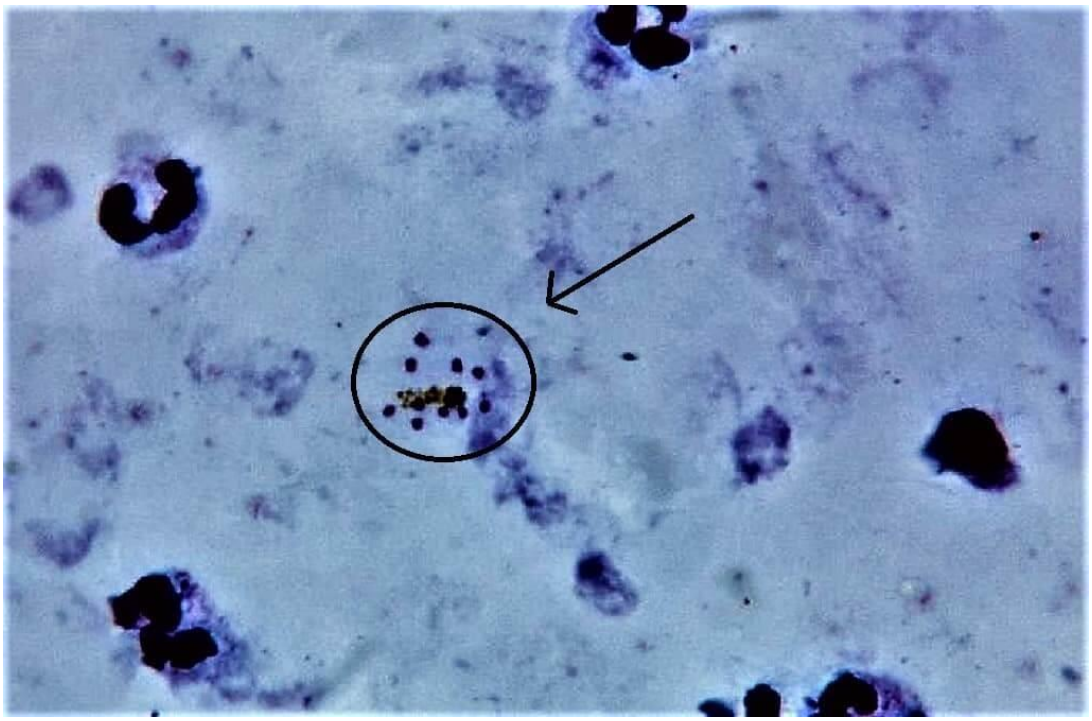
Neutrophils	Bluish Pink
Basophils	Blue

The color of Granules by Leishman Stain

Basophil	Purple black
Eosinophil	Red orange
Neutrophil	Purple
Platelet	Purple

Giemsa stain

Giemsa stain is a mixture of Azure, Methylene blue, and Eosin dye. Giemsa stain is a differential stain that is used to variably stain the various components of the cells and it can be used to study the adherence of pathogenic bacteria to the human cells. It differentially stains the human and bacterial cells and appeared as purple and pink colored bodies respectively.



The Giemsa stain is one of the best stains for malaria and other blood parasites and also satisfactory as a routine bloodstain to stain the Peripheral blood smear for the examinations of blood film under the microscope.

PRINCIPLE OF GIEMSA STAINING

Giemsa stain is commonly used when there is a need to examine the Blood smear for the Parasites but is a good stain for routine examination of blood smear and used to differentiate nuclear and cytoplasmic morphology of the various cells of the blood like Platelets, RBCs, WBCs as well as the parasites. This stain is the most dependable stain for blood parasites, particularly in thick blood smears.

The working principle of the Giemsa stains is the same as described above. As it is a type of Romanowsky stains, it contains both the Acidic and Basic dyes which have the affinity for Basic and Acidic components of the Blood cells respectively. The acidic dye, Eosin & Azure variably stains the Basic components of the cells i.e. the cytoplasm, Granules etc. and the Basic dye, Methylene blue stains the Acidic components, especially the Nucleus of the cell. The stain must be diluted for use with

water buffered to pH 6.8 or 7.2, depending on the specific technique used.

The Standard Operating Procedures (SOPs) are different for almost every laboratory as per the quality of the staining solution & buffer they are using and the purpose of staining. Do not consider this article as the only correct way of staining the Blood smear with Giemsa stain.

