KOMAR UNIVERSITY OF SCIENCE AND TECHNOLOGY

DEPARTMENT OF MEDICAL LABORATORY SCIENCES (MLS)

PRINCIPLES OF HEMATOLOGY (MLS2410L)

LABORATORY MANUAL

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PhD (HEMATOLOGY AND CLINICAL PATHOLOGY)

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Preface

This "Handout" laboratory manual has been prepared mainly in light of three excellent manuals namely Theml, Color Atlas of Hematology, (2004) Thieme, Dacie and Lewis Practical Haematology, 11th edition (2012) and Quick Review Cards for Medical Laboratory Science, 2nd edition (2014). Some modifications have been applied based on the direction and requirements of the Medical Laboratory Sciences (MLS) department curriculum and study program.

This manual composed of 14 exercises in total focused on the basic concepts of hematology and assists students to get necessary knowledge, skills, and practice in this field. In addition, students will learn how to work safely in hematology labs. Each exercise composed of a short background about a specific topic followed by the principle, aims, required material, and procedures needed to perform that exercise. The results of each exercise will be recorded by the students and together with the answers of some review questions will be submitted to the lab instructor in the form of lab reports.

Exercises are written in a way to be easy to follow yet informative to the students. The overall laboratory experience reinforces the concepts of the theoretical lectures and together provides a comprehensive knowledge to the students in the field of physiology.

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Laboratory 1: An Introduction to the Laboratory Practice

General Recommendations for Hematology Laboratory

1. Eating, drinking, smoking, putting hand into the mouth, nares, eyes and ears should be completely and strictly avoided.

2. Laboratory apron, gown, glove, and if necessary cap and mask must be worn.

3. Eyewear, face shield and goggles (in procedures that may generate infectious or hazardous aerosols) should be used.

4. Hands should be washed and sterilized before and after working.

5. Work surfaces should be disinfected properly with a potent disinfectant.

6. Mouth pipetting should be inhibited.

7. Working with dangerous agents must be carried out in a biological safety hood (cabinet).

8. A note book is required for recording notes and demonstrations.

9. You must have "first aid kit" ready in lab to be used in cases required.

10. Do not re-use contaminated, opened or used materials.
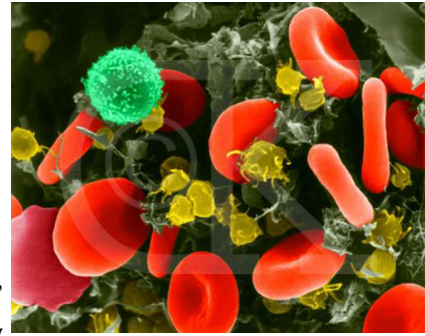
General Hematology Lab Requirements

1. Test Tubes

Different types of collecting sample tubes with different containing additives and/or anticoagulants for various laboratory procedures should be available in the lab.

Classification[1]	Items[2]	Additive	Color[3]		Tube Material[4]	Main Intended Use[5]	Basic Tube size[6] (mm)
Serum Tube	No Additive Tube	/	Red		Glass	Determinations in serum for clinical biochemistry, immunology, and serology	Φ 13x75 Φ 13x100 Φ 16x100
	Pro-coagulation Tube	Clot Activator	Red		Glass/Plastic		
	Gel & Clot Activator Tube	Gel & Activator	Golden		Glass/Plastic		
Plasma Tube	Glucose Tube	Potassium Oxalate/Sodium fluoride or EDTA /Sodium fluoride	Grey		Glass/Plastic	Determinations in stabilised anti-coagulated whole blood or plasma for glucose and lactate testing	Φ 13x75 Φ 13x100
	PT Tube	0.109mol/L or 0.129mol/L Sodium Citrate (1:9)	Light Blue		Glass/Plastic	Determinations in citrated plasma for coagulation testing	Φ 13x75 Φ 13x100
	Heparin Tube	Lithium Heparin, Sodium Heparin	Green		Glass/Plastic	Determinations in heparinised plasma for clinical chemistry	Φ 13x75 Φ 13x100 Φ 16x100
	Gel & Heparin Tube	Gel & Lithium Heparin or Sodium Heparin	Green		Glass/Plastic	For plasma determinatons in chemistry.	Φ 13x100 Φ 16x100
	Gel & EDTA.K2 Tube	Gel & EDTA.K2	Lavender		Glass/Plastic	For use in molecular diagnostic test methods(such as but not limited to PCR).	Φ 13x100 Φ 16x100
Whole Blood Tube	EDTA Tube	EDTA.K2 EDTA.K3	Lavender		Glass/Plastic	Determinations in EDTA whole blood for hematology	Φ 13x75
	ESR Tube	0.109mol/L or 0.129mol/L Sodium Citrate (1:4)	Black		Glass	Blood cell sedimentation rate test	Φ 9x120 Φ 13x75
					Plastic		Φ 13x75

2. Anticoagulants

It refers to any substance that inhibits the blood coagulation. The choice of anticoagulant depends on the type of examination to be carried out, thus they are of many types, which include:

1. EDTA (ethylene diamine tetra acetic acid)

Dipotassium and disodium salts of EDTA prevent the coagulation of blood by combining with calcium.

It has the advantage of preserving the stain ability and morphologic characteristics of leukocytes. EDTA can be used in 2 forms, which are liquid and dry (powder) form.

2. Heparin

It prevents coagulation of blood by interfering with the conversion of prothrombin to thrombin. A 5 mL syringe can be rinsed with 1% heparin solution for highest anticoagulation activity. It has the disadvantage of adversely affecting the leukocyte stain ability.

3. Sodium Citrate

It is not commonly adopted for the preservation of blood for hematologic determination and can be used for blood transfusion.

4. Oxalates (Sodium and Potassium)

They also prevent coagulation of the blood by combining with calcium. They should not be used as an anticoagulant when blood non protein nitrogen and blood urea tests are required.

3. Needles

The size of the needle should be 19-21 gauges in adults and 21-23 gauges in pediatrics to avoid hemolysis during sample drawing from the patient and also during evacuation into the test tubes.

4. Disposables

They include syringe, needle, gauze, swab, gloves, plasters and lancets.

4. Consumables

They include various types of disinfectants and sterilizers such as 5% savlon solution, tincture iodine, or 70% alcohol.

5. Missalenous

They include lab coat, gown, apron, permanent marker, vacuette and tourniquates.

Types of Blood Collection

1. Capillary Blood Collection

Blood is collected from capillary network if few drops of blood are required such as measurements of Hb level, RBC count by microdilution method and preparation of thin blood film. Capillary blood cannot be used for platelet testing.

Major Capillary Blood Sampling Sites

a. Finger prick.

b. Ear lobe.

c. Sides of the heel especially in pediatric and neonatal patients.

2. Venous Blood Collection

Blood is collected from the venous system of the patient when large amount of blood is required. Choose the veins that are large and accessible, and avoid bruised or scarred areas.

Major Venous Blood Sampling Sites

1. Median cubital veins.
2. Cephalic (dorsal hand) veins.
3. Basilic veins.
4. Great Saphenous (foot) veins.
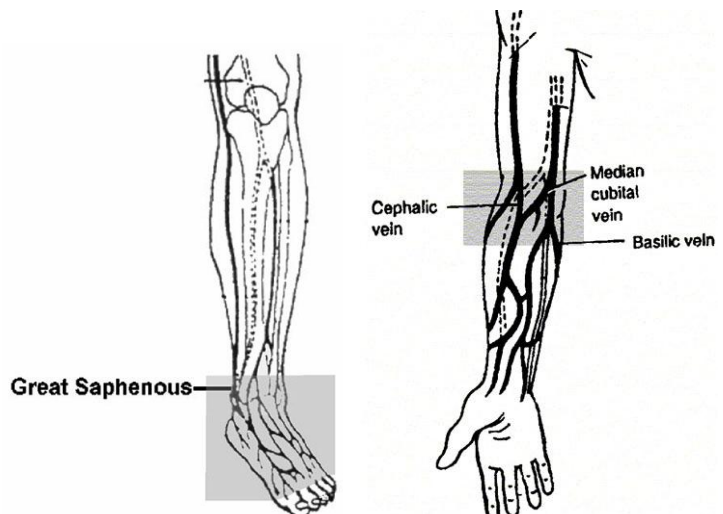
General Cautions/Procedure

1. Be quite with the patient and let him/her feel comfortable.

2. Verify that computer printed labels match requisitions. Check patient identification band against labels and requisition forms. Ask patient for his /her full name, address, identification number, and date of birth.

3. If a fasting specimen or dietary restriction is required, confirm patient has fasted or eliminated food from the diet as ordered by physician.

4. Position the patient on a chair or a bed properly.

5. Choose the appropriate tube for collection.

6. Apply a tourniquet 3-5 inches above the antecubital fossa for not more than 1 minute.

7. Ask the patient to make (clench) a fist without vigorous hand pumping. Select a suitable site for venipuncture.

8. Feel or palpate for any vein to determine its potential size, depth and direction that many be hidden. If still the vein is not visible or palpable, ask the patient to "pump" the hand 3 times and no more, as it may cause hemoconcentration. If all measures fail to palpate a suitable vein, ask for the opinion from another experienced technician.

9. Put on gloves with consideration of latex allergy for the patient.

10. Cleanse the venipuncture site with 70% isopropyl alcohol. Allow the area to dry.

11. Anchor the vein firmly.

12. Enter the skin with the needle at approximately 30 degree angle or less to the arm with the level of the needle up;

    a. Follow the geography of the vein with the needle.

    b. Insert the needle smoothly and fairly rapidly to minimize the patient discomfort.

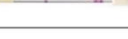   c. If using a syringe, pull back on the barrel with a slow, even tension as blood flows into the syringe. Do not pull back too quickly to avoid hemolysis or collapsing the vein.

    d. If using an evacuated system, as soon as the needle is in the vein, ease the tube forward in the holder as far as it will go, firmly securing the needle holder in place. When the tube has filled, remove it by gasping the end of the tube and pulling gently to withdraw and gently invert tubes containing additive and/or anticoagulant.

13. Draw the proper volume required for each test.

14. Release the tourniquet when blood begins to flow. Never withdraw the needle without removing the tourniquet.

15. Withdraw the needle, and then apply the pressure to the site. Cleaners to be available such as 5% savlon solution, tincture iodine, or 70% alcohol.

16. Apply adhesive bandage strip over cotton ball or gauze to adequately stop the bleeding and to avoid the hematoma.

17. Mix the inverted tube with anticoagulant; do not shake the tubes. Check the condition of the patient. Dispose of the contaminated material in designated containers (sharps container) use Universal precautions.

18. Label the tubes before leaving patients side with:

    a. Patients first and last name.

    b. Unique hospital Identification number and name of the department doing the test.

    c. Date, time and place of collection.

    d. Specimen type (whole blood, serum, plasma, body fluid, genetics, etc).

    e. Initial name of the sample collector.

19. Deliver the tubes of the blood for testing to appropriate laboratory section or central receiving and processing area.

20. Great care being taken to avoid self-injury with the needle. The needle should be removed from the syringe before expelling the blood into the specimen container. The needle should be put directly into a special receptacle for sharp objects without re-sheathing it.

The Order of Blood Sample Collection

The order (sequence) of sample collection should be as indicated below if a patient have more than 1 test been ordered.

Tube Closure Color		Collection Tube
Yellow		Blood Cultures – SPS
Light Blue		Citrate Tube (Light Blue)
Gold / Tiger		Serum Separator Tubes (Gold and Tiger)
Red		Serum Tube (Red)
Orange		Rapid Serum Tube (Orange)
Light Green/Tiger		Plasma Separator Tube
Green		Heparin Tube (Green)
Lavender/Pink		EDTA Tube (Lavender)
Pearl		PPT Separator Tube (Pearl)
Gray		Fluoride Tube (Gray)

Sources of error

1. Difficult finding of a vein.

2. Malpractices of blood collector (phlebotomist).

3. Improper needle size which leads to hemolysis of the blood.

4. Expelling the blood vigorously into a tube.

5. Shaking or mixing the tubes vigorously.

6. Performing blood collection before the alcohol has dried at the collection site.

Consequences of RBC Hemolysis

Hemolysis can falsely increase blood constituents such as potassium, magnesium, iron, LDH, phosphorus, ammonium, and total protein. Because of the extremely important role of potassium in cardiac excitation, elevations due to hemolysis can be problematic, especially for emergency room patients who are at risk of hemolysis during a stressful blood collection.

