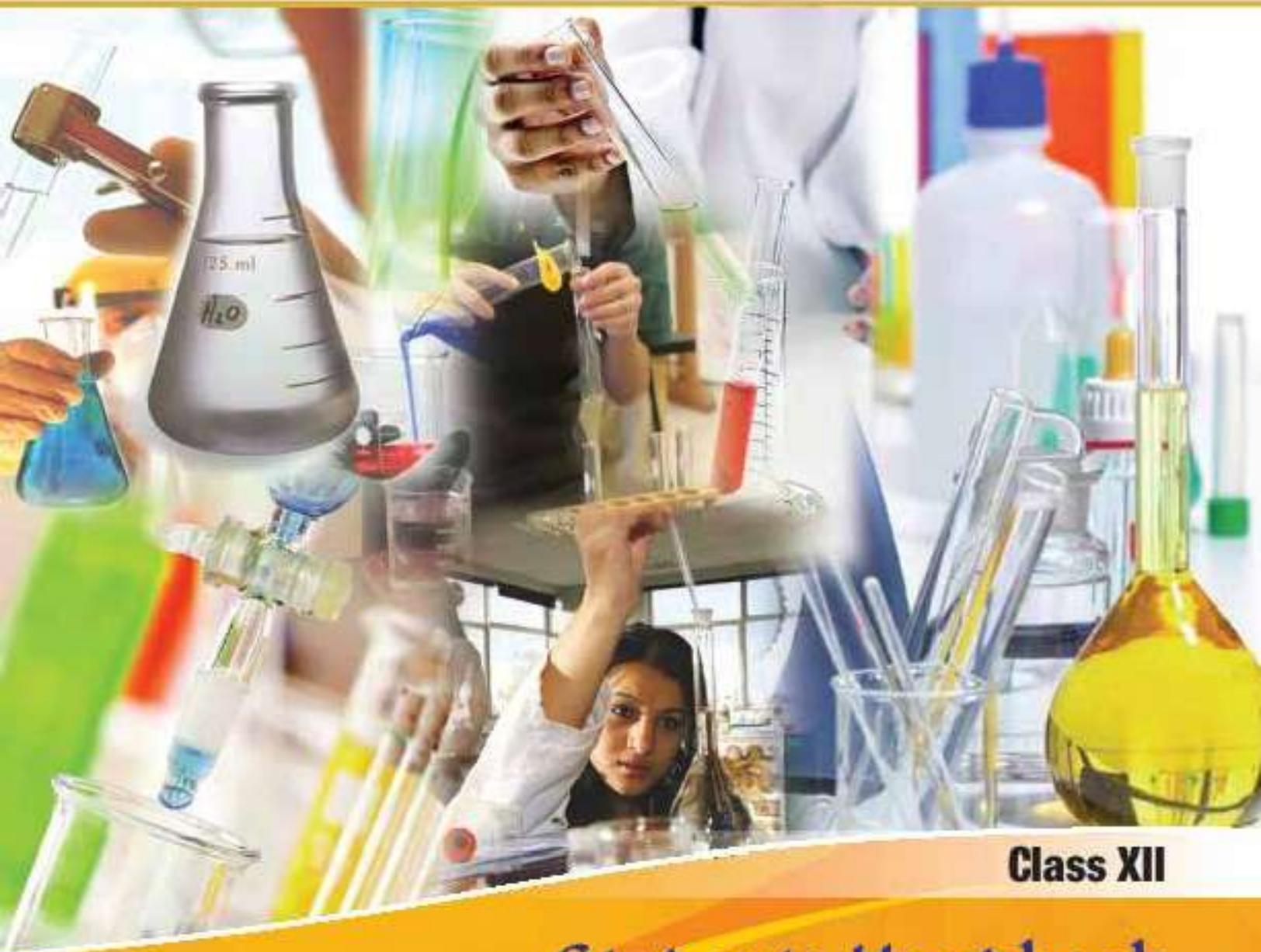


CBSE STUDY MATERIAL 2019



# LABORATORY MEDICINE-II



**Class XII**

## Students Handbook



**Central Board of Secondary Education**

Shiksha Kendra, 2, Community Centre, Preet Vihar, Delhi-110301

## Laboratory Medicine - II

Student Handbook, Class-XII

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## भारत का संविधान

### उद्देशिका

हम, भारत के लोग, भारत को एक सम्पूर्ण प्रभुत्व-संपन्न समाजवादी पंथनिरपेक्ष लोकतंत्रात्मक गणराज्य बनाने के लिए, तथा उसके समस्त नागरिकों को:

सामाजिक, आर्थिक और राजनैतिक न्याय,  
विचार, अभिव्यक्ति, विश्वास, धर्म

और उपासना की स्वतंत्रता,

प्रतिष्ठा और अवसर की समता

प्राप्त कराने के लिए

तथा उन सब में व्यक्ति की गरिमा

और राष्ट्र की एकता और अखंडता

गुनिश्चिन करने वाली बंधुता बढ़ाने के लिए

दृढ़संकल्प होकर अपनी इस संविधान सभा में आज तारीख 26 नवम्बर, 1949 ई० को एतद्वारा इस संविधान को अंगीकृत, अधिनियमित और आत्मार्पित करते हैं।

1. संविधान ( बचपत्तीसवां संशोधन ) अधिनियम, 1976 की धारा 2 द्वारा ( 3.1.1977 ) से " प्रभुत्व संपन्न लोकतंत्रात्मक गणराज्य " के स्थान पर प्रतिस्थापित।
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### भाग 4 क

## मूल कर्तव्य

51 क. मूल कर्तव्य - भारत के प्रत्येक नागरिक का यह कर्तव्य होगा कि वह -

- (क) संविधान का पालन करे और उसके आदर्शों, संस्थाओं, राष्ट्रध्वज और राष्ट्रगान का आदर करे;
- (ख) स्वतंत्रता के लिए हमारे राष्ट्रीय आंदोलन को प्रेरित करने वाले उच्च आदर्शों को हृदय में संजोए रखे और उनका पालन करे;
- (ग) भारत की प्रभुता, एकता और अखंडता की रक्षा करे और उसे अक्षुण्ण रखे;
- (घ) श्रेष्ठ सेवा की रक्षा करे और आह्वान किए जाने पर राष्ट्र सेवा संघा करे;
- (ङ) भारत के सभी लोगों में समरसता और समान भ्रातृत्व की भावना का निर्माण करे जो धर्म, भाषा और प्रदेश या वर्ग पर आधारित सभी भेदभाव से परे हो, ऐसी प्रथाओं का त्याग करे जो स्त्रियों के सम्मान के विरुद्ध हैं;
- (च) हमारी सामाजिक संस्कृति की गौरवशाली परंपरा का महत्त्व समझे और उसका परिरक्षण करे;
- (छ) प्राकृतिक पर्यावरण की जिसके अंतर्गत वन, झील, नदी, और वन्य जीव हैं, रक्षा करे और उसका संवर्धन करे तथा प्राणी मात्र के प्रति दयाभाव रखे;
- (ज) वैज्ञानिक दृष्टिकोण, मानववाद और जनार्जन तथा सुधार की भावना का विकास करे;
- (झ) सार्वजनिक संपत्ति को सुरक्षित रखे और हिंसा से दूर रहे;
- (ञ) व्यक्तिगत और सामूहिक गतिविधियों के सभी क्षेत्रों में उत्कर्ष की ओर बढ़ने का सतत प्रयास करे जिससे राष्ट्र निरंतर बढ़ते हुए प्रयत्न और उपलब्धि की नई उंचाइयों को छू ले;
- (ट) यदि माता-पिता या संरक्षक हैं, छह वर्ष से चौदह वर्ष तक की आयु वाले अपने, यथास्थिति, बालक या प्रतिपाल्य के लिये शिक्षा के अवसर प्रदान करे।

1. संविधान ( छयासीवां संशोधन ) अधिनियम, 2002 की धारा 4 द्वारा प्रतिस्थापित।

## THE CONSTITUTION OF INDIA

### PREAMBLE

**WE, THE PEOPLE OF INDIA**, having solemnly resolved to constitute India into a '**SOVEREIGN SOCIALIST SECULAR DEMOCRATIC REPUBLIC**' and to secure to all its citizens :

**JUSTICE**, social, economic and political;

**LIBERTY** of thought, expression, belief, faith and worship;

**EQUALITY** of status and of opportunity; and to promote among them all

**FRATERNITY** assuring the dignity of the individual and the<sup>2</sup> unity and integrity of the Nation;

**IN OUR CONSTITUENT ASSEMBLY** this twenty-sixth day of November, 1949, do **HEREBY ADOPT, ENACT AND GIVE TO OURSELVES THIS CONSTITUTION.**

---

1. Subs. by the Constitution (Forty-Second Amendment) Act, 1976, sec. 2, for "Sovereign Democratic Republic" (w.e.f. 3.1.1977)

2. Subs. by the Constitution (Forty-Second Amendment) Act, 1976, sec. 2, for "unity of the Nation" (w.e.f. 3.1.1977)

---

## THE CONSTITUTION OF INDIA

### Chapter IV A

### FUNDAMENTAL DUTIES

#### ARTICLE 51A

**Fundamental Duties** - It shall be the duty of every citizen of India-

- (a) to abide by the Constitution and respect its ideals and institutions, the National Flag and the National Anthem;
- (b) to cherish and follow the noble ideals which inspired our national struggle for freedom;
- (c) to uphold and protect the sovereignty, unity and integrity of India;
- (d) to defend the country and render national service when called upon to do so;
- (e) to promote harmony and the spirit of common brotherhood amongst all the people of India transcending religious, linguistic and regional or sectional diversities; to renounce practices derogatory to the dignity of women;
- (f) to value and preserve the rich heritage of our composite culture;
- (g) to protect and improve the natural environment including forests, lakes, rivers, wild life and to have compassion for living creatures;
- (h) to develop the scientific temper, humanism and the spirit of inquiry and reform;
- (i) to safeguard public property and to abjure violence;
- (j) to strive towards excellence in all spheres of individual and collective activity so that the nation constantly rises to higher levels of endeavour and achievement;
- <sup>1</sup>(k) who is a parent or guardian to provide opportunities for education to his/her child or, as the case may be, ward between age of 6 and 14 years.