MATERIALS REQUIRED FOR THE GIEMSA STAIN

- Giemsa Stain (Stock Solution)
- Microscopic Glass Slide
- Phosphate buffer (pH 7.2)
- Graduated pipettes
- Measuring cylinder
- Distilled Water
- Pasteur pipette
- Coplin Jar
- Blood Specimen – The specimen used usually consists of fresh whole blood collected by finger puncture (capillary puncture) or the EDTA anticoagulated whole blood, collected by venipuncture and it should be less than 1-hour old for better results. The blood specimen can also be collected in Heparin or Sodium Citrate for the identification of parasites.

Nowadays, commercially prepared Giemsa stain solution is used in most of the laboratories which are then diluted in various ratios for different purposes. However, you can easily prepare it in the laboratory. Follow the link to learn more….

Similarly, the commercially prepared phosphate buffer solution is used in laboratories which can be adjusted as per the pH required using HCl or NaOH solution. However, you can easily prepare it in the laboratory.
Follow the link to learn more…

PROCEDURE OF GIEMSA STAINING

The Procedure of Giemsa staining varies as per the purpose of staining that means whether the staining is done for the examination of Blood cells or to find the Parasites in the blood smear and accordingly the Blood smears are prepared as Thin Blood films or Thick blood films. Here, I'm explaining the procedure of staining 3 different types of smears as the thin smear, Thick smear and the Combination of Thick and Thick smear on the same slide…. So let's start with staining the thin blood smear.

Staining the Thin blood smear with Giemsa stain

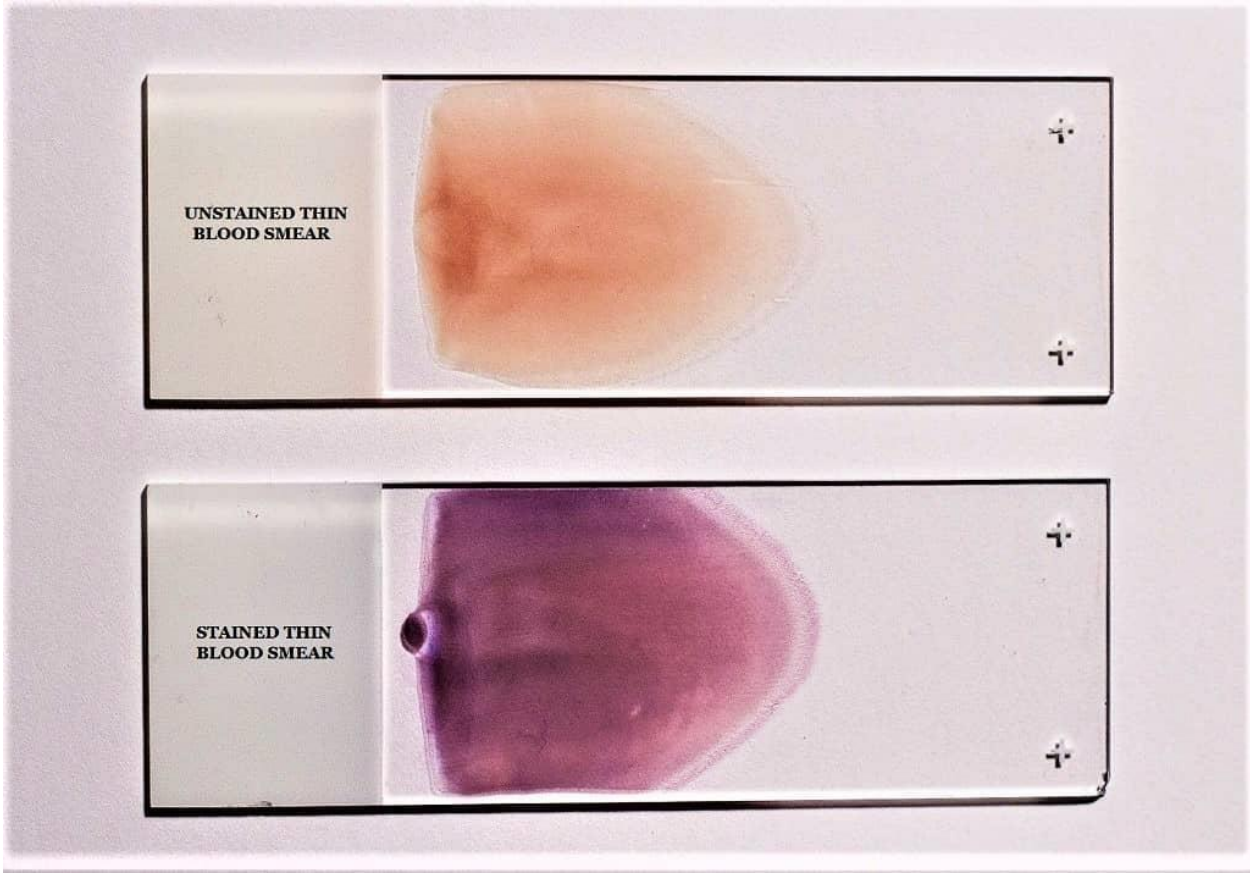
⇒ Prepare a thin blood smear on a clean and dry microscopic glass slide and air dry it.