Figure 2.1 Venepuncture in adults
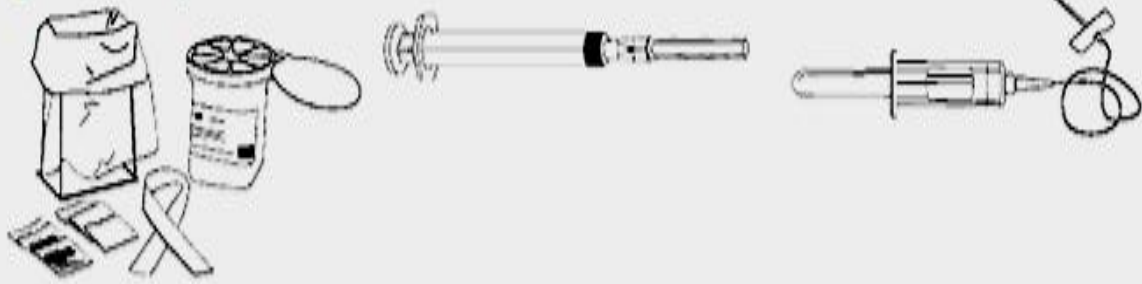
1. Assemble equipment and include needle and syringe or vacuum tube, depending on which is to be used.

2. Perform hand hygiene (if using soap and water, dry hands with single-use towels).

3. Identify and prepare the patient.

Median cubital vein

Ulnar nerve

Ulnar artery

Basilic vein
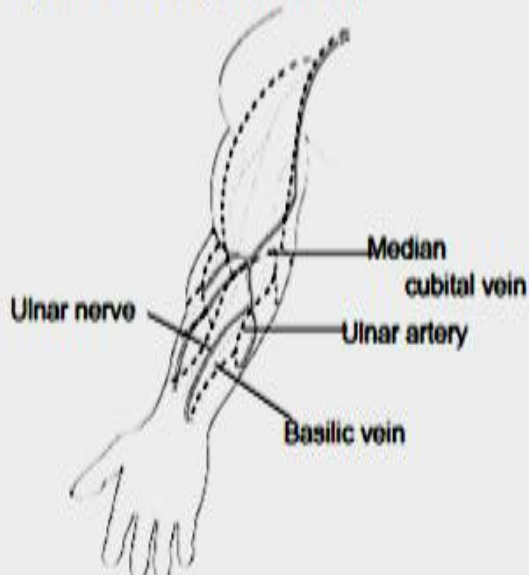
4. Select the site, preferably at the antecubital area (i.e. the bend of the elbow). Warming the arm with a hot pack, or hanging the hand down may make it easier to see the veins. Palpate the area to locate the anatomic landmarks. DO NOT touch the site once alcohol or other antiseptic has been applied.

5. Apply a tourniquet, about 4–5 finger widths above the selected venepuncture site.

6. Ask the patient to form a fist so that the veins are more prominent.

7. Put on well-fitting, non-sterile gloves.

8. Disinfect the site using 70% isopropyl alcohol for 30 seconds and allow to dry completely (30 seconds).

9. Anchor the vein by holding the patient's arm and placing a thumb BELOW the venepuncture site.

10. Enter the vein swiftly at a 30 degree angle.

11. Once sufficient blood has been collected, release the tourniquet BEFORE withdrawing the needle.

12. Withdraw the needle gently and then give the patient a clean gauze or dry cotton-wool ball to apply to the site with gentle pressure.

13. Discard the used needle and syringe or blood-sampling device into a puncture-resistant container.

14. Check the label and forms for accuracy.

15. Discard sharps and broken glass into the sharps container. Place items that can drip blood or body fluids into the infectious waste.

16. Remove gloves and place them in the general waste. Perform hand hygiene. If using soap and water, dry hands with single-use towels.

Figure 2. 2 Filling tubes
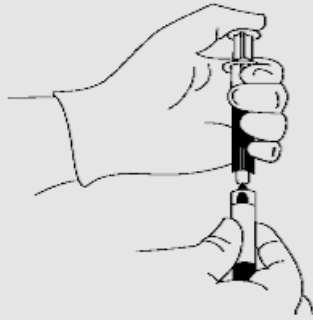
1. If the tube does not have a rubber stopper, press the plunger in slowly to reduce haemolysis (this is safer than removing the needle).

2. Place the stopper in the tube.
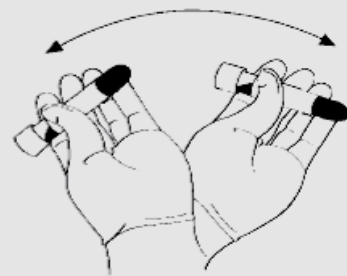
3. Following laboratory instructions, invert the sample gently to mix the additives with the blood before dispatch.

Laboratory 2: Total Erythrocyte Counting

Aims of the Test

To enumerate the total number of red blood cells (RBC) of a given blood sample.
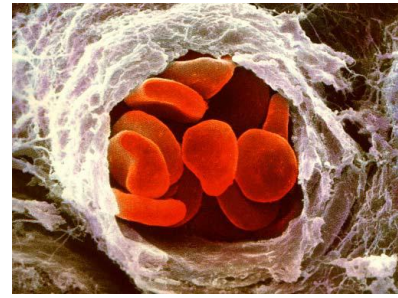
Significance

• It performs some functions such as transportation of O_2 and CO_2.

• A decrease in RBC accounts for less hemoglobin i.e., anemia.

• An increase in RBC is referred as Polycythemia.

Methods

1. Photoelectric Counting Method.

2. Electronic Counting Method.

3. Hemocytometer (Neubauer) Counting Method.

1. Photoelectric Counting Method

It is not reliable in anemia's patients because there may be a variation in shape, Hb content and the size of RBC.

2. Electronic Counting Method

The accuracy of this method is much greater than other methods, but the high cost of some units precludes their use in most hospital laboratories.

3. Hemocytometer (Neubauer) Counting Method

Materials

1. Hemocytometer chamber.

2. Cover slip.

3. Light microscope.

4. RBC pipette.

5. RBC diluting fluid (Haeyem's solution or Physiological saline 0.85% Nacl).

Procedure

1. Blood should be carefully (gentle suction) drawn to the 0.5 mark of the RBC pipette, and excess blood clinging to the exterior of the pipette is removed by wiping it with a piece of cloth or tissue.

2. An isotonic solution (Normal saline or Hayem's solution) should be drawn to the 101 mark to dilute the blood.

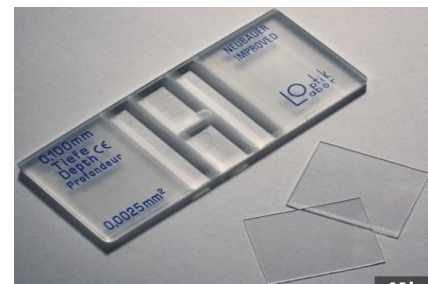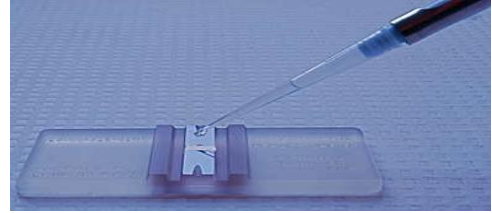3. The blood and diluting fluid are mixed by shaking the pipette vigorously in a horizontal position for 2 - 3 minutes (to ensure complete hemolysis of WBC).

areas of the grid where WBC are counted

4. Several drops (2 - 4 drops) of mixed fluid are discarded and the end of the pipette is dried with a piece of lint-free absorbent material.

5. The tip of the pipette is touched to the side of the hemocytometer chamber (without any hesitation) and a drop of a fluid will run under the cover glass.
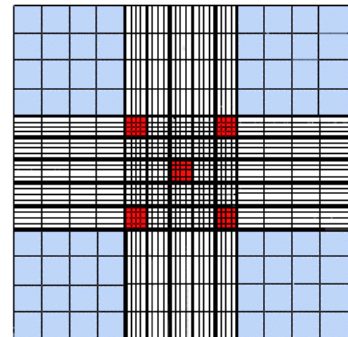
areas of the grid where RBC are counted

6. Wait for about 2 - 3 minutes as erythrocytes require settling time to assume a single level.

7. Total number of cells in 5 squares in the center of counting chamber is determined under the high dry objective of the microscope (40X).
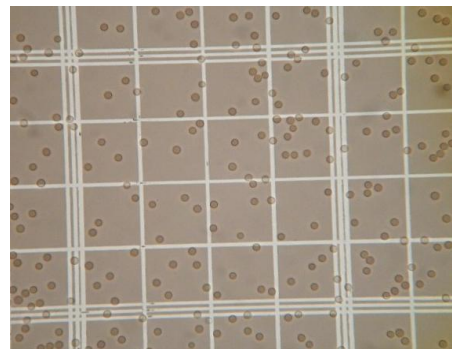
8. To avoid duplicate counting of a single cell, you must count only those cells that touch the lower and left boundaries.

Counting 2 sides in L shape
(i.e. count the Cells settle on the Top and left sides
(colored sides)
and exclude the cells on the right and the bottom sides)

Counting is Zigzag in all squares

Blue cells is counted
gray cells isnot counted
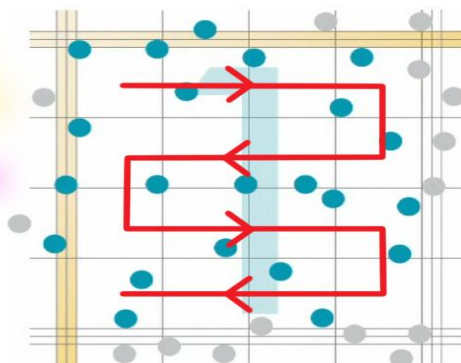
Calculation

RBC/µL or mm^3= No. of cells in 5 squares (80 small squares) x dilution no. x reciprocal of volume.

Dilution No. = 0.5: 100 = 200

Each square contains 16 small squares, and each small square has an area of 1/400 mm^3 and a depth of 1/10 mm. A total of 80 squares are included in the counting, i.e. 1/10 x 1/400 x 80 = 1/50.

RBC/µL = No. x 200 x 50

RBC/µL = No. x 10,000
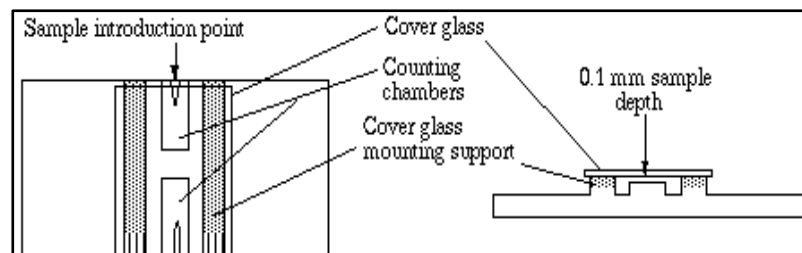
Notes

-The inherent error is about ± 20

-1 µL = 1 mm^3 = 0.001 mL

Normal RBC Range in Human

-Male: 4.7 to 6.1 million cells per µL.

-Female: 4.2 to 5.4 million cells per µL.

❖ The above ranges are common measurements for results of these tests.
❖ Normal value ranges may vary slightly among different laboratories as different labs use different measurements or methods.
❖ Normal value ranges may vary slightly among different individuals (usually the result affected by age, sex, physical activity, or health condition).