---

1. Subs. by the Constitution (Eighty - Sixth Amendment) Act, 2002

## *Preface*

I am very pleased to present the first edition of the text book in for the laboratory part of medical diagnostics. This is a new vocational course from Central board of Secondary Education. This is a part of the dream project of our Prime minister Mr. Narendra Modi

For skilling India. This is to ensure that all students who pass their 12th board examinations shall have the capability to get gainful employment because of their skill set.

This book has been written by Competent persons actively working in the various field of laboratory medicine -which includes histopathology, cytology, hematology, clinical pathology, microbiology, blood banking etc. They are professors, writers, practising doctors and academicians.

This book shall have contents that shall cover the complete course curriculum for classes 11th to 12th for the areas of Medical Diagnostics. The writeup of the book is fairly simple and shall help the student update his knowledge in the subject including all recent developments. He shall be able to self judge /asses his own competency through a set of questions given for self assessment.

I would like to thank CBSE vocational Unit, who had been the driving force behind the development of this book. who has pains taxingly devoted so much of her time in ensuring that it comes along in this fine form. My team of subject expert specially to mention from Safdarjung Hospitals. The current text has been prepared keeping in view the current requirements of the students and the latest updates in the relevant areas in a concise manner using simple language for increasing the comprehension.

Constructive and helpful suggestions from readers for the improvement of the book are welcome.

Chairman, CBSE

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## HAEMATOLOGY LAB (Procen & Investigation)

### Overview

- 3.1 Introduction
- 3.2 Haematology Lab Instruments
- 3.3 Collection of Blood samples
- 3.4 Preparation of Blood smears
- 3.5 Reagents - Preparation and their uses
- 3.6 Staining methods
- 3.7 Measurements or Quantitative Analysis
- 3.8 Anaemia
- 3.9 Haemostasis
- 3.10 Bone Marrow Aspiration / Biopsy
- 3.11 Lab safety

### 3.1 INTRODUCTION

The Haematology laboratory was started few centuries ago when blood cells were measured, counted and examined manually with the aid of stains and microscope. Now a day however blood samples are commonly analyzed by multi parameter, automated analysers. Automation has increased precision and accuracy in the identification, classification and counting of cells

Laboratory investigation of hemostasis has also advanced significantly. From the earlier evaluation which included platelet count, bleeding time etc., now it has moved on to include Prothrombin Time with INR, Partial Thromboplastin Time, Thrombin Time, Fibrinogen, individual coagulation factor assays, platelet function study on automated instruments.

#### Test performed in Haematology lab includes.

1. CBC - HB, TLC, DLC, RBC, PCV, PLATELET COUNT, MCV, MCH, MCHC
2. Erythrocyte Sedimentation Rate (ESR)
3. Peripheral smear for morphology
4. Bone Marrow mororphological study
5. Coagulation study



6. Immuno flowcytometric analysis e.g. cell identification in leukemia.
7. Other tests - LE cell, test for haemolytic anemia(e.g. Osmotic fragility test)

## 3.2 HEMATOLOGY LAB INSTRUMENTS

### (A) CENTRIFUGE :

This instrument is used to separate a solution or mixture into sediment and supernatant by using required speed. Some precautions while using it are.

- a) The buckets should be balanced equally with correct weight and size of the tubes .
- b) Centrifuge should always be covered when in use.
- c) It should be kept on firm and hard base.



### (B) MICROSCOPE :

This instrument helps us to examine tiny objects which cannot be visualized with the naked eye. It is a delicate instrument and needs utmost care.

- a) Cleaning of objective and eyepiece should be done regularly and they should be kept free from dust. The optical part is cleaned to remove grease using soft cloth or lens paper.
- b) Hold the microscope firmly while moving it to prevent the lenses from dropping down.
- c) Exposure to sunlight should be avoided and it should be kept at room temperature.
- d) After one uses oil immersion, one must always clean the oil from the objective.



### (C) AUTOMATED CELL COUNTERS :

These can be semiautomated or fully automated and are of two types (3 part and 5 part)

These are multichannel instruments used for cell counting and are based on the principles of electrical impedance, light scattering and flowcytometry. The cell counts in blood include RBC, WBC and PLATELETS, along with measurement of Hemoglobin and RBC indices. Sophisticated instruments can also detect abnormal cells.

**Proper care and handling by trained staff is mandatory which includes:**

- a) A quality control programme which is run everyday



- b) Annual calibration and regular decontamination by the manufacturing company.
- c) Daily maintenance

## COAGULATION ANALYSER

Coagulation tests like Prothrombin time, thromboplastin time, thrombin time, fibrinogen etc and coagulation factor assays are performed on these analysers Maintenance of instrument requires cleaning, daily Quality Control runs, maintaining optimum temperatures for room and instrument.

### Exercise

1. What are the precautions one should take while using a centrifuge?
2. What is the principle behind automated cell counters?

## 3.3 COLLECTION OF BLOOD SAMPLES

### 3.3.1 Anticoagulants

Anticoagulants are chemicals which when mixed with blood prevent clotting of blood. This is important since whole blood (or unclotted blood) is required for many investigations.

It would be helpful to revise the clotting mechanism briefly before we study about the various anticoagulants. Thromboplastin released in blood converts prothrombin to thrombin. This conversion also requires the presence of calcium ions. The thrombin so formed, then further converts fibrinogen (soluble) into fibrin clot (insoluble). This reaction too requires the presence of calcium ions. With this overview, we can now study the various anticoagulants, their mechanism of action and their uses.

#### 1. E.D.T.A (ethylenediaminetetra-acetic acid)

EDTA acts by chelating calcium molecules in blood. Potassium, sodium, and Lithium salts of EDTA can be used. However the recommended salt is dipotassium salt at a concentration of  $1.5 \pm 0.25$  mg /ml of blood. Tripotassium salt produces some changes in RBC parameters like an increase in MCV and a decrease in PCV after some time of keeping blood. Such changes are negligible with dipotassium salt. EDTA in excess of 2mg/ml may produce changes in RBCs irrespective of the salt used. An increased MCHC and a decreased PCV (by manual method ) may be seen. Excessive EDTA may also lead to a spuriously high platelet count (platelets may swell and then disintegrate into fragments which are counted as platelets)

#### 2. TRISODIUM CITRATE

This anticoagulant is best used for coagulation studies in 1: 9 ratios (1 vol of sodium citrate



(32g/l) to 9 vol of blood). For ESR (Westergren's method), 1 vol of sodium citrate solution is added to 4 vol of blood.

### 3. Heparin

One may use lithium or sodium salt of heparin for gas analysis and biochemistry test. Chance of RBC lysis is minimum with this anticoagulant and hence it is best used for osmotic fragility test and immunophenotyping. However there are certain disadvantages with heparin-it gives the background a faint blue colouration in Romanowsky stained smears particularly when abnormal proteins are present. It also causes clumping of leucocytes and platelets and so can lead to their erroneous estimation.

**4. Double oxalates** - These are a combination of ammonium (3 parts) and potassium oxalate (2 parts).

Their anticoagulant action is due to their capacity to precipitate out calcium as insoluble oxalate. This anticoagulant is not generally used for routine haematology investigations.

### 3.3.2 Specimen Collection:

Proper blood sample collection is the first step to ensure reliable and accurate results from clinical laboratory testing.

#### Methods:

1. By Venipuncture
2. By Skin puncture
3. From indwelling catheters

#### 3.3.2.1 Venipuncture:

The most common technique used to obtain a blood specimen is venipuncture. There are two ways to collect blood.

1. Syringe method.
2. Evacuated tube collection system.

The patient is first identified by name, OP/IP number or any other unique ID number.