If you don't know how to prepare it, check it out here, the preparation of thin blood Smear

⇒ Firstly fix out the air-dried thin blood smear in the absolute methanol by dipping the blood smear quickly (two dips) in a Coplin jar containing absolute methanol.

⇒ Take out the slide from the Coplin jar and let it air dry.

⇒ **Diluting the Giemsa Stain for Thin Blood smear:** For staining the thin blood smear the Giemsa stain is used in 1:20. To make 1:20 dilution of Giemsa stain add 2 ml of stock solution of Giemsa stain to 40 ml of phosphate buffer solution in a clean Coplin jar. You can also use the Distilled water instead of buffer but the results may vary.



STAINED & UNSTAINED THIN BLOOD SMEAR

⇒ Now, stain the Methanol fixed Blood smear with diluted Giemsa stain (1:20, v/v) for 20 min. For this, put the slide in the Coplin jar containing the diluted Giemsa stain or you can also put it on a staining rack or on any flat surface with the smear side up and pour the stain over the smear so that it equally covers the smear.

⇒ Now Wash out the stained slides by quickly dipping, once or twice, the slide in and out of a Coplin jar containing buffered water or Distilled water.

Do not overexpose the smear to buffered water (phosphate buffer solution). Excessive washing may decolorize blood smear.

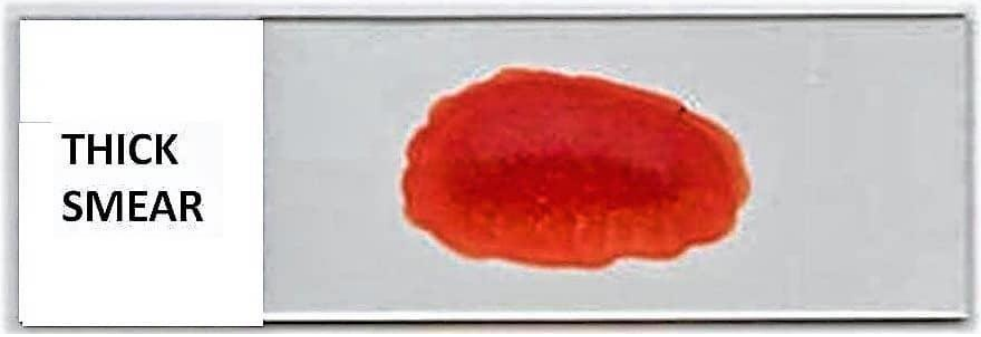
⇒ Let the smear dry well in the air.

Staining the Thick blood smear with Giemsa stain

⇒ Prepare a thick blood smear on a clean and dry microscopic glass slide and air dry it.

⇒ If you don't know how to prepare it, check out the preparation of Thick blood Smear

⇒Do not dry the thick blood smear in an incubator or by heat, because this will fix the blood smear onto the slide and interfere with the lysis of RBCs.