Cases of Higher- numbers of RBCs (Erythrocytosis)

1. Cigarette smoking.

2. Problem with heart's structure and function (congenital heart disease).

3. Dehydration (severe diarrhoea).

4. Kidney tumor (renal cell carcinoma).

5. Low blood oxygen level (hypoxia).

 6. Scarring or thickening of the lungs (pulmonary fibrosis).

7. Bone marrow disease (polycythemia vera).

8. Taking some drugs (gentamicin and methyldopa).

Cases of Lower- numbers of RBCs (Erythrocytopenia)

1. Anaemia.

 2. Bleeding.

 3. Bone marrow failure (radiation, toxins, or tumor).

 4. Deficiency erythropoietin hormone that caused by kidney disease.

 5. RBC destruction (hemolysis) due to transfusion or blood vessel injury.

 6. Cancer (leukemia and multiple myeloma).

 7. Malnutrition and nutrition deficiencies such as iron, copper, folic acid, vitamin B6, or vitamin B_{12}.

 8. Pregnancy.

 9. Taking some drugs (chemotherapy, chloramphenicol and quinidine).

Risks during RBC counting

-There is very little risk involved with having your blood taken for RBC counting.

-Veins and arteries vary in size from one patient to another and from one side of the body to the other.

-Taking blood from some people may be more difficult than from others.

Other risks associated with having blood drawn are slight, but may include:

 1. Excessive bleeding.

 2. Fainting or feeling light-headed.

 3. Hematoma (blood accumulating under the skin).

 4. Infection (a slight risk any time the skin is broken).

Laboratory 3: Total Leukocyte Counting

Aims of the Test

To enumerate the total number of leukocyte (White Blood Cell: WBC) of a given blood sample.
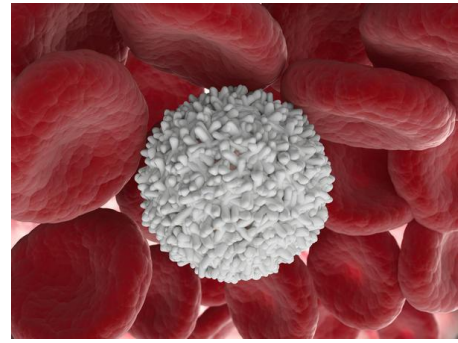
Significance

The normal WBC performs some important physiological functions. The chief function of it is imparting immunity to the body.

Therefore, the decrease or increase in circulating WBC indicates physiological condition i.e., increase in WBC indicates inflammation, whereas decrease in WBC may indicate AIDS.

Materials

1. Hemocytometer chamber.

2. Cover slip.

3. Light microscope.

4. WBC pipette.

5. WBC diluting fluid (1% HCl or 1% Glacic Asetic Acid)[1ml GAA+1ml MB +100ml DW].

Method

Hemocytometer (Neubauer) Counting Method

Procedure

1. Blood should be carefully (gentle suction) drawn to the 0.5 mark of the WBC pipette, and excess blood clinging to the exterior of the pipette is removed by wiping it with a piece of cheesecloth.

2. WBC reagent (Glacial acetic acid 3%) should be drawn to the 11 mark to dilute the blood.

3. The blood and diluting fluid are mixed by shaking the pipette vigorously in a horizontal position for 2-3 minutes (to ensure complete hemolysis of RBC).
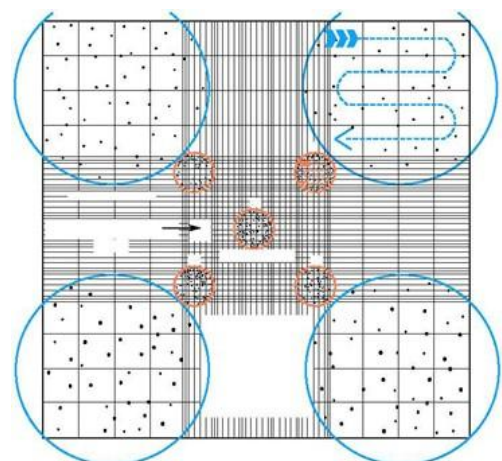
4. Several drops (2-4 drops) of mixed fluid are discarded and the end of the pipette is dried with a piece of lint-free absorbent material.

5. The tip of the pipette is touched to the side of the hemocytometer counting chamber (without any hesitation) and a drop of a fluid will run under the cover glass.

6. Wait for about 2-3 minutes as leukocytes require settling

time to assume a single level.

7. Total number of cells in 4 squares at the corner of counting chamber is determined under the low objective of the microscope (10X).

8. To avoid duplicate counting of a single cell, you must count only those cells that touch the lower and left boundaries.
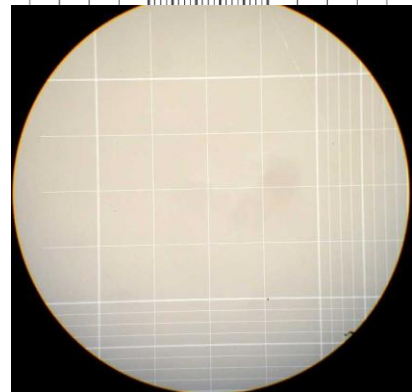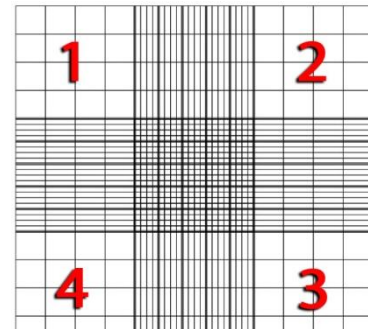
Calculation

WBC/µL or mm^3= No. of cells in 4 squares (64 small squares)/4 x dilution No. x reciprocal of chamber depth.

Dilution No. = 0.5: 10= 20

Depth of the chamber = 1/10 mm.

WBC/µL = No. /4 x 20 x 10

WBC/µL= No. x 50
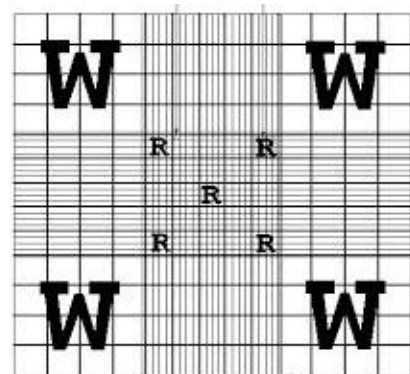
Note

The inherent error is about ± 10

Interpretation of Leukocyte Counts

Physiological factors to be considered in the interpretation of leukocyte counts include:

1. Age.

2. Degree of excitement and muscular activity at the time of blood collection.

3. Stage of pregnancy.

4. Stage of estrus cycle.

5. Stage of digestion.

Inherent Error in Total Erythrocyte and Leukocyte Counting

1. Errors in dilution, counting, calculation and calibration.

2. Improper shaking of the pipette after dilution.

3. Contamination of diluting fluids with yeast, mold, or other cells.

4. Failure to wipe excess blood from the end of the pipette.

5. Drying of the sample during or prior to counting.

6. Overflow of the fluid in to the moat.

7. Failure to allow the cells to settle into a single plane prior to counting.

8. Failure to discharge diluting fluid from the capillary prior to charging the chamber.

9. Chipped pipettes.

10. Inadequate cleaning of the glass wares.

11. Failure to focus microscope up and down during counting.

Advantages of the Bead (red and white) in the Blood Diluting Pipettes

1. Differentiating RBC pipette from WBC pipette.

2. Enhance mixing of the blood.

Cases of High- numbers of WBCs (Leukocytosis)

1. Blood disease (anaemia).

2. Certain drugs or medications (corticosteroides).

3. Cigarette smoking.

4. Infections (Salmonellosis).

5. Inflammatory disease (rheumatoid arthritis or allergy)

6. Cancer (leukemia).

7. Severe mental or physical stress.

8. Tissue damage (burns).

Cases of Low- numbers of WBCs (Leukocytopenia)

1. Bone marrow deficiency or failure (infection or tumor).

2. Cancer treating drugs (chemotherapy) or other medicines (antibiotics and sulphonamides).

3. Certain autoimmune disorders (lupus erythematosus).

4. Disease of the liver or spleen.

5. Radiation treatment (cancer).

6. Certain viral illnesses (HIV).

7. Cancers that damage the bone marrow (multiple myeloma).

Risks during WBC Counting

Same as those of RBC count.

Laboratory 4: Hemoglobin Determination

Background

- Hemoglobin (Hb or Hgb) is the iron-containing protein in the red blood cells.

- Hb test is performed for routine health screenings or if a disease or toxicity is suspected.

- Low Hb value means low RBC count which mainly related to anemia (especially iron deficiency anaemia).

- Hb value is usually expressed as grams/dL or grams/100 mL of blood or by percentages.
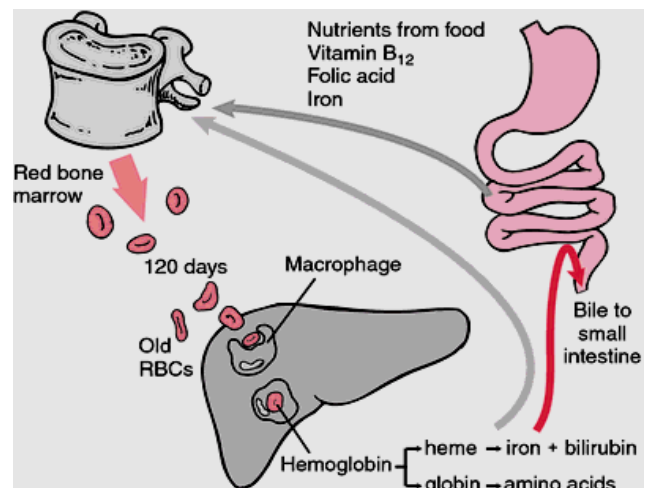
Notes

- 1 dL = 100 mL = 100 cubic cm= 0.1 L

- 1 dL = 100,000 μL = 100,000 cubic mm

Aims of the Test

To determine the amount of Hb present in 100 ml of blood of a given sample.

Significance

a. It serves as an index of blood condition.

b. If the Hb content falls below the normal levels, it indicates anemia, or pregnancy (physiological).

c. If it increases to the normal value, it indicates polycythemia, decrease in O2 supply, heart disease, emphysema etc.

Methods

1. Manual

a. Cyanmethaemoglobin method.

b. Acid haematin method.

2. Automated

a. Coulter counter.

b. Strip based technique.
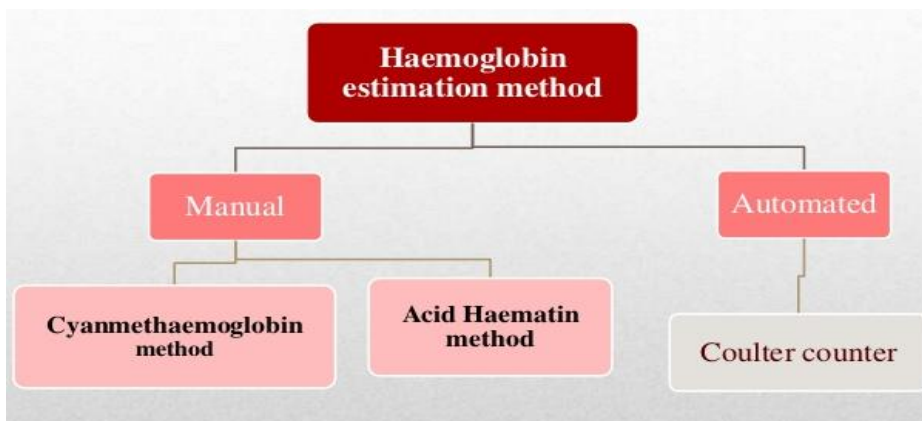
Acid Hematin Method

Materials

Sahli set.

Parts of Sahli Set

1. Sahli standard (color comparator) for matching color.

2. Hydrochloric acid (HCl 0.1N) for hemolyzing of RBC and converting blood to acid hematin.

3. Graduated hemometer tube.

4. Hemometer or Sahli's pipette with rubber tube and plastic mouth piece for sucking of the blood.

5. Glass rod or stirring rod for mixing.

6. Small brush for cleaning.

7. Dropper for HCl suction.

Notes

1. We also need distilled water (DW) for diluting of acid hematin and matching to the standard color.

2. 0.1 N HCl is prepared by mixing 0.8 mL concentrated HCl with 99.2 mL DW.