The veins of the antecubital fossa are usually selected for routine venipuncture.

**3.3.2.2 Tourniquet application:** It should never be left for more than 1 minute. For prolonged application re- apply tourniquet after the site has been cleaned and just prior to insertion of needle.



### 3.3.2.3 Cleansing the venipuncture site:

The site is cleansed thoroughly with 70% isopropanol from inside out. Dry the area with sterile gauze or allow it to air dry.

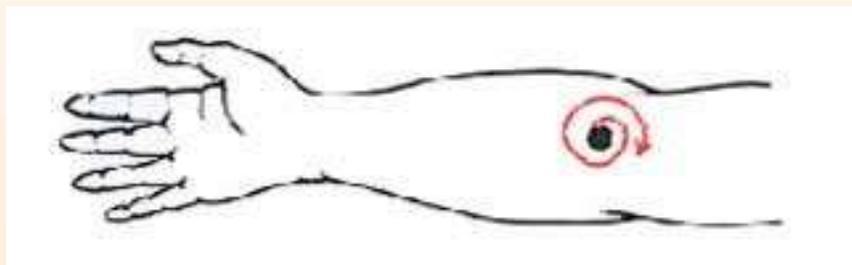


Fig. a

### COLLECTION OF BLOOD:

When the tourniquet is tightened, veins become prominent. Tourniquet should not be applied for prolonged period as this could lead to haemoconcentration. If the veins are not visible the patient is asked to exercise the fingers or the forearm by flexion and extension. Thumb of left hand is placed over the vein just below the point of entrance and slight traction is applied to fix the vein. The syringe is taken in the right hand and the needle with the bevel uppermost is inserted at an angle of about  $30^\circ$  to the skin and pushed in firmly and steadily, care being taken not to pass right through the vein to avoid haematoma formation. When the needle has entered the vein, a slight pull on the piston is applied to draw blood into the syringe. When the necessary amount has been withdrawn, the tourniquet is released and the needle is quickly withdrawn. A piece of cotton wool is placed over the puncture and pressure is applied over it. The patient is then asked to keep the pressure for a while to prevent bleeding from the wound. The blood collected is immediately transferred to an appropriate container after first removing the needle from the syringe. When collecting in multiple tubes, correct order is very important. The following order is recommended.

- [1] Sterile blood culture tubes.
- [2] Tubes containing anticoagulants [Collecting Citrated specimens first followed by Heparin, EDTA and Oxalate / Fluoride]
- [3] Non additive tubes.



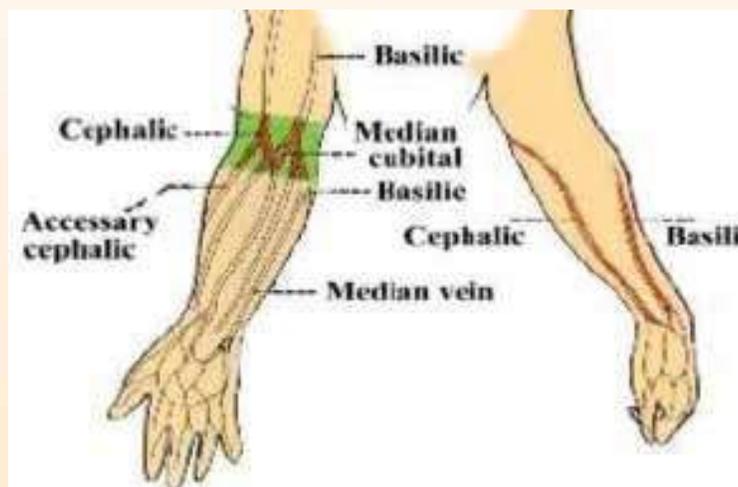


Fig b



Fig c

## Evacuated Tube collection system

Collection of samples in evacuated tube is becoming popular now a day. One requires glass or plastic tube under defined vacuum, needle holder and a needle for such a system. The tubes are available with and without anticoagulant. The rear end of the needle can pierce the cap of the evacuated tube and multiple tubes can be filled one after other.

## 2. Skin puncture:

It may sometimes be difficult to obtain venous blood sample. (e.g. in obese and in newborns). In such cases skin puncture can be done and capillary blood collected. Usually only small amounts of blood can be collected by this technique and so a limited number of tests can be performed. Preferred method of blood collection still remains venous blood.

Finger or ear lobe is usually selected for sample collection. Palmer surface of distal digits of third or fourth finger, 2 - 3 mm lateral to the nail-bed is selected for finger prick. In case of infants one can select the area over the heel or the great toe. The central planter area and the posterior curvature should not be selected as there is risk of injury or infection to the underlying bones. A stab is made by a sterile lancet after cleaning the selected site (by alcohol or spirit). The first drop of blood is wiped away and thereafter blood is collected. There should be free flow of blood. One should remember not to squeeze the cut or else erroneous results will be obtained. After adequate sample has been collected, a dry cotton swab is pressed on the cut till bleeding stops.



Hb, PCV and RBC are higher in capillary blood than venous blood, however platelet count can be lower in capillary blood (as platelets may adhere to the skin puncture site.)

### 3. Collection from Indwelling catheters:

This method is used only in certain special situations.

Collection of blood in a patient who has an I/V line When an intravenous solution is being administered in a patient's arm, blood should be drawn from the opposite arm. If an intravenous infusion is running in both arms, samples may be drawn after the intravenous infusion is turned off for at least two minutes before venipuncture and applying the tourniquet below the intravenous infusion site.

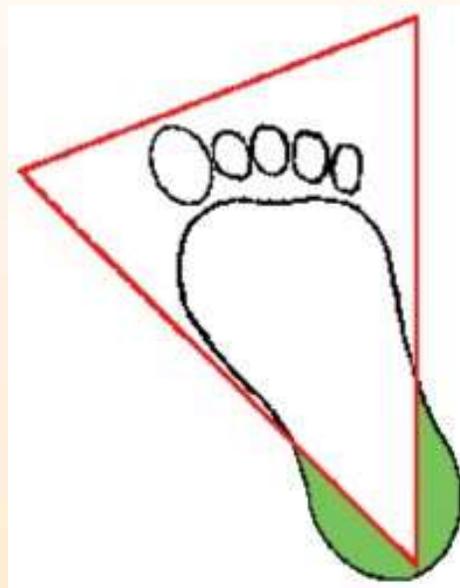


Fig d

### Advantage of Evacuated Tube system

1. Adequate sample is ensured (vacuum in the tube controls the amount of blood entering the tube.)
2. Correct ratio of anticoagulant to blood is ensured.
3. This is a closed system and spillage of blood and hence any Bio-hazard is thus avoided.
4. Large amounts of blood (in multiple tubes) can be collected with minimum discomfort to patient.

### Exercise

1. What is an anti-coagulant?
2. Which is the most commonly used anticoagulant for routine haematological Studies?
3. Which anticoagulant is commonly used for osmotic fragility test and why?
4. What are the disadvantages of using heparin as an anticoagulant?
5. What is the preferred site for routine venipuncture?
6. Why should the tourniquet not be left for more than 1 minute during sample collection?
7. Explain the process of sample collection by venipuncture?
8. Enlist the order of sample collection tubes when transferring blood after venipuncture?
9. Enumerate few advantages of using evacuated tube system for blood collection.



## 3.4 PREPARATION OF BLOOD SMEARS

Blood films should be made as early as possible after collection of blood sample.

### METHODS OF MAKING A BLOOD FILM;

EDTA blood or fresh blood without any anticoagulant can be used for making blood films. One should make blood films as soon as possible after collection of blood. Clean glass slides (75mm x 25mm and around 1 mm in thickness) are taken and a drop of blood is put on the slide about 1cm from one end in the centre line of slide. A spreader is then placed in front of the drop. At an angle of 30° to the slide. Spreader is then moved back so that the drop of blood spreads out along the line of contact. Next the Spreader is moved forward with steady movement so that a film about 3 cm in length is made.

Labeling of slides is then done.

### Characteristics of a Proper Wedge Film:

The well - prepared blood film should have the following characteristic:

1. Two third the length of the glass slide should be covered by the film.
2. Film should be narrower than the slide for better examination of side edges.
3. A homogeneous spread should be displayed with a gradual transition from thick to thin areas and with no deformities.
4. It should end in a slightly curved feathered end.
5. The film should be thin to allow proper fixation during the staining procedure. Thick areas appear dark green or gray or are washed off during staining.
6. It should contain at least 10 low - power fields in which 50% of the erythrocytes do not overlap. Single erythrocytes should have a well preserved central pale area.