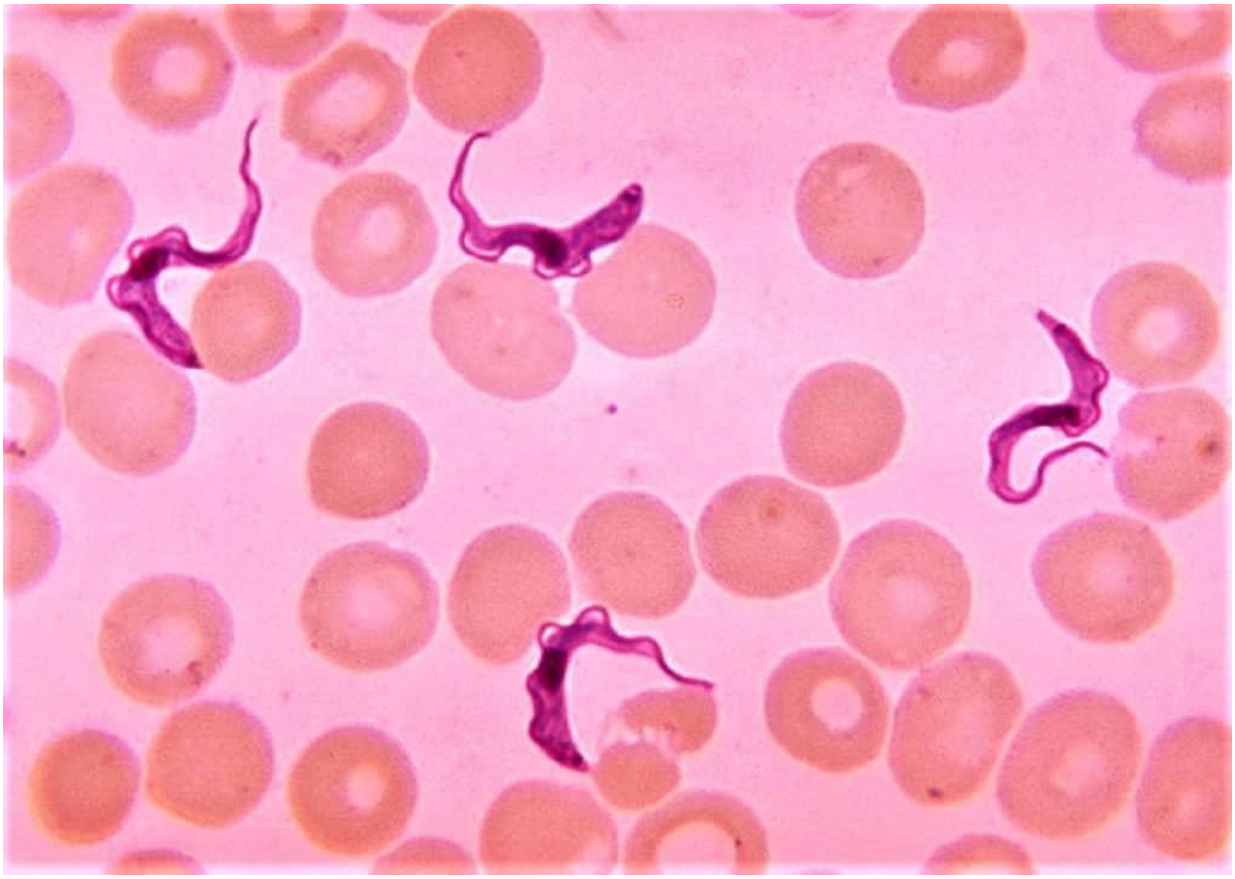
THICK BLOOD SMEAR / THICK BLOOD FILM

⇒ **Diluting the Giemsa Stain for Thick Blood smear:** For staining the thick blood smear the Giemsa stain is used in 1:20. To make a 1:50 dilution of Giemsa stain, add 1 ml of stock solution of Giemsa stain to 49 ml of phosphate buffer solution in a clean Coplin jar. You can also use the Distilled water instead of buffer but the results may vary.

⇒ Now, stain the Air-dried Blood smear with diluted Giemsa stain (1:50, v/v) for 50 min. For this, put the slide in the Coplin jar containing the diluted Giemsa stain or you can also stain it on a staining rack or on any flat surface with the smear side up and pour the stain over the smear so that it equally covers the smear.

⇒ Wash out the Blood smear by placing it in the buffered water or Distilled water for 3 to 5 min.

⇒ Let the smear dry well in the air.