Procedure

1. Add hydrochloric acid (HCl) to the mark 2 of graduated capillary tube.

2. Fill the capillary pipette to the 20 mm^3 mark with well mixed, unclotted sample by gentle suction.

3. Wipe off all the blood adhering to the outer surface of the pipette with cotton wool.

4. Expel the blood into the acid solution in the graduated tube.

5. Rinse out the pipette with the solution and thoroughly mix the tube contents with glass stirrer.

6. Remove it from the bright light and allow it to stand for 5 minutes for maximum color development.

7. Dilute it with DW, mixing thoroughly at each addition, until the color matches that of the standard (it is in the form of a tinted glass rod which does not fade).

8. The resulting brownish-yellow mixture is matched with a standard in a colorimeter, and from this determines the hemoglobin content of the blood.
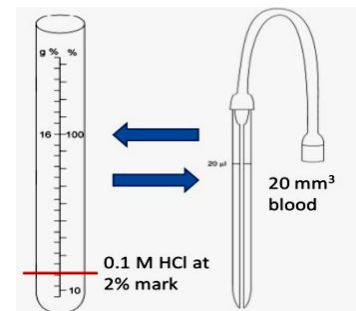
Notes

1. The intensity of the brown color depends on the amount of acid hematin produced, which in turn depends on the amount of hemoglobin in the blood sample.

2. The inherent error in this method is less than 10%.

Sources of Error

1. Non hemoglobin substances normally present in the plasma, such as protein and lipids.

2. Usually it is difficult to match the color of the sample accurately to the brown color of the standard.

3. The variation in the ability of individual operators in matching colors is the common source of error.

4. Some inactive forms of Hb present in the blood, such as methemoglobin, sulfhemoglobin and carboxyhemoglobin, which are not converted in acid solutions into hematin, are not included in values obtained by this technique.

5. Improper mixing of blood in the comparator.

6. Pipetting error during blood suction.

Normal Hb Values

Individuals	g/dL	%
Women	12.5-15	83-110
Man	14-17	97-124
Newborn infants	17-23	97-138

Abnormal Values of Hb

Increased Hemoglobin	Decreased Hemoglobin
•High altitude due to low Oxygen tension. •Obstructive lung disease. •Congestive Heart disease due to hypoxia. •Polycythemia (increased RBCs). •Smoking.	•Anemia. •Parasitic infestations. •Drugs. •Lead poisoning. •Iron deficiency. •Copper deficiency.. because copper is necessary for the formation of protein-ceruloplasmin which converts ferric to ferrous. •Kidney disease.. because formation of erythropoietin is decreased by kidney.

Laboratory 5: Packed Cell Volume Determination
Background

- Literally, the word hematocrit means the separation of the blood, and in the laboratory this is most readily accomplished by centrifugation.

- PCV is the volume percentage (%) of erythrocytes in the blood.

Synonyms

1. Hematocrit (Ht or HCT).

2. Erythrocyte Volume Fraction (EVF).

Aims of the Test

To determine the hematocrit value for a given blood sample.

Principles of the Test

Blood compartment is separated into three parts using capillary tube in a hematocrit centrifuge.

Significance

· Packed Cell Volume (PCV) = erythrocyte mass; anemia when PCV falls dawn.

· Buffy coat; white to gray layer above PCV. It will give number of WBC (0.5mm to1.5mm). Leukopenia or leukocytosis.

· Plasma content: usually about 55%, Yellowish in color. Degree of yellowness indicates icterus (jaundice).

Methods

1. Microhematocrit (capillary tube) method.

2. Macrohematocrit (wintrobe) method.

3. Automated estimation of PCV.

Microhematocrit Test
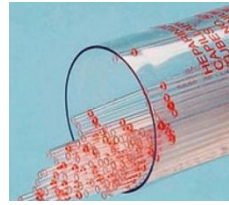
Requirements

1. Capillary tube (Microhematocrit tube) with anticoagulant (red tip).

2. Special clay or sealing clay.

3. Microhematocrit chart (Reader).

4. Hematocrit centrifuge.

Procedure

1. Fill about ¾ of the capillary tube with blood.

2. Clean and dry the outside of the tube carefully with a piece of gauze.

3. Seal the opposite end of the tube with special clay or by heating in a flame.

4. Place the sealed tubes in a hematocrit centrifuge, replace the cover, tighten securely and spun for 4 minutes at 10,000 rounds per minute (rpm).

The sealed end should face the outside rim of the centrifuge in order to avoid the breakage of the tube. You have to use 2 tubes at the same time in opposite direction to balance the centrifuge.

5. After centrifugation, carefully place the tube on a special microhematocrit reader for determining the percentage of RBC.

The base line (0%) of the reader should intersect base of red cells. The top line (100%) of the reader should intersect top of plasma.
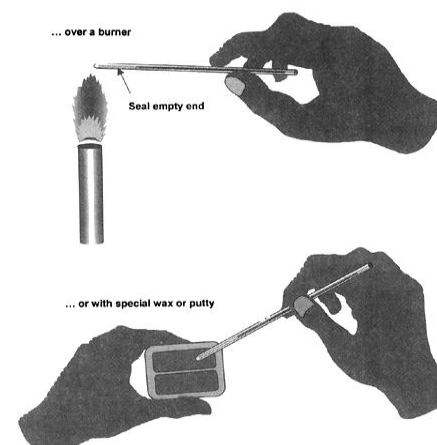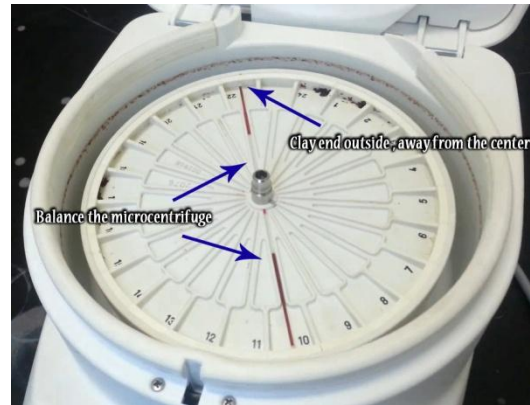
Disadvantages of the Technique

1. A special reader is required for determining the PCV values.

2. A special centrifuge is required.

3. It has an error of $\pm\ 0.5\%$.

Separated Layers

1. After centrifugation, blood is separated into 3 distinct parts including:

a. The mass of erythrocytes at the bottom (packed cell volume).

b. A grey or white layer of leukocytes and thrombocytes immediately above the red cell mass (Buffy coat).

c. The blood plasma (slight yellow color).
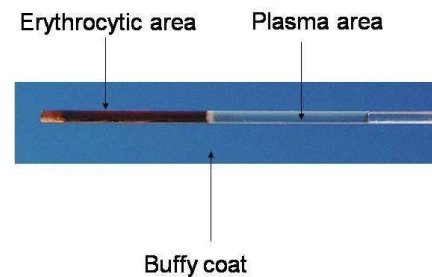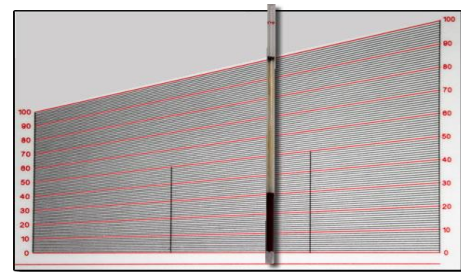
Notes

1. Utilized venous blood must be preserved with an anticoagulant that will not cause distortion in cells.

2. Recommended anticoagulant is EDTA, but care must be taken not to use it in excess, as the PCV will be decreased.

Normal PCV Values

1. Male : 40-52%

2. Female : 36-48%
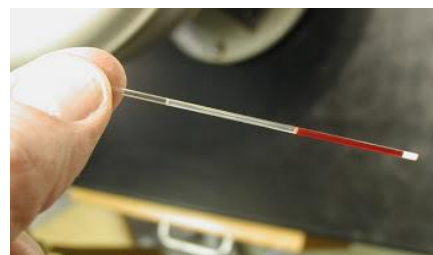
Cases of Increased Hematocrit

Raised PCV reflects hemoconcentration, which may be due to:

1. Reduced plasma volume:

   a. Dehydration (burning and diarrhoea).

   b. Acute   pancreatitis   (haemorrhagic pancreatitis).

   c. Medication (diuretic therapy).
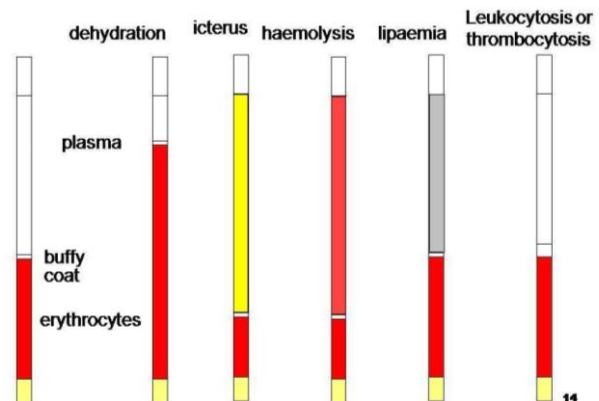
2. Increased red cell mass:

   a. Primary (polycythaemia).

   b. Secondary (chronic lung disease, smoking, altitude, tumours).

Cases of Decreased Hematocrit

1. Blood disorders (Anaemia and leukaemia).

2. Menstruating and Pregnant women.

3. Patients with chronic kidney disease.

4. Tumor (multiple myeloma of bone marrow).

Sources of Error

1. Haemolysed blood.

2. Improper shaked venous blood.

3. Inadequate volume of blood.

4. Improper sealing.

5. Not enough centrifugation and separating of layers.

Laboratory 6: Erythrocyte Indices or Blood Cell Indices Measurements

Background

- It is the test that provides information about the Hb content and size of red blood cells.

- Abnormal values indicate the presence of anemia and which type of anemia it is.

Aims of the Tests

To determine or classify various types of anemia.

Objectives

-To be able to calculate MCV, MCH, and MCHC from red blood cell count, hematocrit and hemoglobin concentration

-To compare with normal ranges of the blood indices of different domestic animals.

Significance

Here one can classify red blood cells as normo, hypo and hyperchromic anemia. Apart from these facts, the size of red blood cell as normo, micro and macrocytosis can be classified.

Erythrocyte Indices Tests

1. MCV.

2. MCH.

3. MCHC.

1. Mean Corpuscular Volume (MCV)

- It expresses the average size of the RBC.

- The calculation is as below:

 MCV = (PCV ÷ RBC) x 10

- The result expresses in femtoliter (fL)

- Normal range = 80 - 100 fL

Interpretation

1. Normal MCV: normocytic

2. Increased MCV: macrocytic

3. Decreased MCV: microcytic
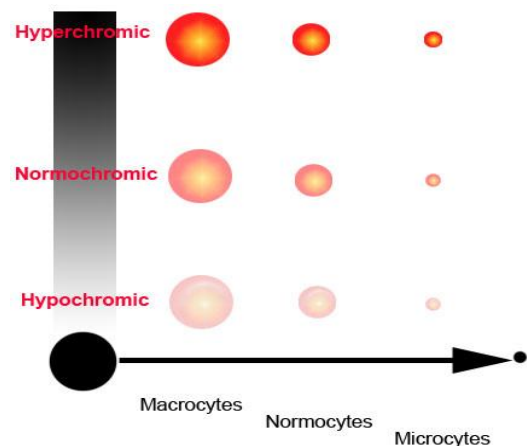
2. Mean Corpuscular Hemoglobin (MCH)

It is the average amount of Hb per red blood cell.

The calculation is as follows:

MCH = (Hb ÷ RBC) x 10

The result expresses in picogram (pg).