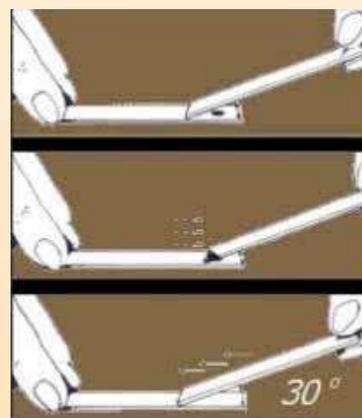


Fig. e

### Exercise

1. Describe method of making a good blood smear?
2. What are the characteristic of a good wedge film?



## 3.5 REAGENTS - PREPARATION AND THEIR USES

'Romanowsky' stains are made up of combinations of acid and basic dyes and various types are available e.g. Leishman's, Giemsa's and Wright's Jenner's stains. Methylene blue is used as the basic stain, and eosin as the acid stain. Some stains use toluidine blue and azure II.

### Leishman's stain:

#### Preparation:

0.15gms of powdered stain is dissolved in 100ml of acetone free methyl alcohol. Crystals are grounded to powder in a mortar and alcohol is added a little at a time until the stain is dissolved. The stain can be used in an hour but improves with time provided it is kept in a glass stoppered bottle.

### Giemsa Stain:

#### Preparation:

This stain is prepared from Giemsa Powder.

Giemsa Powder: 0.3 gms

Glycerine : 25.0ml

Acetone - free Methyl alcohol: 25.0ml.

The solution is diluted before use by adding 1 ml. to 10 ml. [or 1 drop/ml] of dist. water.

### Wright's Stain:

#### Preparation:

Dissolve 0.2gms of stain powder in 100ml of Acetone free methyl Alcohol. The solution is allowed to stand for few days before using.

Field's stain: Usually used for staining thick blood film. Thick blood film is required to detect parasites like microfilaria and malaria parasite.

### Preparation:

#### Solution A

Methylene blue 0.8gms

Azure 0.5gms

Disodium Hydrogen phosphate



[Anhydrous] 5.0gms

Pot. Dihydrogen phosphate

[Anhydrous] 6.25gms

Distilled water 500.00 ml.

## Solution B

Eosin [Yellow Eosin, water soluble] 1gms.

Disodium hydrogen phosphate [Anhydrous] 5 Gms.

Pot. Dihydrogen phosphate [Anhydrous] 6.25gms

Distilled water 500.00ml

## Preparation of solution:

The phosphate salts are first dissolved in water and then the stains are added. Azure I is grounded in a mortar with phosphate solution to dissolve it well. Then it is kept for 24 hours and filtered before being used. The stain should be filtered again if a scum appears on the surface of the stain or if the dye precipitates on the stained film. The stains are kept in covered jars of such a size that the depth of the solution is maintained at about 3 inches, the level being maintained by the addition of fresh stain as necessary. Eosin solution should be discarded if it becomes greenish.

Buffer Water: - pH - 7.2

## DILUTING FLUIDS

### A. RBC Diluting fluid:

#### 1. Dacie's formol citrate Solution

1ml of 40% formaldehyde

900ml of 3% w/v trisodium citrate.

This is Cheap, easy to make, and red cell shape is maintained. Formaldehyde prevents growth of bacteria and fungus.

#### 2. Hayem's fluid:

Mercuric chloride - 0.5gms - Prevents growth of bacteria and fungus.

Sodium chloride - 1.0gms

Sodium sulphate - 5.0gms



Distilled water - 200ml

The fluid has to be renewed frequently to avoid RBC clumping.

### 3. Toisson's fluid:

Sodium chloride - 1.0gms

Sodium sulphate - 8.0gms

Methyl Violet - 0.025gms

Neutral Glycerine - 30ml

Distilled water - 200ml

Because of Glycerine, RBC tend to settle on the surface of the counting chamber very slowly

### 4. Gower's Solution:

Sodium Sulphate - 12.5gms

Acetic Acid - 33.3ml

Distilled water - 200ml

Rouleaux formation is inhibited in this fluid (perhaps more than others.)

### B. WBC Diluting fluid:

#### 1. Turks Diluting fluid:

Glacial acetic acid 1.5ml

1% solution of Gentian violet in water 1ml

Distilled water 98ml

Thymol - (pinch) - prevent the growth of fungus.

Gentian violet stains the nuclei of leukocytes. Glacial acetic acid lyses the red cells.

#### 2. Hingleman's solution:

Yellow eosin 0.5gms

95% phenol 0.5ml

Formalin 0.5ml

Distilled water 99ml

This fluid is used for staining Eosinophils.



## C. Platelet Diluting fluid:

Formol Citrate solution:

1% formalin in 3% tri sodium citrate.

0 -2 drops of 1% Brilliant Cresyl blue - to stain the platelets

### Exercise

1. What are Romanowsky stains?
2. What is the use of Field stain?
3. Which is a better RBC diluting fluid if one wants to prevent rouleux formation?
4. What is the function of glacial acetic acid in Turk's (WBC) fluid?

## 3.6 STAINING METHODS

### STAINING OF THE BLOOD FILMS:

#### Romanowsky stains:

The blood cells contain acid as well as basic structures. As already mentioned the stain has two type of dye - Basic dye and an Acid dye. Basic substances are acidophilic [phile = like] and so stain with the acid dye. Acidic substances are basophilic and stain with the basic dye. Nucleic acids of the nuclei are basophilic and stain blue. The acidic basophil granules contain acidic heparin and other substances and stain blue. Haemoglobin is basic and thus acidophilic, staining red.

#### Steps for staining:

1. Preparation of the blood film (using wedge technique )
2. Air drying the smear
3. Fixation of the smear

Cells must first be fixed to the slide with pure Acetone free methonal alone or in solution with the dye. Fixation prevents the RBC from hemolyzing, prevents degenerative autolytic changes in the cells and allows storing the smears for longer duration. No staining occurs during this step. After fixation, addition of a buffer solution changes the pH of the solution and ionizes the reactants to initiate staining process.

4. Staining: It can be done two ways: -
  - Manual - By rack method or by dip method.

#### Rack Method:

This uses rods overlying a sink. Glass slides are held in horizontal position on the rods during staining.



## Technique:

1. Films should be air dried
2. Stain solution [Leishman etc.,] is spread over the slide till the top surface is flooded.
3. Wait for 2 to 3 minutes
4. Add double the volume of buffer water  
Mix by rocking or blowing with a help of a Pasteur pipette and wait for 7 to 10 minutes. Then washing is done by flooding the film with distilled water. This should be completed in 2 to 3 sec. If washing is prolonged, the stain will get removed.
5. The staining mixture is cleaned from the back of the slide with the help of a tissue or gauze and the slide is air dried by standing in a rack.

## Criteria for a good stain:

The well stained film is reddish brown

Microscopically RBCs are stained pink, WBC nuclei are purple blue and platelets are purple blue.

Some of the problems encountered during staining:

**Excessive blue stain is seen with** Thick film, prolonged staining, inadequate washing and too high an alkalinity of stain or diluent. **Remedial actions are** Using less stain or more diluents, staining for a shorter time and changing to a buffer with a low pH

**Excessive pink stain is seen with** Insufficient staining, Prolonged washing, Mounting the cover slips before drying, too high an acidity of stain or buffer or methyl alcohol and the dye with improper polychromes (Try another lot)

**Precipitate on the film occurs with** Dirty slides, Drying of film during staining procedure, Improper washing of the slides, Improper Filtration of the stain and presence of dust on the slide or smear.

## Exercise

1. What is the principle behind staining is romanowsky stains?
2. Write briefly about the steps in staining the blood films?
3. Describe the criteria for a well stained blood film?
4. What are the reasons for excessive blue stain?
5. What precautions one must keep in mind to prevent precipitate on the stained film?



## 3.7 MEASUREMENTS OR QUANTITATIVE ANALYSIS

### The Haemocytometer:

This is an instrument used for counting blood cells. It consists of two pipettes and a counting chamber. The pipette with a smaller bulb is used for W.B.C. counts, while the one with the larger bulb is used for R.B.C. counts. The counting chamber that is most commonly used is the Levy Chamber with the IMPROVED NEUBAUER ruling.

### 3.7.1 R.B.C COUNT

#### Method:

1. Red cell counting is performed with the pipette with the large bulb. This has a red glass bead inside it. It has three marks, 0.5, 1 and 101. The blood is drawn up to the mark 0.5 tip is wiped clear followed by the diluting fluid which is drawn up to the 101 mark. The pipette is rotated rapidly between the fingers to allow the fluid to mix well. The glass bead helps in mixing of the fluid and the blood.

The dilution of blood contained is 1/200.

2. Charge the Counting Chamber:
3. Allow the cells to settle for 2 to 3 minutes and then count the RBCs in the central square.
4. With the help of low power [10X] the ruling is first focused and then the counting is done under the 40X objective.
5. The basic formula to be used for calculation is as follows:

$$\text{No. of cells / cu.mm} = \frac{\text{No. of cells counted} \times \text{Dilution}}{\text{Chamber depth} \times \text{chamber area}}$$

Referance range: varies with age and gender.