THICK BLOOD SMEAR SHOWING TRYPANOSOMA SPECIES

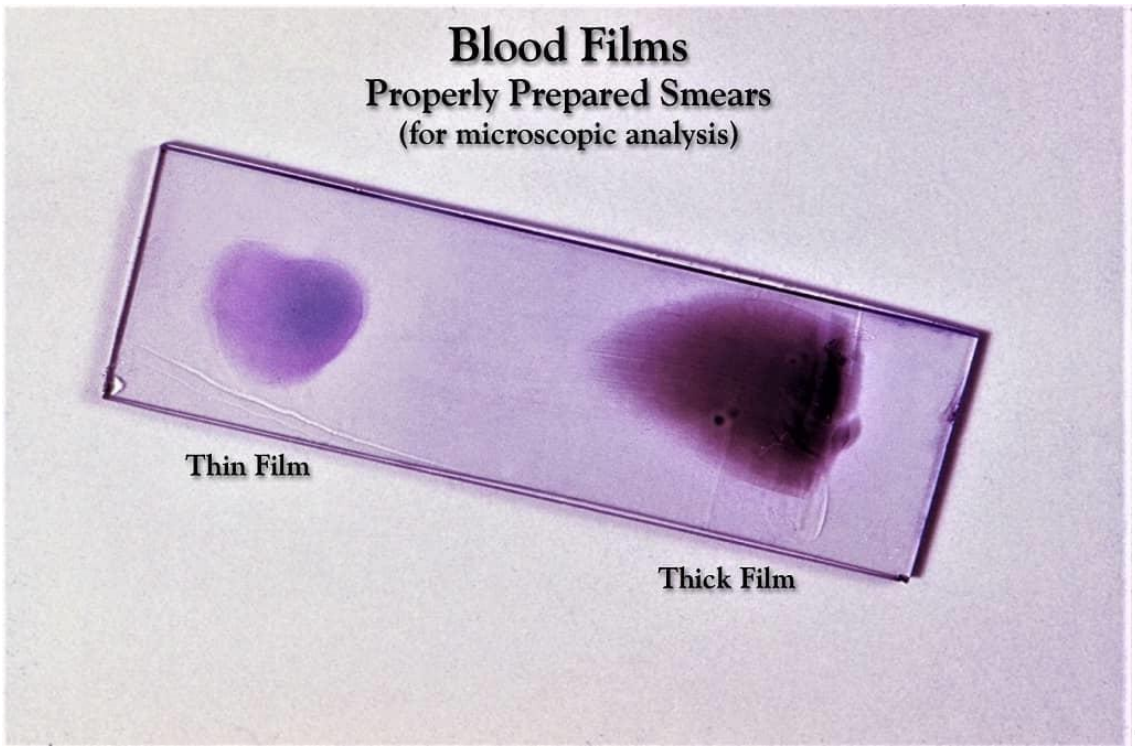
Staining the Thin and thick blood smear on the same slide with Giemsa stain

⇒ Prepare a thin and thick smear of the specimen on the same slide which can be done by dividing the slides into two parts and then making the thin smear on one side and thick on the other side. Allow the smear to air dry.

⇒ Now, carefully fix the air-dried thin blood smear in absolute methanol by dipping the thin smear side quickly (2-3 dips) in a Coplin jar containing absolute methanol. Be sure that the Methanol or its fumes get to the thick film which is done by slightly tilting the slide.

⇒ Take out the slide from the Coplin jar and let it air dry with the thick smear up. Be sure that the slide is thoroughly dried on both the thin & thick smear side before staining.

⇒ **Diluting the Giemsa Stain for Thin and thick blood smear on the same slide:** For staining the Thick & thin blood smear on the same slide the Giemsa stain is used in 1:50. To make a 1:50 dilution of Giemsa stain, add 1 ml of stock solution of Giemsa stain to 49 ml of phosphate buffer solution in a clean Coplin jar. You can also use the Distilled water instead of buffer but the results may vary.