Normal range: 27-31 pg/cell

Interpretation

- Normal MCH : normochromic

- Decreased MCH : hypochromic

- Increased MCH : hyperchromic

3. Mean Corpuscular Hemoglobin Concentration (MCHC)

- It is the average concentration of Hb relative to volume of red blood cells.

- The calculation is as below:

MCHC = (Hb ÷PCV) x 100

- The result is expressed in g/dL

- Normal range = 32 – 36 g/dL

Interpretation

- Normal MCHC: normochromic

- Decreased MCHC: hypochromic

- Increased MCHC : hyperchromic

Parameter	Definition	Units	Formula	Example
Mean Corpuscular Volume (MCV)	Average volume of the RBC	Femtoliters (fL) or 10^{-15} Liters	$MCV= \dfrac{Hematocrit(\%) \times 10}{RBC\ (\times 10^{12}/L)}$	$MCV= \dfrac{42 \times 10}{4.2} = 100\ fL$
Mean Corpuscular Hemoglobin (MCH)	Average weight of Hb in the RBC	Picograms (pg) or 10^{-12} grams	$MCH= \dfrac{Hb\ (g/dL) \times 10}{RBC\ (\times 10^{12}/L)}$	$MCH= \dfrac{12.5 \times 10}{4.1} = 30.5$
Mean Corpuscular Hemoglobin concentration (MCHC)	Average concentration of Hb in the RBC volume	Grams/deciliter (g/dL)	$MCHC= \dfrac{Hb\ (g/dL) \times 100}{Hematocrit\ (\%)}$	$MCHC= \dfrac{12.5 \times 100}{37} = 34$

Laboratory 7: Erythrocyte Sedimentation Rate (ESR)

Aims of the Test

To determine the rate at which RBCs are falling (sedimenting) during a given period of time.

Principle

The distance that the erythrocytes fall during a given period of time when blood to which anticoagulant has been added in a tube placed in a vertical position.

Significance

· It is not a specific test, but reflects change in plasma protein accompanying most of acute and chronic infection.

· Some pathological condition causes rouleax formation.

· The greater the ESR reading, the more the severity of pathological condition.

· During TB and rheumatic disease ESR increases drastically.
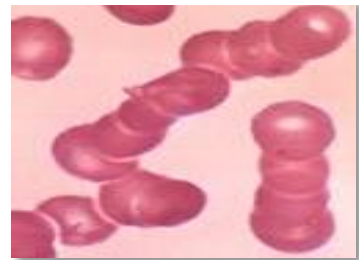
Methods

1. Manually (Westergren or Wintrobe method).

2. Automatically (ESR analyzer).

Wintrobe Method

Materials

Wintrobe set which composed of:

1. Wintrobe tube (pipette), calibrated in mm.

2. Wintrobe rack.

-A special timer is also required.
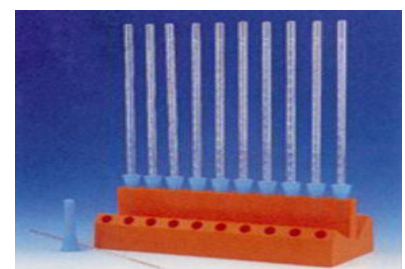
Procedure

1. Well mixed venous blood containing anticoagulant is filled into a Wintrobe tube.

2. Put the tube in a sedimentation rack (Westergren) that holds it perpendicular to the surface of the tube.
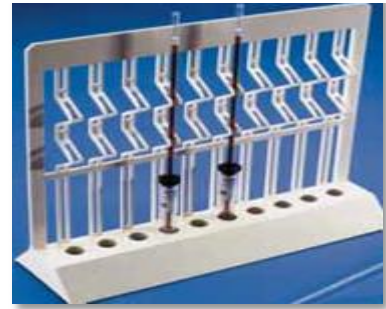
3. The rack should be placed in an isolated area in the laboratory that is free from vibrations.

4. The filled tube in a rack is allowed to stand for exactly 1 hour.

5. Lastly the level of the top of the erythrocyte column is recorded as the number of millimeters of fall per hour (mL/hr).
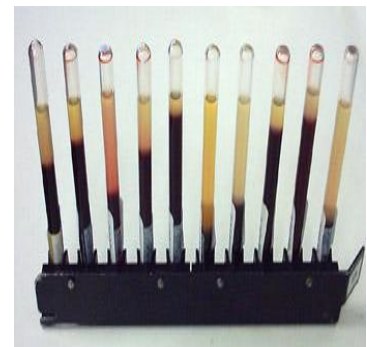
Notes

- Care must be taken to ensure that a standard amount of anticoagulant was used, as high anticoagulant make erythrocytes to sink faster because they become heavier than the normal.

- Since temperature may influence ESR, every attempt should be made always to regulate the room temperature near 20^0C.

- This process depends to some extent on plasma viscosity that alters when inflammatory proteins are present.

- The speed with which erythrocytes fall in the blood of normal individuals is relatively slow, but in those with inflammatory diseases in which there is tissue necrosis and degeneration; the speed is increased, which may be due to the activation of coagulative cascades and changes that occur in the physiochemical properties of the erythrocyte surfaces and the plasma.

Sources of Error

- Incorrect using of anticoagulants.

- Presence of substances such as dirt, alcohol or ether within the Wintrobe tube.

- Not placing the tube absolutely vertical to the table.

- Presence of air babbles in the tube.

- Hemolysis of the blood sample.

- Refrigeration of the blood sample.

- Old blood sample.

An Elevated ESR may be found in:

- Pregnancy and Menstruation.
- Acute and chronic infections.
- Rheumatic fever and Rheumatoid arthritis.
- Myocardial infection.
- Nephrosis and Acute hepatitis.
- Tuberculosis.
- Hypothyroidism.

A Decreased ESR will be Present in:

- Polycythemia.

- Congestive heart failure.

- Hypofibrinogenemia.

- The presence of red blood cell abnormalities (poikilocytosis, spherocytes, and sickle cells).

Laboratory 8: Differential Cell Count (DCC)
Background

It means determining the exact number and/or the percentage of each type of white blood cell that is present in an individual stained blood smear.
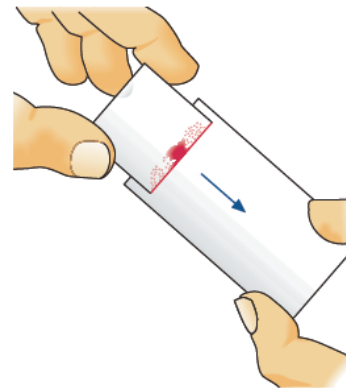
Synonyms

1. White Blood Cell Count (WBCC).

2. Complete Blood Count (CBC).

3. Differential Leukocyte Count (DLC).

Aims of the Test

1. To check general health condition.

2. To help diagnose the diseases that affect one or more type of WBCs.

3. It is useful in confirming the diagnosis of specific neoplastic disorders (lymphoma and leukemia).

Procedure

A. Blood Smear Preparation

1. Put a drop of blood on the end of a clean glass slide use hematocrit tube.

2. Hold another slide (or cover slip) firmly in a horizontal position (an angle of about 30-45^{0}) just in front of a drop of blood.

3. Pull the horizontal slide somewhat back ward in order to make the drop of blood to run along the line of contact between the 2 slides.

4. Push the horizontal slide gently but firmly along the surface of the first slide to the far end.

5. Dry rapidly by waving in the air or using a blower.
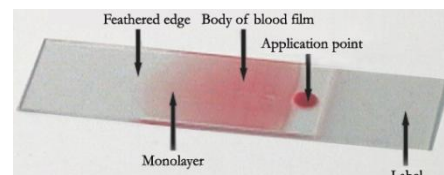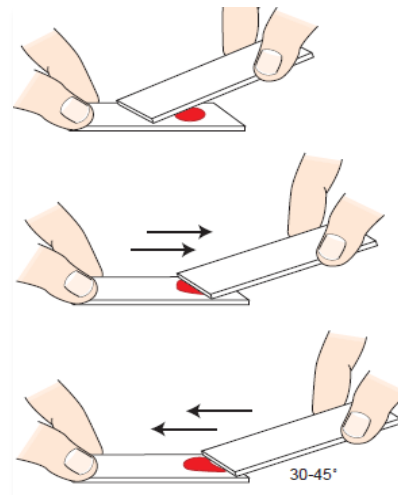
Types of the Blood Smear

1. Thin blood smear: It is mainly useful for detection of cell morphology and differential cell count.

2. Thick blood smear: It is most useful for detecting of blood parasites (especially malaria), because they examine a larger sample of blood, even if few numbers of parasites are available in the blood at the time the test. Additionally, the percentage of red blood cells that are infected (parasite density) and what type of parasites is present can be detected.
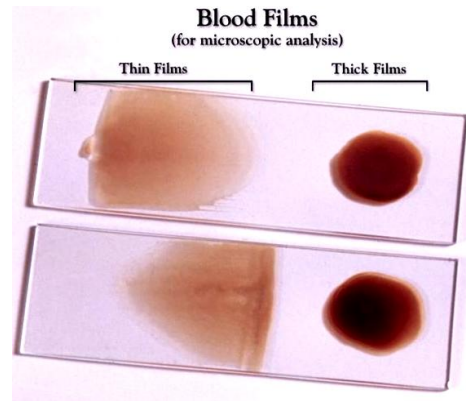
B. Staining of the blood smear

Different types of stains in current use

1. Wright Stain: It is a mixture of eosin (red) and methylene blue dyes, used primarily to stain blood and bone marrow aspirate smears.

2. Giemsa Stain: It is used in cytogenetics and for the histopathological diagnosis of malaria. Giemsa's solution is a mixture of methylene blue, eosin, and Azure B.

3. Wright-Giemsa Stain: It is a combination of Wright and Giemsa stains.

4. May-Grünwald Stain: It produces a more intense coloration, also takes a longer time to perform. May-Grünwald and Giemsa are stable for five years. The bottles must be kept closed. The advised storage temperature is 18 - 30°C.

5. Leishman Stain: It provides excellent staining quality. It is generally used to differentiate and identify leukocytes, malaria parasites, and trypanosomas.

Procedure of Giemsa Stain

1. Immerse the blood film in absolute methanol (99%) for 5 minutes to fix the film.

2. Drain the methanol and stand the slide upright to dry.

3. Immerse the slide in working solution (1: 10) for 15 minutes or (1:20) for 30 minutes.

4. Wash the slide with D.W. to remove excess stain.

5. Place it in an upright position to dry completely.

6. Mount with DPX, and put cover slip.

7. Examine the slide under oil immersion lens (HPF, 100X).

FIGURE A-2. Preparation of thin and thick blood films

1
Whenever possible, use separate slides for thick and thin films.

2
Thin film (a): Bring a clean spreader slide, held at a 45-degree angle, toward the drop of blood on the specimen slide.

3
Thin film (b): Wait until the blood spreads along the entire width of the spreader slide.

4
Thin film (c): While holding the spreader slide at the same angle, push it forward rapidly and smoothly.

5
Thick film: Using the corner of a clean spreader slide, spread the drop of blood in a circle the size of a dime (diameter 1–2 cm). Do not make the smear too thick or it will fall off the slide (you should be able to read newsprint through it).

6
Wait until the thin and thick films are completely dry. Fix the thin film with 100% (absolute) methanol. Do not fix the thick film.

7
If both the thin and thick films must be made on the same slide, fix only the thin film with 100% (absolute) methanol. Do not fix the thick film.

8
When the thin and thick films are completely dry, stain them. Thick smears might take ≥1–2 hours to dry. Protect unstained blood smears from excessive heat, moisture, and insects by storing in a covered box.

Examination of Stained Blood Smear

•   Inspect the smear under low power to note distribution of cells and select an area where the cells do not overlap.

•   Switch to oil immersion objective for further examination.