Adult male: 4.5 - 5.5 millions /cumm

Adult female: 3.8 - 4.8 millions /cumm

#### Erythrocytosis:

### 3.7.2 W.B.C. COUNT

1. The same principle used for Total R.B.C. count also applies here W.B.C. are present in much smaller numbers than R.B.C., therefore the dilution required is much less.
2. The leukocyte pipette is smaller than the red cell pipette and is marked 0.5, 1 and 11.
3. Steps:



1. The special W.B.C. pipette is used.
2. Blood is drawn up the 0.5 mark and the tip is wiped clean.
3. Diluting fluid is drawn to the 11 mark, [ making a dilution of 1 in 20]
4. The pipette is well shaken at right angles to its axis to mix the fluid and cells.
5. After discarding the first few drops, the count chamber is charged as described earlier.

The cells are counted using either the Low Power [10 x objective] lens and 10x eyepiece or [40x objective] lens and a 5 x eyepiece.

The cells in the four large corner squares of the Neubauer ruling [each 1 mm. square] are counted.

**Basic formula:-**

Total cells in cu.mm =  $\frac{\text{No. of white cells counted} \times \text{Dilution factor}}{\text{Depth factor} \times \text{Area counted}}$

**Referance range** (for WBC COUNT ):  $4 - 10 \times 10^3 / \text{cumm}$

**Leucocytosis** : is the count higher than the ref. range. It is seen in infection, injury, cancers etc.

**Leucopenia** : is the count lower than ref. range. It is seen in aplastic anemia, sepsis, megaloblastic anemia etc.

### 3.7.3 PLATELET COUNT

**PLATELETS:**

They are small and colorless and moderately refractile, in unstained preparations. They may be oval, spherical or elongated in shape and do not have any nucleus. Accurate platelet count requires far greater care because of the nature of platelets. It is not uncommon to obtain a falsely low count or a falsely high count. To avoid it syringes and EDTA tubes should be perfectly clean, diluting fluid must be fresh, kept in a glass stoppered bottle at 20 - 40°C and filtered daily before use.

**DILUTING FLUID**

Formol citrate solution which is 1 percent formalin in 3 percent trisodium citrate solution is used. One or two drops of 1 percent brilliant cresyl blue may be added. The fluid is stored in the refrigerator or else made fresh before use.

**METHOD:** 0.02 ml. [20cumm] of the blood should be diluted with 1.98ml of the diluting fluid.



It is mixed well for 2 minutes before charging the Improved Neubauer Counting Chamber. Charged chamber is placed in petridish with some moist filter paper (this prevents drying and keeps the air moist under the petridish). It is left undisturbed for 10 minutes. Platelets settle on the surface of the counting chamber. Using the 40x objective and 10 X eye piece with the condensor racked down the platelets are seen as highly refractile particles. Count the platelets in one or more sq. mm. It is important for greater accuracy to count at least 100 platelets.

**Calculation: -**

$$\text{Total cells / cu.mm} = \frac{\text{No. of cells counted} \times \text{Dilution factor}}{\text{Area counted} \times \text{Depth factor}}$$

**Referance range** for platelet count:  $150 - 400 \times 10^3 / \text{cumm}$

**Thrombocytopenia:** A decrease in platelet count is called thrombocytopenia.

It is seen in following conditions: ITP, Megaloblastic anemia, aplastic anemia, acute leukemia etc.

**Thrombocytosis :** An increase in platelet count is called as thrombocytosis.

It is seen in Iron deficiency anemia, after trauma, essential thrombocythemia etc.

### 3.7.4 RETICULOCYTE COUNT

**PRINCIPLE:**

Reticulocyte is a juvenile RBC, contains small amounts of RNA and ribosome and detected by incubating with supravital stains like 1% Brilliant cresyl blue or 1% New Methylene blue.

**SAMPLE:**

EDTA blood.

**REAGENTS AND EQUIPMENTS:**

Brilliant Cresyl Blue - 1gm

Normal saline - 100 ml.

**PROCEDURE:**

Add equal volumes of well mixed blood and freshly filtered reagent, mix and incubate the tube at  $37^{\circ}\text{C}$  for 15 to 20 min. Make thin smear from well mixed fluid.

Air dry and count the number of Reticulocyte for 1000 RBC's under oil Immersion.



## CALCULATION:

$$\text{Retic \%} = \frac{N \times 100}{1000}$$

## QUALITY CONTROL:

Duplicate measurements and checking with peripheral smear and RBC morphology.

## INTERPRETATION:

Reticulocytosis (increased reticulocyte count) is seen in iron deficiency anemia on treatment, Megaloblastic anemia on treatment and Hemolytic anemias.

Reticulocytopenia (decreased reticulocyte count) is observed in Aplastic anemia and PRCA (Pure red cell Aplasia)

Errors may occur if

Reticulocytes are counted in less than 1000 RBC's, there is delay in counting and Pappenheimer /Heinz bodies are confused with Reticulocytes and erroneously counted.

## REFERENCE RANGE:

1. Adults: 0.5 - 2.5%
2. Infants (upto 1 yr) & full term (cord blood) : 2.0 - 5%
3. Children > 1yr: 0.5 - 2.5%

## 3.7.5 ABSOLUTE EOSINOPHIL COUNT

**Eosinophilia** : If the absolute eosinophil count is more than 440 / ul it is called Eosinophilia.

Some conditions in which eosinophillia is seen :

1. Allergic reactions
2. Parasitic infections.
3. Brucellosis
4. Certain Leukemias.

**Eosinopenia** : If the count is less than 40 / ul is called Eosinopenia.

## CONDITIONS:

1. Hyperadrenalism [Cushings disease]
2. Shock
3. Administration of Adrenocorticosteroids [ACTH]



## SPECIMEN REQUIRED

EDTA blood.

## PRINCIPLE

Dilution of Blood is done in a special diluting fluid which stains the eosinophils and removes the red cells. These cell are counted under low power (10X) in a known volume of fluid with the help of a neubauer counting chamber.

## REQUIREMENTS

- 1) Microscope
- 2) Improved Neubauer chamber or Fuch Rosenthal counting chamber
- 3) Diluting fluid : (Hingleman's solution)

## PROCEDURE

- 1) Pipette 0.36 ml of diluting fluid in a test tube.
- 2) Add 0.04 ml of blood (Hb pipette may be used twice).
- 3) Mix and keep for 10 minutes.
- 4) After thorough mixing charge the counting chamber.
- 5) Let it stand under a moist petri dish for about 2 to 3 minutes.
- 6) Count the cells under low power objective with reduced light.

If improved neubauer counting chamber is used, count cells in all nine squares

## CALCULATIONS

Total number of eosinophils, cu mm ( $\mu\text{l}$ )  
=  $\frac{\text{Number of cells counted} \times 10}{0.9}$  (dilution)  
0.9 (volume of fluid)

## NOTE

- a) Dilution = 10  
b) Volume of fluid = area counted X depth  
= 9 sq.mm X 0.1  
= 0.9

## PRECAUTIONS

- Eosinophils disintegrate in the diluting fluid, hence the count should not be delayed for more than 30 minutes after diluting the blood.



**REFERENCE RANGE:** 40-440 cells / ul

**ADDITIONAL INFORMATION** If total leucocyte count and differential leucocyte count are known then one can calculate absolute count as follows

$$\% \text{ Eosinophils} = \frac{\text{Absolute count} \times 100}{\text{Total leukocyte count}}$$

### Exercise

1. What can be the sources of error while doing manual RBC count?
2. What is the normal range for total leucocyte count?
3. What is leucocytosis?
4. What are the causes of falsely high manual platelet count?
5. In a manual platelet count, why should we leave the counting chamber for some time after charging?
6. What is the function of Brilliant Cresyl Blue in Formol-Citrate solution used for estimating platelet count?
7. What is thrombocytopenia? Enumerate two conditions in which it is seen.
8. What are reticulocytes? How is a reticulocyte count done?
9. What are causes of increased reticulocyte count?
10. What can be the sources of error in reticulocyte counting?
11. What is the normal range of Absolute eosinophil count?
12. List few causes of eosinophilia?

### 3.7.6 HEMOGLOBIN ESTIMATION

Hemoglobin is a metalloprotein with the primary function of carrying oxygen from lungs to tissue and carbondi-oxide from tissues to lungs. Each molecule of haemoglobin has four polypeptide chains, each chain having one heme group. Hemoglobin estimation is done generally to detect anemias. It is also used for the diagnosis of primary and secondary polycythemia.

Hemoglobin estimation: Hemoglobin may be estimated by various methods based on different principles like Colorimetric Method, Specific gravity Method, Chemical method and Gasometric method

#### 1. Sahli Method or Acid Haematin Method:

**Principle:** Haemoglobin is converted to acid haematin by the addition of N/10 or 0.1 N hydrochloric acid and the resulting brown colour is compared with standard brown glass



reference blocks. The intensity of the brown colour depends on the amount of acid haematin, which in turn, is proportional to the amount of haemoglobin in the blood sample. Protein, lipid, bilirubin, methaemoglobin, carboxy - haemoglobin and sulfhaemoglobin influence the depth of colour. Acid haematin is in colloidal suspension and so cannot be used in the photometer or colorimeter which require optically clear solutions.