THIN AND THICK BLOOD SMEAR ON THE SAME SLIDE

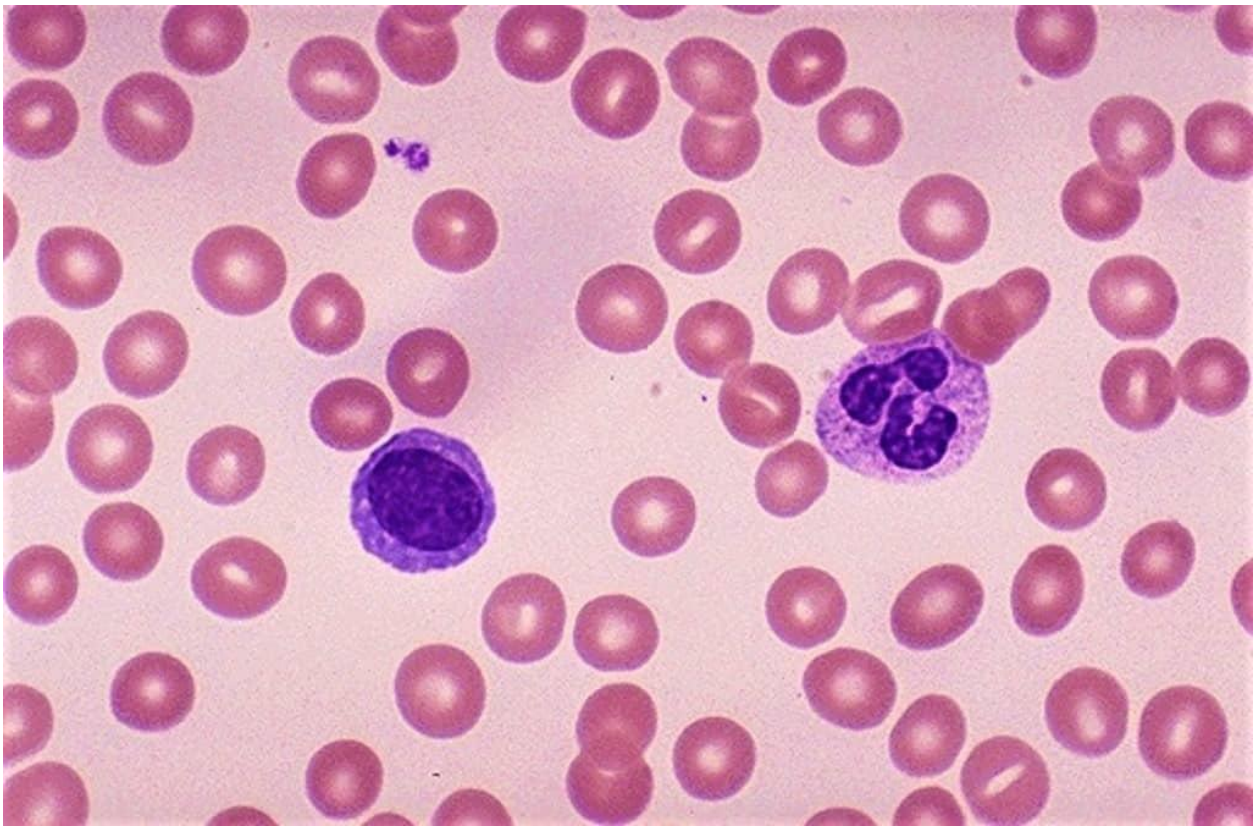
⇒ Now, stain the Air-dried Blood smear with diluted Giemsa stain (1:50, v/v) for 50 min. For this, put the slide in the Coplin jar containing the diluted Giemsa stain or you can also stain it on a staining rack or on any flat surface with the smear side up and pour the stain over the smear so that it equally covers the smear.

⇒ Place the slide in the Coplin jar in a way that the thick smear should be down to prevent the debris of RBCs from falling down onto the thin blood smear.

⇒ Now it's the crucial step of rinsing the slide…… Rinse the thin smear side by quickly dipping the smear in and out of a Coplin jar of phosphate buffer (one or two dips) and then Wash the thick smear side for 3 to 5 min using phosphate buffer or distilled water. Be sure that the thick smear is immersed but do not allow the water to cover any part of the thin smear. Otherwise, the thin smear will get decolorized.

⇒ Let the smear dry well in the air.

OBSERVATIONS OF GIEMSA STAINED SMEAR UNDER THE MICROSCOPE



The various blood cells will be observed under the microscope as follows:
NORMAL BLOOD SMEAR UNDER THE MICROSCOPE

⇒ **Re Blood Cells:** Pink color.

⇒ **Neutrophils:** Reddish Purple nuclei with pink cytoplasm.

⇒ **Eosinophils:** Purple nuclei, faintly pink cytoplasm & Red to Orange granules.

⇒ **Basophils:** Purple nuclei, Blue coarse granules.

⇒ **Lymphocytes:** Dark blue nucleus with Light blue cytoplasm.

⇒ **Monocytes:** Pink cytoplasm with a Purple color nucleus.

⇒ **Platelets:** Violet to Purple color granules.