Feathered Edge   **Monolayer area**   **Body area**
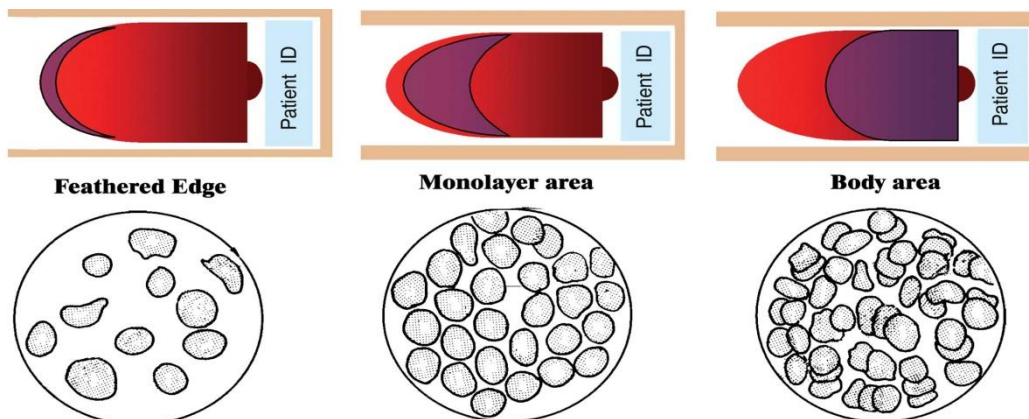
- The examination should starts at the thin end (tail part) of the smear.

- Fields selected for examination should be those in which erythrocytes are well separated and the leukocytes thinly spread.

- Meander system should be carried out as it avoids recounting of the same field.

- Avoid fields in which erythrocytes are stacked up or aggregated.

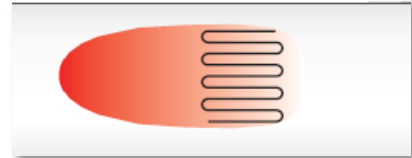- Enumerate at least 100 leukokytes and classify them according to their staining reactions, nuclear morphology and the characterization of any cytoplasmic granules that may occur.

- Tabulate the individual cells in columns on a prepared sheet of paper.

- Express the values of each cell as a percentage.

The Criteria of a Good Blood Smear

1. Smooth and an even appearance.

2. The smear has long straight borders.

3. The RBCs are distributed in a single layer in a major part of the smear.
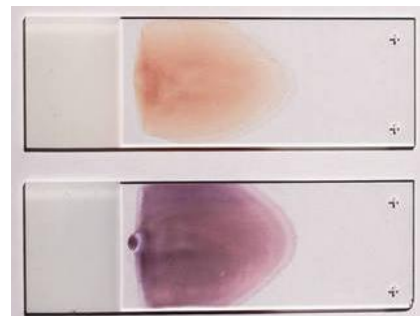
General Causes of Poor Staining

1. Buffer and tap water are of not proper PH.

2. Improper stain (wrong preparation and filtration).

3. Too thick smear.

4. Insufficient washing.

5. Too long fixation period.

6. Excessive staining time.

7. Not enough stain placed on the slide.

Cell Appearances after Staining

1. Erythrocytes: Pink-tan.

2. Leukocyte nuclear chromatin: Stains magenta.

3. Lymphocytes: Clear sky blue cytoplasm with red purple granules.

4. Monocytes: Mosaic of pink and pale blue cytoplasm with azure granules.

5. Neutrophils: Light purplish-pink or lavender granules in cytoplasm.

6. Eosinophils: Bright red or reddish-orange granules in cytoplasm.

7. Basophils: Deep purple and violet black granules in cytoplasm.

8. Platelets: Clearly demarcated red-purple granules in light blue cytoplasm.

General Cautions during Staining

1. The buffer must be of proper PH in order to provide the best differential staining (Buffer that is too alkaline causing the cells to become too blue, while too acidic buffer leads to little or no staining the nuclei of the cell).

2. Avoid excessive washing of the stained film as it will decolorize the film.

3. Do not dry films in an incubator or by heat as it will distort the film.

4. Air drying of the film should be done in horizontal position.

5. Both Wright and Giemsa stains are flammable; keep away from heat, sparks, or flames.

Classification of Leukocytes

- The circulating blood inside the vascular system composed of plasma and blood cells.

- The blood cells are RBCs, WBCs and platelets.

- Normal WBCs are divided into: polymorphonuclear leukocytes (or granulocytes) and mononuclear cells.

1. Polymorphonuclear Leukocytes

a. Neutrophils

Mature Neutrophils

Mature neutrophils have either a monolobular nucleus or its nucleus may have up to 5 lobes that are joined by thin strands giving it a segmented appearance. In properly stained blood film, neutrophils have faintly acidophilic cytoplasmic granule with deeply basophilic nucleus.

Band Neutrophils

-Absent or very low in normal peripheral blood.

-Its cytoplasm is less granular than mature form and nucleus is sausage-shaped, kidney, bean or coiled with no lobulation (indentation).

-An increased number of band cells are referred to as a left shift.

b. Eosinophils

-It is readily recognized in stained blood smears by the presence of numerous, small, circular, orange-reddish granules in the cytoplasm which may cause the cell to bulge.

-The nucleus is frequently non segmented and the cytoplasm is faint or sky blue which may have vacuoles.

- Their number in circulation fluctuates throughout the day, seasonally, and during menstruation. It rises in response to allergies, parasitic infections, collagen diseases, and disease of the spleen and central nervous system.
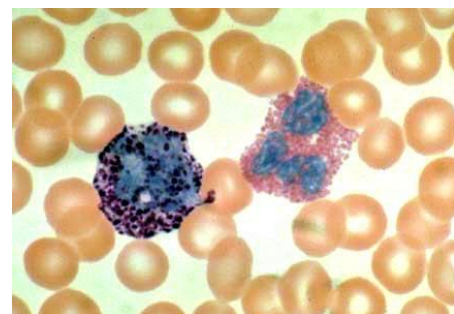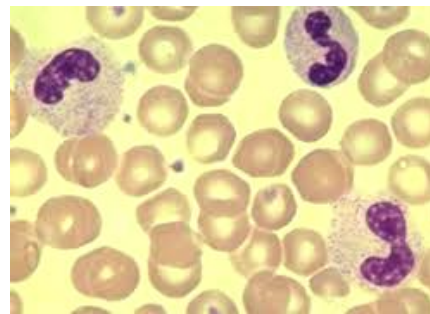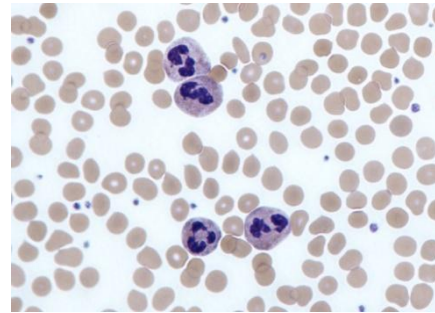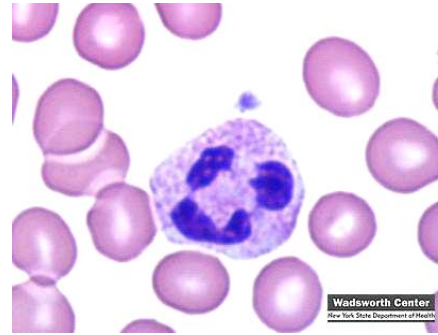
c. Basophils

-Rarely seen in normal peripheral blood smear.

-It occurs only rarely in the blood of the dog and cat but most commonly seen in horse.

-It is characteristically has purplish staining granules that scattered throughout the cytoplasm and over the surface of the nucleus.
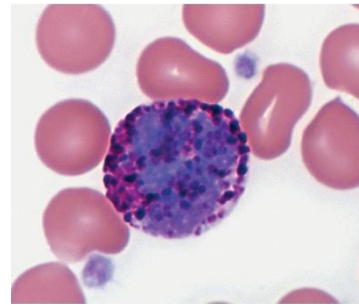
2. Mononuclear leukocytes

a. Lymphocytes

1. Small Lymphocytes

It is the commonest form that has a small size (less than 10 µm in diameter) with large, almost circular or slightly indented nucleus and a narrow peripheral zone of blue-stained cytoplasm. The cytoplasm may contain large, dark-blue or red (azurophilic) granules.
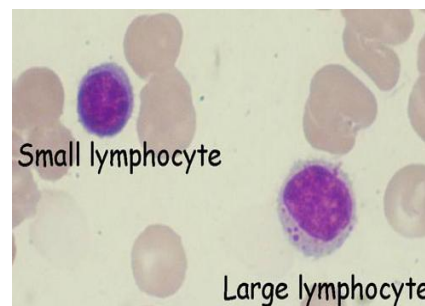
2. Large lymphocytes

It has proportionally more cytoplasm, which stains pale-blue and may contains small vacuoles, thus causing some confusion with monocytes.

b. Monocytes

-It is the largest cell in the leukocyte series.

-In stained blood smears; their nuclei are more varied morphologically, being oval, elliptical, kidney or horse-shoe shaped or even segmented.

-The cytoplasm is faintly granular, stains basophilic and may have a vacuolated or foamy appearance.

Normal WBC Values in Human

-Neutrophil: 45% to 65%

-Band neutrophil: 0% to 3%

-Eosinophil: 1% to 4%

-Basophil: 0.5% to 1%

-Lymphocyte: 20% to 40%

-Monocyte: 2% to 8%

Normal White Blood Count
(Average percentage values for an adult*)
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Laboratory 9: Blood (ABO System) and Rh Grouping Systems

Blood Grouping

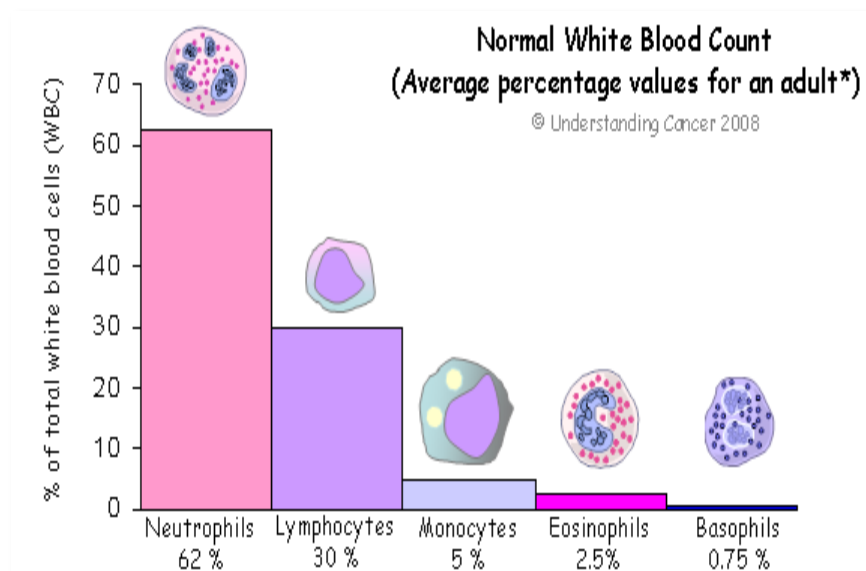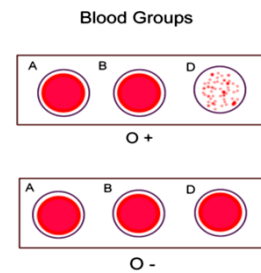Red blood cells of human and animals exhibit a number of polymorphic antigens on their surfaces correspond to allelic series at a number of loci referred to blood groups.

Benefits of Blood Grouping

Blood groups are of practical importance with regards to:

1. Blood transfusion.

2. Organ transplantation.

3. In a number of specific hemolytic diseases.

4. In Forensics.

The ABO system

- The ABO blood group system is the most important blood type system in human blood transfusion.

- It is characterized by three alleles IA, IB and IO that located on chromosome 9.

- IA and IB are dominant, while IO is recessive to both.