The Sahli Haemoglobinometer consists of a standard brown glass mounted on a comparator and graduated tube. A special pipette to measure out 20 cu.mm of blood is supplied with the instrument. The graduation on the tube varies with the different modifications. The original ones show 17.3gms. as equal to 100 per cent. The tubes commonly used now are square with graduations in per cent on one side and grams per 100ml. on the other.

**Method:** Place N/10 (made by mixing 1ml conc. HCL and 99ml of distilled water) hydrochloric acid in the tube up to the lowest mark. Draw blood up to the 20 cu.mm mark in the pipette and transfer it to the acid in the tube. Rinse the pipette well by drawing up some of the acid and re - expressing it. Mix the acid and blood by shaking the tube well, and allow the tube to stand for at least 10 minutes to allow the brown colour to develop. (About 98 percent of the colour has developed at the end of 10 minutes. Then the solution is diluted with distilled water by adding a few drops at a time until the colour of this solution matches with the glass plates in the comparator. The solution is mixed well after each addition of distilled water with the glass rod provided. The matching should be only against natural light. The level of the fluid is noted at its lower meniscus after taking out the glass rod and the reading corresponding to this level on the scale is read in grams per 100 ml. If only a percentage is given on the tube, this should be converted into grams percent. Carboxy Hb, meth Hb and sulf Hb are not converted to acid haematin by this method and hence not measured. The brown colour so formed is not stable and so one should not delay in taking the reading.

## 2. Colorimetric Method:

This is based on measuring the colour of hemoglobin or its derivative. They are all based on Beers Law, which states that the optical density [depth of colour] of a coloured solution is directly proportional to the concentration of the coloured material in the solution.

**Cyanmeth Hemoglobin Method:** This is the preferred method of determining haemoglobin in most parts of the country. It is possible to make up stable known standards for comparison.

**Principle:** The haemoglobin is first converted to methaemoglobin and then to cyanmethaemoglobin by the addition of Sodium or Potassium cyanide and potassium ferricyanide.



## Reagents:

Cyanmethaemoglobin solution.

Sodium Bicarbonate 1gm

Potassium ferricyanide 200gms

Potassium cyanide 50 mg

Make up to 1000ml with distilled H<sub>2</sub>O Store solution in a brown bottle in the refrigerator.

## Standard Haemoglobin solution:

Preparation of standard curve that can be used to determine Grams per cent of Haemoglobin of whole blood.

- [1] Pipette 5ml of standard Haemoglobin solution into cuvette.
- [2] Pipette 2.5ml of standard Haemoglobin solution into a second cuvette and exactly 2.5ml of Cyanmethaemoglobin reagent.
- [3] Prepare a blank by adding 5 ml of cyanmethaemoglobin reagent to a third cuvette.
- [4] Place blank in the instrument. Set the wave length at 540 or use a 540nm filter [Green]. Set transmission at 100 per cent or zero optical density.
- [5] Record values of the diluted and undiluted standards read against the blank
- [6] Prepare a graph: Use optical density values and the equivalent gm. per cent Hb. values to plot construction of graph.
- [7] Determination of Haemoglobin:
  1. Pipette 5 ml. of cyanmethaemoglobin reagent into cuvette.
  2. Add 20 $\mu$ l of whole blood.
  3. Read optical density and record value from chart.

**Note** - Except sulf Hb, all forms of Haemoglobin are converted to cyanmeth Hb and hence measured by this method.

## REFERENCE RANGE:

Males: 13 to 17g/dl

Women: 12 to 15 g/dl

## Exercise

1. Describe briefly Acid Haematin Method for the estimation of Haemoglobin.
2. Which is the preferred method for Haemoglobin estimation and why? Describe it briefly.



## 3.7.7 PCV & ERYTHROCYTE INDICES

### Definition of PCV [Haematocrit]:

It is defined as the volume of packed red cells in a given sample of blood and is expressed as a percentage of the total volume of blood.

It is used as screening test for anemia. Along with estimation of hemoglobin and RBC counts it enables the calculation of absolute indices.

### Methods:

1. Wintrobe's method
2. Automated cell counter method
3. Micro haematocrit method

### Wintrobe's method:

The Wintrobe's tube is 11cms long glass tube with an internal diameter of 2.5mm and is calibrated from 1mm to 105 - 110mm. and holds 1ml of blood.



Figure 1

### Sample required:

Venous blood collected in EDTA.

### Method:

Fill the Wintrobe's tube with blood using Pastuer pipette, upto 100mm mark starting from the bottom and gradually withdrawing the pipette as blood is expressed to avoid air bubbles.

Centrifuge the tube at 3000 rpm for 30minutes

Note the upper level of the column of red cells. This gives the PCV and is expressed as percent of total volume of blood.

After centrifugation 3 definite layers can be seen.

1. Lower most is the Layer of packed red cells.
2. Thin layer of WBC and platelets is just above the red cells and is called buffycoat.
3. Plasma forms the uppermost layer.

### REFERENCE RANGE:

Male: 40 - 50 %

Female: 36 - 46 %



## ERYTHROCYTE INDICES

Based on the results of hemoglobin, PCV and total red cell count several indices are derived, which give quantitative information about the red blood cells. These are called absolute values or Erythrocyte indices.

### Three basic indices are :

1. M.C.V. [Mean corpuscular volume]

It is the average cell volume of red blood cells

$$\text{M.C.V} = \frac{\text{P.C.V} \times 10}{\text{R.B.C. in millions}}$$

This is expressed in femtolitre

Reference range: [Normocytic] : 83 - 101 fl

### Clinical application:

Microcytic :less than 80 fl : Low MCV is found in Iron deficiency anemia and Thalassemias.

Macrocytic : more than 100 fl.: High MCV is found in conditions like Megaloblastic anemia because of Vitamin B12 and Folate deficiency etc.

2. M.C.H [Mean corpuscular hemoglobin]

It signifies the average haemoglobin in a red cell.

$$\text{M.C.H} = \frac{\text{Hb [grams/dl]} \times 10}{\text{R.B.C. in millions}}$$

This is expressed in Picograms.

Reference ranges:[Normochromia] 27-32 Picograms.

### Clinical application:

Decreased value of MCH i.e. less than 26pg is Hypochromia and is found in Iron deficiency and thalassemias & increased value of M.C.H more than 32pg is seen in macrocytic anaemia.

3. M.C.H.C [Mean corpus cular Hemoglobin concentration]

It is the average concentration of Hb in a red blood cell.

$$\text{M.C.H.C} = \frac{\text{Hb [grams/dL]} \times 100}{\text{PCV}}$$

Reference ranges: 31.5-34.5 [grams/dL]



## Clinical application:

High value of MCHC is seen in Spherocytosis & decreased value of MCHC is found in hypochromic anaemia.

## Exercise

1. Describe Wintrobe's tube and explain the Wintrobe's method for the estimation of PCV.
2. What are various Red Cell Indices? Give their clinical significance.

## 3.7.8 ERYTHROCYTE SEDIMENTATION RATE [E.S.R.]

Sedimentation is defined as settlement of red cells to the bottom with an upper plasma layer when anti coagulated blood is kept undisturbed for a period of time. There are three stages in which this occurs:

- 1) The stage of aggregation - This is the first stage when the red cells form rouleaux and is the most important stage in sedimentation.
- 2) Stage of sedimentation - is the phase of actual falling of the cells, the larger the aggregates formed in stage I, the faster the rate of fall. This is related to both weights to surface area.
- 3) The stage of packing - is the final one when individual cells and aggregates slow down due to crowding.

The distance the cells have fallen in a given period of time is measured and reported. The reading is usually taken after one hour.

There are two commonly used methods of measuring the E.S.R.

- 1) Westergren's Method
- 2) Wintrobe's Method

**Westergren Method: Is the recommended method.**

## SAMPLE

Fasting blood sample is collected in trisodium citrate (32g/l). Four volumes of blood is added to one volume of tri sodium citrate solution.

(Blood can also be collected in EDTA and diluted as one volume of tri sodium citrate solution (32g/l) to four volumes of blood).

## REQUIREMENTS

- 1) Westergren's ESR tube - It is straight glass / transparent plastic tube 30cm in length, about 2.55mm in diameter and graduated over lower 20cm.



- 2) Stand for holding the tube
- 3) Timer or watch

## PROCEDURE

- 1) Fill the westergren tube exactly upto zero mark by means of a rubber bulb (avoid air bubbles).
- 2) Place the tube upright in the stand. It should fit evenly into the groove of the stand.
- 3) Allow the tube to stand for exactly one hour (at room temperature - 18-25°C)
- 4) Exactly after one hour, level to which the red cell column has fallen is noted.
- 5) The results are reported in terms of mm/after 1st hour.

The test should preferably be done within four hours of collection of samples.

Wash the tubes as early as possible, under running tap water. Rinse in deionized water and dry in the incubator between 40°C - 50°C.