- The associated anti-A and anti-B antibodies are usually IgM antibodies, which are not able to pass through the placenta to the fetal blood circulation.
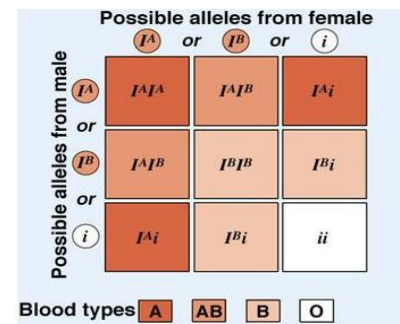
- Anti-A and anti-B antibodies are produced in the first years of life by sensitization to environmental substances, such as food, bacteria, and viruses.

- O-type individuals can produce IgG-type ABO antibodies.

- ABO blood types are also present in some other animals like rodents and apes.

Rh (Rho) Grouping

Rh is the most important blood group system after ABO in transfusion medicine.

Antigens of Rh Grouping

-It refers only to presence or absence of *antigen D* on the RBC surfaces that termed "D-positive" and "D- negative.

-Antibody produced through exposure to D antigen by transfusion or pregnancy.

Significance of Rh

-When the mother is Rh-negative and the father is Rh-positive, the fetus can inherit the Rh factor from the father.

-This makes the fetus Rh-positive too.

-Problems can arise when the fetus's blood has the Rh factor and the mother's blood does not.

-A mother who is Rh-negative may develop antibodies to an Rh-positive baby when a small amount of the baby's blood mixes with the mother's blood (at birth).

1. Rh+ father.

2. Rh− mother carrying her first Rh+ fetus. Rh antigens from the developing fetus can enter the mother's blood during delivery.

3. In response to the fetal Rh antigens, the mother will produce anti-Rh antibodies.

4. If the woman becomes pregnant with another Rh+ fetus, her anti-Rh antibodies will cross the placenta and damage fetal red blood cells.
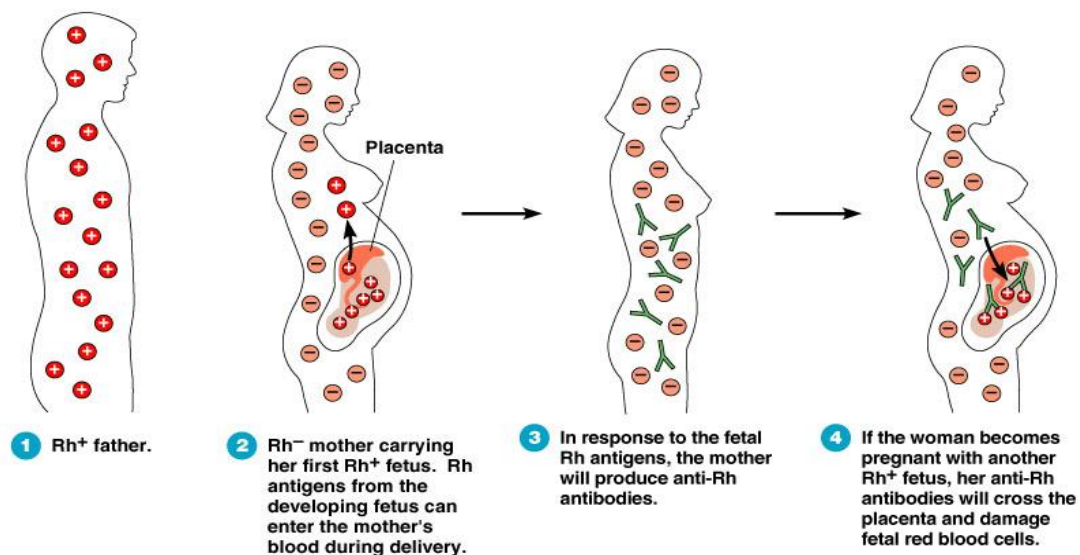
-The mother's body may make antibodies to the Rh antigens.

-The mother antibodies may cross the placenta and attack the baby's blood in the next pregnancy.

-Such an attack breaks down the fetus's RBCs and creating hemolytic anemia.

-It can become severe enough to cause serious illness, brain damage, or even death in the fetus or newborn.

-When any incompatibility is detected, the mother often receives an injection (IgG anti-D antibodies [Rho(D) Immune Globulin]) at 28 weeks gestation and at birth to avoid the development of antibodies toward the fetus.

Blood Transfusion

1. Blood Donation

Blood Type	Antigen (RBC Membrane)	Antibody (plasma)	Can Receive Blood from	Can Donate Blood to
A (40%)		Anti-B antibodies	A, O	A, AB
B (10%)		Anti-A antibodies	B, O	B, AB
AB (4%)		No antibodies	A, B, AB, O	AB
O (46%)		Both Anti- A and Anti-B antibodies	O	O, A, B, AB

-Group A can donate blood to A and AB.

-Group B can donate blood to B and AB.

-Group AB can donate blood to AB.

-Group O can donate blood to A, B, AB, and O.

2. Blood Receiving

-Group A can receive blood from A and O.

-Group B can receive blood from B and O.

-Group AB can receive blood from A, B, O and AB.

-Group O can receive blood from O.

Notes

1. Rh-negative can donate or receive blood only from Rh-negative.

2. Rh-posative can donate or receive blood only from Rh-posative.

Materials Needed for Blood and Rh Grouping

1. Glass slide or special card.

2. Glass rod or wooden stick.

3. Antiserum (Anti A, B and D).

Procedure of Blood (ABO System) and Rh Grouping

1. Divide a clean glass slide into 3compartments and label them A, B and D.

2. Put a small drop of blood in to each compartment.

3. Put small drop of anti A, anti B and anti D into the center of each compartment (near to the previous drop of blood).

4. Mix the blood with antisera at each compartment by using different wooden sticks (or glass rod).

5. Examine the reactions for the presence or absence of agglutination.
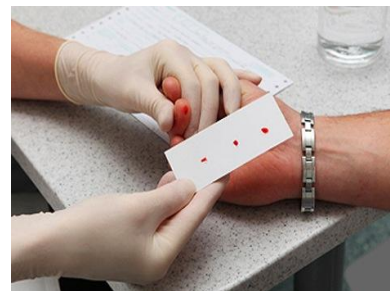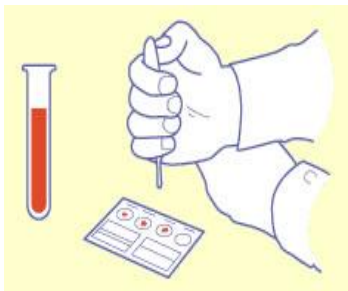
Blood group	Anti A	Anti B	Anti D
A	Agglutination	No agglutination	-
B	No agglutination	Agglutination	-
AB	Agglutination	Agglutination	-
O	No agglutination	No agglutination	-
Rh +	-	-	Agglutination
Rh -	-	-	No agglutination

Sources of Error

1. Contaminated reagents.

2. Reagent deterioration (expired or bad storage).

3. Use of wrong sera.

4. Autoagglutinins or abnormal serum proteins coating RBCs.

5. Incorrect anti-serum to cell ratio.

6. Using same stick to mix different blood samples.

Laboratory 10: Platelets Counting and Abnormalities

-Platelets or thrombocytes are smallest blood cells that are derived from megakaryocytes cytoplasmic fragmentation in the bone marrow.

-Platelets are found only in mammals as small fragments between the red cells containing small purple-staining granules.
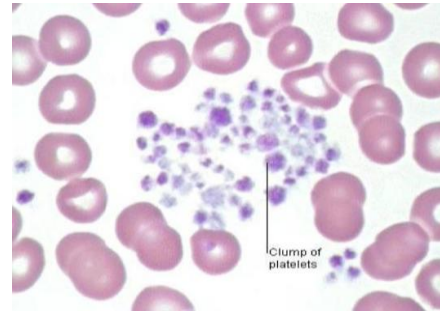
-EDTA anticoagulant minimizes platelet clumping.

-They have a tendency to extrude hair like filaments from their membranes especially during activation.

Morphology of Thrombocytes

-They are unnucleated (incapable of cell division or mitosis like RBC).

-They may be round, discoid, flat, spheroid, elongated, oval or rod shaped.

-The cytoplasm stained light blue hyalomere (clear) with red or purple granules.

Thrombocyte Counting

1. Manually by using a haemocytometer.

2. Automatically by using platelet analyzer (Coulter Counter).

Hemocytometery Method

Aims of the Test

-To investigate abnormal skin and mucosal bleeding which can occur when the platelet count is very low.

-Platelet counts are also performed when patients are being treated with cytotoxic drugs or other drugs which may cause thrombocytopenia.

Materials

-Hemocytometer counting chamber.

-1% Ammoium oxalate to hemolyzed erythrocytes.

-EDTA anticoagulated venous blood.

-Capillary blood should not be used because platelets clump as the blood is being collected.

Procedure

1. Pipette 990µl of 1% ammonium oxalate into a clean, dry test tube.

2. Add 10µl well mixed venous blood.

3. Leave at room temperature for 10 minutes to allow the platelets to settle down.

4. Use micropipette tip to take 20 µl from the prepared solution and the tip is touched to the side of the hemocytometer chamber (without any hesitation) till a fluid will run under the cover glass.

5. Mount the hemacytometer on the microscope and total number of cells in 1 large square on a corner (25 small squares) is determined under the high dry objective power (40X).

6. To avoid duplicate counting of a single cell, you must count only those cells that touch the lower and left boundaries. Platelets appear greenish, not refractile.

Calculation

Cells/mm^3 = $\underline{\text{Total number of cells counted x Reciprocal of dilution}}$

Number of squares counted x Area of each square x Depth of the solution

Example

Total number of cells= 230

Dilution 1:100

Number of squares counted:1

Area of each square: 1 mm^3

Depth of solution: 0.1mm

Cells/mm^3= $\underline{\quad 230 \text{X} 100 \quad}$

$\qquad$ 1 X 1 mm^2 x 0.1mm

= 230,000/mm^3 (µL) or 230 x 10^3/L

Normal Value

The normal number of platelets in the blood is 150,000 - 400,000 platelets/µl.

Cases of Increased Thrombocytes (Thrombocytosis)

1. Inflammation.

2. Hyposplenism (decreased function of the spleen).

3. Splenectomy.

4. Asplenia (absence of normal spleen function).

5. Iron deficiency anemia or hemorrhage.

6. Bacterial diseases, including pneumonia, sepsis, meningitis, urinary tract infections.

7. Osteosarcoma.

Cases of Decreased Thrombocytes (Thrombocytopenia)

1. Dehydration.

2. Vitamin B$_{12}$ deficiency.

3. Leukemia.

4. Liver failure.

5. Systemic viral or bacterial infection.

6. Dengue fever.

Laboratory 11: Reticulocyte Counting
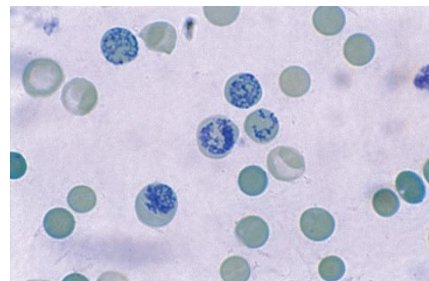
-Reticulocytes are immature red blood cells without nucleus.

-They develop and mature in the bone marrow, then circulate for about a day in the blood stream before developing into mature RBC.

-Reticulocytes appear slightly bluer and larger than other red cells when looked at with the normal Romanowsky stain.

-They are called reticulocytes because of a reticular (mesh-like) network of ribosomal RNA.

Reticulocyte Counting

Aims of the Test

1. To determine the functioning of bone marrow and responding adequately to the body's need.

2. To help detect and distinguish between different types of anemia.

3. To monitor response to treatment (such as that for iron-deficiency anemia).

4. To monitor bone marrow function following treatments (such as chemotherapy).

5. To monitor function following a bone marrow transplant.
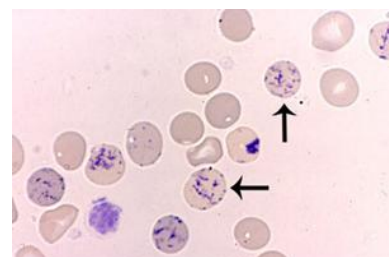
Method

New methylene blue staining.