## Factors Influencing Sedimentation:

1. Fibrinogen, Globulin, Cholesterol: These factors increase the sedimentation rate
2. RBC count - Higher the RBC count lower the ESR
3. Sex: It is generally greater in women.
4. Pregnancy - An increased ESR is seen from the 3rd month of pregnancy and returns to normal 4 weeks after delivery

## Laboratory factors which influence ESR:

1. Temperature : ESR is increased at higher temperature
2. Time: The sedimentation is maximum in first 4 hrs of collection of sample hence test should preferably do within this time.
3. Anticoagulant: Heparin, Oxalate, are not suitable. Citrate in 3.8 percent solution is preferable
4. Length of the ESR tube: ESR is greater with longer tubes.
5. Inclination of the tube: Deviation from the vertical increases the ESR. A 3 degree tilt from vertical can lead to an increase in ESR by as much as 30%.

## Importance of ESR:

1. ESR is not diagnostic of any specific disease.
2. It is used to check the progress of the disease.
3. ESR is markedly raised in tuberculosis, Rheumatic fever etc.,



## REFERENCE RANGE IN HEALTH : (20+-3o C)

Age (yrs) 95% upper limit (mm/hr)

### Men

17 -50 Yrs 10 or less

51 - 60 Yrs 12 or less

61 - 70 Yrs 14 or less

> 70 Yrs about 30 or less

### Women

17 - 50 Yrs 12 or less

51 -60 Yrs 19 or less

61 - 70 Yrs 20 or less

> 70 Yrs about 35 or less

## Exercise

1. What is ESR? What are the stages in ESR?
2. What is the preferred method for estimation of ESR? Describe it briefly.
3. What are the factors influencing ESR?

## 3.7.9 DIFFERENTIAL COUNT

It is the estimation of the percentage of different types of WBC's in blood.

### SAMPLE:

Sample is collected in EDTA.

### METHOD:

Manual method: done on a stained slide

### EQUIPMENT:

- Microscope
- Differential cell counter
- Slides
- Spreader slides



## REAGENTS:

- Leishman' s stain
- Phosphate buffer

## TECHNIQUE

For the differential count, the best area of the smear is where red cells show some overlap.

An uneven distribution of WBC 's is seen with the central thicker portion being occupied by smaller cells like lymphocytes and the edges and the tail being occupied by larger cells like monocytes, eosinophils etc.

## REFERENCE RANGE:

- Neutrophils : 40-80% (2000 -7000)
- Lymphocytes : 20-40% (1000 -3000)
- Eosinophils : 1-6% (40-440)
- Monocytes : 2-10% (200-1000)
- Basophils : 1-2% (20-100)
- Qualities of good film. - There should be a thick and thin portion and a gradual transition form one to another. - The film should have a smooth even appearance, and be free from ridges, waves and holes. - In an optimal thickness film, there should be some overlap of red cells in much of the film and even distribution and separation of red cells towards the tail.
- 200 cell count is done by two independent observers, each on two films prepared from same sample

## CORRECTING THE COUNT FOR NUCLEATED RBCs:

When nucleated RBCs are present in perepheral blood, they can give rise to falsely high TLC values. Hence a correction has to be done to get the actual TLC.

- Care is taken to differentiate small lymphocytes from NRBCs
- When NRBCs are present in significant no. Eg. > 5/100 WBCs, their absolute No. is expressed as other nucleated cells and a correction reported as follows:

$$\text{Corrected WBCs} = \frac{\text{Total WBC} \times 100}{100 + \text{Number of NRBC}/100\text{WBC}}$$



fig. f



## OBSERVATIONS

**Neutrophils: (Polymorphonuclear Leukocyte)**

Measures about  $12\ \mu\text{m}$  in diameter, nucleus stains deep purple in color, lobes connected by delicate filament. The number of lobes varies from two to five. Cytoplasm is colorless and contains tiny, tan to pink granules. Constitute 40 - 80% of WBC in adults.

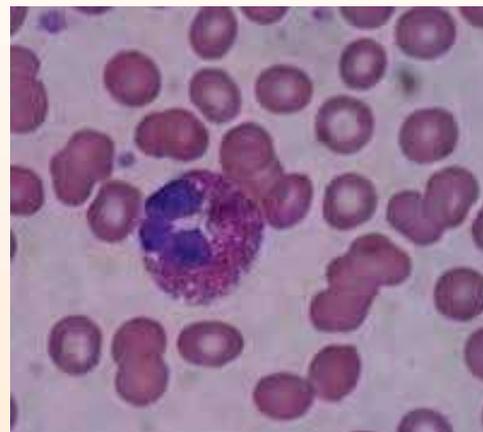


fig.g

## Eosinophils:

Measures about  $13\ \mu\text{m}$  in diameter. Cytoplasm contains large round or oval orangeophilic / bright red granules. The cytoplasm is colorless. The nuclear lobes are less deeply stained, usually bi or tri lobed. (Often spectacle shaped). Constitute 1- 6% of WBC in adults

## Basophil:

Nucleus is less segmented usually indented or partially lobulated, the granules are large, deep purple, often mask the nucleus. Constitute 0 - 1 % of WBCs in adults.

## Monocyte:

Is the largest among the normal blood cells in adults ,  $14 - 20\ \mu\text{m}$  in size, contain single nucleus, partially lobulated, deeply indented or horse shoe shaped, round or oval with delicate lacy chromatin surrounded by ground glass / gray blue cytoplasm and contains fine red to purple granules. Constitute 2 - 10 % of WBCs.

## Lymphocytes:

Lymphocytes are small mononuclear cells with a thin rim of cytoplasm. Nuclei are uniform in size and slightly larger than RBCs. Nuclear chromatin is homogenous with clumping at the periphery.

Sources of Errors while performing a differential count

- Mechanical errors
- Error of random distribution.
- Under / over staining / washing etc.



fig.h



## Variation in leucocytes:

When the total leucocyte count is more than 10,000/cumm, it is called leucocytosis. On the other hand if the total leucocyte count is below 4000/cumm, it is termed as leucopenia.

## Variation in specific leucocytes:

### NEUTROPHILS

**Neutrophilia:** It refers to an increase in the number/percentage of neutrophils in the blood.

#### It is seen commonly in:

Acute infections

Acute blood loss

Corticosteroid therapy

Chronic myeloid leukemia

Pregnancy

Heavy exercise

**Neutropenia:** refers to a decrease in the number of neutrophils in the blood.

#### It is seen in:

Viral infections e.g. measles, dengue e.t.c.

Suppression of bone marrow by irradiation or by drugs

Aplastic anaemia, megaloblastic anaemia

**Eosinophilia:** In a condition wherein the number / percentage of eosinophils is increased in blood.

#### It is seen in:

- (1) Allergic disorders: Eg. Bronchial asthma, Urticaria, Drug sensitivity, seasonal rhinitis (hay fever) etc.
- (2) Skin disorders: Eczema, Pemphigus and Atopic dermatitis.
- (3) Parasitic infestations (with tissue invasion) Eg. Trichinosis, Tape worm,)
- (4) Pulmonary infiltration with Eosinophilia syndrome.
- (5) Blood disease: CML, hodgkin's disease, polycythaemia vera.
- (6) Miscellaneous: After splenectomy, sarcoidosis.



## Basophilis

An increase in basophils in the peripheral blood above the normal reference range is called basophilia.

It is seen in

1. Allergic reactions
2. CML
3. Hypothyroidism
4. Polycythemia vera
5. Following splenectomy
6. Chronic Idiopathic Myelofibrosis

## Monocytes:

Monocytosis It is an increase in monocyte number / percentage count above normal reference range

It is seen in:

1. Bacterial infections like tuberculosis, syphilis.
2. Rickettsial and Protozoal infections like malaria, kala-azar.
3. Viral infections.
4. Haematopoietic diseases like CMML, Myeloproliferative disorders, monocytic leukemia, multiple myeloma.
5. Sarcoidosis, ulcerative colitis.
6. Connective tissue disorders.
7. During recovery from acute infections.

## Lymphocytes

### Lymphocytosis:

It is an increase in lymphocyte count above normal reference range

It is seen in

1. Tuberculosis
2. Cytomeglovirus infection
3. Infectious Mononucleosis



4. Brucecellosis
5. Chronic Lymphocytic leukemia
6. Lymphoma

## Exercise

1. Describe the morphology of a polymorphonuclear leucocyte? List a few conditions where neutrophillia is seen.
2. Describe an eosinophil. Enumerate few conditions in which eosinophilia are seen?
3. Describe a lymphocyte.
4. Enumerate few conditions in which lymphocytosis may be seen?

## 3.7.10 LE CELL

In autoimmune disorders (like SLE) antinuclear antibodies appear in the serum of the patient. These antibodies have the capacity to lyse the nuclei of neutrophils and then phagocytosed by other normal neutrophils. The cell membrane however needs to be broken down (chemically or mechanically) for these antibodies to act on the nuclei. A buffy coat smear is prepared which is stained by leishman stain and LE cells are looked for. These are neutrophils with a spherical large pale purple homogenous opaque mass (LE body) in their cytoplasm. Nuclei of the neutrophils usually pushed to the periphery. Rarely an eosinophil or a monocyte may be the ingesting cell.