Materials

New Methylene Blue Stain (NMB)

-It is a supra vital stain that used for the staining of immature red blood cells (reticulocytes).

-The stain makes precipitation of residual ribosomal RNA within the reticulocytes.

-As a result, the RNA appears as a reticulum within the stained reticulocytes.

Procedure

1. Place equal volumes of well mixed anticoagulated blood (usually 100 μL from each) and NMB into a small vial (This allows the reticulocytes adequate time to take up the stain).

2. Mix well and incubate at 37°C for 5 minutes in a water bath.

3. Make a smear and air dry.

4. Mount with DPX, cover slip and view.

Safety

NMB is toxic. Skin contact or inhalation should be avoided.

Calculation

Reticulocyte (%) = [Number of Reticulocytes ÷ Number of total RBC] X 100

Staining Result

Normal erythrocytes: stain light greenish-blue.

Reticulocytes: stain deep blue and are sharply outlined.

Normal Values

-Normal healthy young adults, reticulocytes comprise 0.3 - 1% of the total number of erythrocytes.

-In young infants, the percentage is 2 - 4 times higher than for young adults.

Sources of Error

1. Equal volumes of blood and stain give optimum staining conditions. An excess of blood causes the reticulum to understain. An excess of stain usually obscures the reticulum.

2. Crenated erythrocytes and rouleaux formation make an accurate count difficult to perform.

3. Stain precipitated on erythrocytes causes them to appear as reticulocytes

4. The dye solution should have adequate time to penetrate the cell and stain the reticulum.

Cases of Increased Reticulocytes (Reticulocytosis)

1. Bleeding: If an individual bleeds (hemorrhage), then the number of reticulocytes will rise a few days later in an attempt to compensate for the red cell loss.

2. Hemolytic anemia: In this condition, anemia is caused by increased destruction of RBCs. The bone marrow increases RBC production to compensate, resulting in a high reticulocyte count.

3. Hemolytic disease of the newborn.

Cases of Decreased Reticulocytes (Reticulocytopenia)

1. Iron deficiency anemia.

2. Pernicious anemia (folic acid deficiency).

3. A plastic anemia.

4. Radiation therapy.

5. Bone marrow failure (such as in case of infection or cancer).

6. Severe kidney disease (cause a low level of erythropoietin).

Laboratory 12: Erythrocyte Hemolysis Assessments and Direct Coomb's Test

Coomb's Test (The Antiglobulin Test)

It refers to clinical blood tests that used in immunohematology to find certain antibodies which cause autoimmune hemolysis of RBCs.
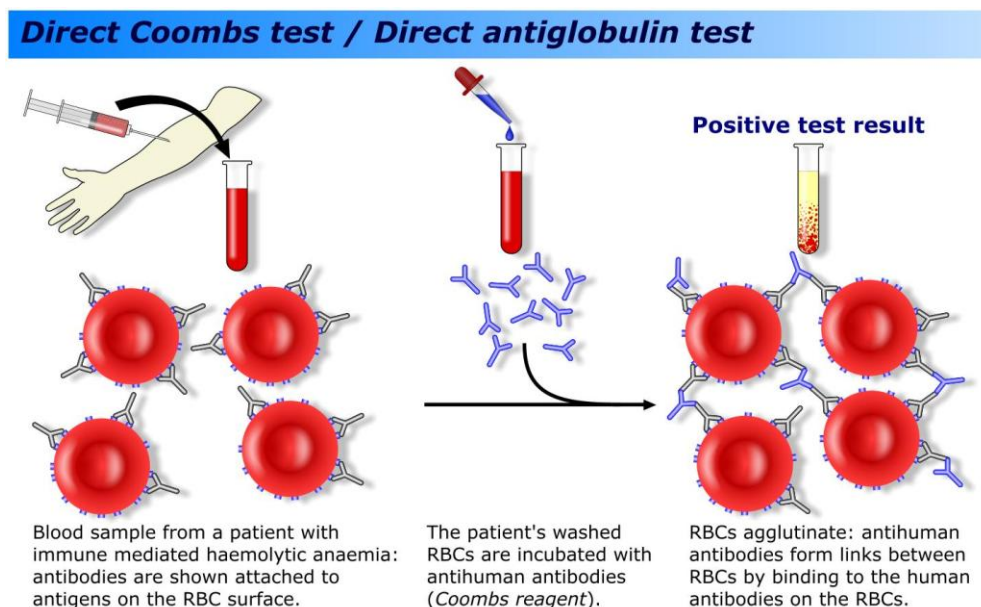
Types of Coomb's Test

1. Direct Coomb's Test

-The patient's cells, after careful washing are tested for sensitization that has occurred in vivo (inside the body).

-This test is done on the newborn's blood sample, usually in the setting of a newborn with jaundice.

-The test is looking for foreign antibodies that are already adhered to the infant's RBCs, a potential cause of hemolysis.

-A positive DAT may also be caused by the presence of allo-antibodies (e.g. owing to a delayed hemolytic transfusion reaction), so details of any transfusion in the past months must be checked for.

Direct Coombs test / Direct antiglobulin test

Positive test result

Blood sample from a patient with immune mediated haemolytic anaemia: antibodies are shown attached to antigens on the RBC surface.

The patient's washed RBCs are incubated with antihuman antibodies (*Coombs reagent*).

RBCs agglutinate: antihuman antibodies form links between RBCs by binding to the human antibodies on the RBCs.

2. Indirect Coomb's Test

Normal RBCs are incubated with a serum suspected of containing an antibody and subsequently tested, after washing for in vitro-bound antibody.

Aims of the Test

-To detect incomplete Rh antibodies.

-IgG antibodies sensitizing RBCs but cannot agglutinate RBCs suspended in saline, while IgM antibodies agglutinate saline-suspended RBCs completely.

Principle of the Test

-In certain diseases or conditions, an individual's blood may contain IgG antibodies that can specifically bind to antigens on the RBC surface membrane.

-RBCs coated with complement or IgG antibodies do not agglutinate directly when centrifuged. These cells are said to be sensitized with IgG or complement.

-In order for agglutination to occur an additional antibody, which reacts with the Fc portion of the IgG antibody, or with the C3b or C3d component of complement, must be added to the system.

-Because antibodies are gamma globulins, they can form bridges between RBCs sensitized with antibodies and cause them to agglutinate.

Materials

Phosphate Buffered Saline (PBS): NaCl 0.9%, pH 7.0 ± 0.2 at 22ºC ± 1ºC.

IgG sensitized red cells

Inert antibody serum

Weak anti-D

Water bath or dry heat incubator equilibrated to 37ºC ± 2ºC.

Coomb's cell washer

Low Ionic Strength Solution (LISS)

Procedure

1. Prepare 5% cell saline suspension of the cells to be tested.
2. Label 3 tubes as T, PC and NC. In the tube labeled as T (Test), take 2 drops of 5% saline cell suspension to be tested.

3. In a test tube, labeled as PC (Positive control), take 1 drop of anti D sera and 1 drop of Rh +ve pooled cells.

4. In a test tube, labeled as NC (Negative control), take 1 drop of normal saline and one drop of Rh +ve pooled cells.

5. Add 2 drops of Anti human globulin to each of the tubes.

6. Mix well and centrifuge for 1 minute at 1500 rpm.

7. Resuspend the cells by gentle agitation and examine macroscopically and microscopically for agglutination.

When the Test is Required?

When there is an evidence of anemia or hemolysis.
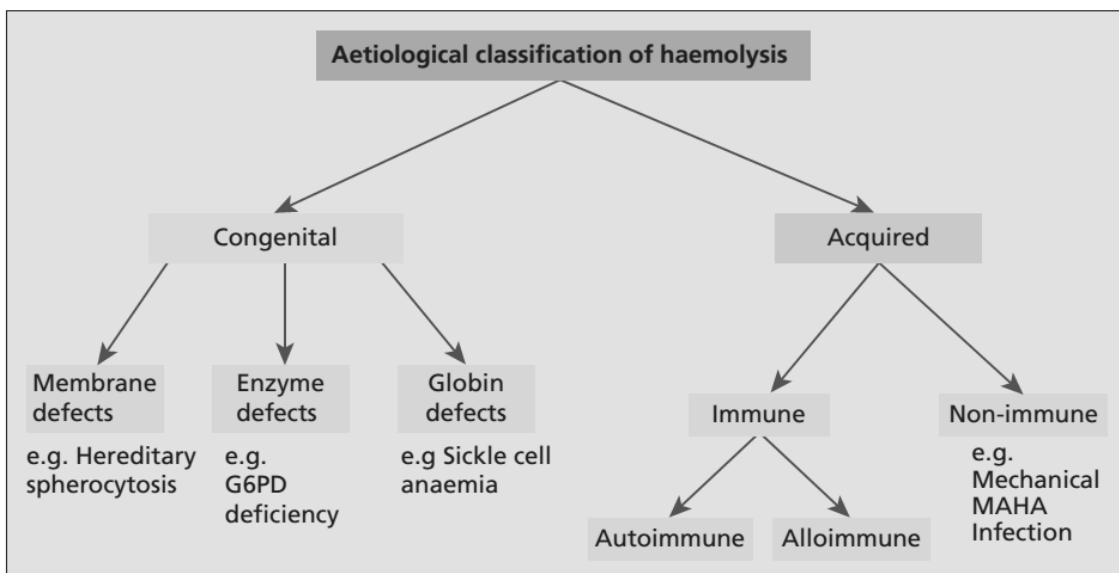
Appendix: Normal Reference Ranges

Red blood cell count
Men	$5.0 \pm 0.5 \times 10^{12}$/l
Women	$4.3 \pm 0.5 \times 10^{12}$/l

Haemoglobin concentration[a]
Men	150 ± 20 g/l
Women	135 ± 15 g/l

Packed cell volume (PCV) or Haematocrit (Hct)
Men	0.45 ± 0.05 (l/l)
Women	0.41 ± 0.05 (l/l)

Mean cell volume (MCV)
Men and women	92 ± 9 fl

Mean cell haemoglobin (MCH)
Men and women	29.5 ± 2.5 pg

Mean cell haemoglobin concentration (MCHC)
Men and women	330 ± 15 g/l

Red cell distribution width (RDW)
As coefficient of variation (CV)	$12.8 \pm 1.2\%$
As standard deviation (SD)	42.5 ± 3.5 fl

Red cell diameter (mean values)
Dry films	6.7–7.7 µm

White blood cell count	$4.0–10.0 \times 10^9$/l
Differential white cell count	
Neutrophils	$2.0–7.0 \times 10^9$/l (40–80%)
Lymphocytes	$1.0–3.0 \times 10^9$/l (20–40%)
Monocytes	$0.2–1.0 \times 10^9$/l (2–10%)
Eosinophils	$0.02–0.5 \times 10^9$/l (1–6%)
Basophils	$0.02–0.1 \times 10^9$/l (<1–2%)

Lymphocyte subsets (approximations from ranges in published data)
CD3	$0.6–2.5 \times 10^9$/l (60–85%)
CD4	$0.4–1.5 \times 10^9$/l (30–50%)
CD8	$0.2–1.1 \times 10^9$/l (10–35%)
CD4/CD8 ratio	0.7–3.5

Blood volume (normalized to 'ideal weight')
Red cell volume
Men	30 ± 5 ml/kg
Women	25 ± 5 ml/kg
Plasma volume	45 ± 5 ml/kg
Total blood volume	70 ± 10 ml/kg
Red cell lifespan	120 ± 30 days

Serum iron	
Men and women	10–30 µmol/l (0.6–1.7 mg/l)
Total iron-binding capacity	47–70 µmol/l (2.5–4.0 mg/l)
Transferrin saturation	16–50%
Serum ferritin concentration	
Men	15–300 µg/l (median 100 µg/l)
Women	15–200 µg/l (median 40 µg/l)

HbA$_2$	2.2–3.5%
HbF	<1.0%
Methaemoglobin	<2.0%

Bleeding time[b]	
Ivy's method	2–7 min
Template method	2.5–9.5 min
Thrombin time	15–19 s