**Sample type:** Whole blood

**Materials Required:** Glass Beads, Centrifuge, Rubber Bungs, Centrifuge, Microscope, test tubes (10x 100mm), Glass slides, Leishman stain, Vortex Mixer.

### Procedure:

5ml of blood is taken into glass test tube. After adding 5 rubber beads the test tube is stoppered. The test tube is rotated on a vortex mixer at 50 rpm for 30 minutes. The tubes are placed at 37°C for 10 minutes. The contents of the tube are transferred to a Wintrobe tube which is centrifuged for 30 minutes at 3000RPM. Smears are made from the buffy coat, dried, fixed in methanol and then stained using Lieshman Stain. Examination of films is done under high power and then under oil immersion.

### Interpretation:

One should report LE cell positive only if one can find several LE cells.

Both false positive and false negative results can occur.



Now a days specific and sensitive immunological methods are available for the detection of these antibodies and hence those tests have superseded the LE Cell test.

## Exercise

1. Describe a LE cell?

### 3.7.11. DETERMINATION OF OSMOTIC FRAGILITY OF RED BLOOD CELLS

Structure of red cell membrane is such that the membrane restricts entry of solutions but allows water to pass through it so if red cells are placed in a hypotonic solutions (where concentration of sodium is less  $<0.55\%$  w/v saline) then endosmosis takes place leading to swelling of red cells and ultimately haemolysis. If the red cells are kept in isotonic solution, then the red cells show no change. In this test decreasing concentrations of sodium chloride are made ( $0.9\%$  w/v to  $0\%$ ) and red cells are suspended in these different concentrations. With the help of a photometer, the degree of haemolysis is measured and a fragility curve is plotted.

Increased Osmotic fragility is seen in conditions such as hereditary spherocytosis.

Decreased Osmotic fragility is seen in conditions seen as iron deficiency and thalassemia.

## Exercise

What is the principle behind osmotic fragility test? Write in brief.

## 3.8 ANAEMIA

### 3.8.1 HAEMOPOISIS

The production of blood cells from Haematopoietic stem cells is called Haematopoiesis.

The cell of origin is called the pluripotent stem cell. The Stem cells have the capacity to proliferate and produce more stem cells. The stem cells also have the capacity to differentiate into progenitor cells. The progenitor cells are of two types- Common lymphoid progenitor cells and Common myeloid progenitor cells. The common lymphoid progenitor cell gives rise to precursors for B cells, T cells and natural killer cells. On the other hand the common myeloid progenitor cell gives rise to three types of committed stem cells (also called CFU or colony forming units) which can differentiate along the erythroid/ megakaryocytic, granulocyte/ macrophage and eosinophilic pathways. The committed stem cells divide and ultimately differentiate into the precursors of various mature cells like myeloblast, proerythroblast, monoblast, eosinophiloblast, basophiloblast and megakaryoblast. From these will then arise mature cells -neutrophil, RBCs, monocyte/macrophage,



eosinophils, basophils, and platelets. Cytokines or haematopoietic growth factors help in the proliferation and differentiation of stem cells. Haematopoiesis starts in the third week of intra-uterine life in the yolk sac. From 3rd month of intra-uterine life, haematopoiesis starts in the liver and continues till shortly before birth. From fourth month, haematopoiesis also starts in the bone marrow. At birth, haematopoiesis is almost restricted to the marrow and that in liver almost subsides. Marrow throughout the skeleton is active till the age of puberty, however only the marrow in the ribs, skull, vertebrae, pelvis and proximal regions of humerus and femur retains activity and is red by the age of 18. Marrow in rest of the bones becomes inactive.

In the bone marrow, under the influence of erythropoietin, the committed stem cells divide and differentiate into proerythroblast. These are the first cells which can be recognised as belonging to the erythroid series. These then give rise to basophilic erythroblast which give rise to polychromatic erythroblast which further gives rise to orthochromatic erythroblast which ultimately gives rise to reticulocyte. Reticulocytes are released in blood circulation and mature into red blood cells. As the cells pass from one stage to other the amount of haemoglobin in the cell increases, the cell size decreases and the nucleus becomes smaller. Reticulocytes are anucleate cells, similar to RBCs, except that they have polyribosomes in their cytoplasm.

## ERYTHROCYTES

These are anucleate cells

They are biconcave in shape which allows for greater flexibility.

Size: Their diameter varies from 6.0 - 8.5  $\mu$ m (In well stained smears they are roughly the same size the size of the nucleus of a small lymphocyte). Their outer periphery is thicker than the inner portion and so in well stained films the central area shows one third pallor.

Life Span of RBCs is 120 days.

Function of RBCs is to carry oxygen and carbon di-oxide.

### 3.8.2 ANAEMIA - APPROACH

Anemia - Anemia is defined as decrease in oxygen carrying capacity of blood. In practice, decrease in hemoglobin is considered as anemia. Evaluation of Anemia is based on clinical history, examination and lab findings.

One should talk to the patient and ask about any history of exposure to drugs, chemicals, any change in bowel habits, fever, kidney dysfunction, early graying of hair or skin changes. Family history of bleeding disorders should also be asked. Sometimes patient may also complain of breathlessness, tiredness and fainting spells.



After a thorough history, one should examine the patient and look for jaundice, any lymph node enlargement, spleen or liver enlargement, changes in nails and sternal tenderness.

Tests are very essential in establishing the diagnosis of Anaemia. Laboratory test shows low haemoglobin. In addition one may also find low TLC, low Platelet Count and abnormal cells. Retic Count may be increased or decreased depending on the cause of anaemia.

**Based on absolute values, anaemia can be classified as:**

Microcytic, Macrocytic, Normocytic.

In microcytic anaemias,  $MCV < 80$  fl. It may be accompanied by low MCH and MCHC and then is called microcytic hypochromic anaemia. If one examines the peripheral blood film, microcytic cells are seen. Common clinical conditions where this is seen are Iron deficiency anaemia and thalassemia.

In macrocytic anaemias on the other hand,  $MCV > 100$  fl. There is usually an increased MCH with a normal MCHC. Peripheral blood examination in such cases shows macrocytes. Macrocytic anaemia could be because of vitamin B12 and/or folic acid deficiency (then it is labelled as megaloblastic anaemia)

Or it could be due to other causes like liver disease, alcohol intake, hypothyroidism, aplastic anaemia and accelerated erythropoiesis. Some drugs such as cytotoxic drugs, immunosuppressants and anticonvulsants can also cause macrocytic anaemias.

In Normocytic anaemias, the MCV is normal. These are usually accompanied by normal MCH and MCHC. There may be however a reduction in RBC Count. Peripheral blood film reveals relatively normal appearing red cells. Such anaemias are found in chronic diseases and after acute blood loss.

A brief mention is made here of Haemolytic anaemias. These anaemias are marked by increased red cell destruction. Various tests such as the reticulocyte count, serum unconjugated bilirubin, serum LDH, serum haptoglobin, urine haemoglobin, urine haemosiderin and urine urobilinogen help detect such anaemias. Conditions where they are found are hereditary spherocytosis, autoimmune immune haemolytic anaemias, G6PD deficiency etc.

## Exercise

1. What is haematopoiesis?
2. List all the organs where all haematopoiesis takes place in the intrauterine life?
3. What are the stages in the development of red blood cells?
4. Describe the red blood cells briefly?
5. What is anaemia? How does one classify it based on absolute indices?
6. Fill in the blanks:



- A. Microcytes are found in\_\_\_\_\_.
- B. In macrocytic anaemias MCV is\_\_\_\_\_.
- C. Deficiency of Vitamin B 12 leads to\_\_\_\_\_anaemia.
- D. Serum unconjugated bilirubin is increased in\_\_\_\_\_anaemia.
- E. In haemolytic anaemias the reticulocyte counts is\_\_\_\_\_.
- F. Haemolytic anaemia is seen in conditions such as\_\_\_\_\_and\_\_\_\_\_.
- G. Excessive alcohol intake usually leads to\_\_\_\_\_anaemia.

## 3.9 HAEMOSTASIS

Human beings have their own mechanism to keep blood in fluid state physiologically and save themselves from the dangers of thrombosis and haemorrhage. Injury to the blood vessel starts repair mechanism or thrombogenesis.

**The various components are discussed below;**

**BLOOD VESSEL:** The integrity of blood vessel wall helps in normal blood flow. An intact endothelium maintains the flow of blood and saves from thrombogenic influence of subendothelium and releases a few anti -thrombotic factors.

Damage to vessel exposes the subendothelial connective tissue which is thrombogenic and has important role in initiating coagulations as well as thrombosis.

**PLATELETS:** Endothelial cell injury, plays an important role through Platelet adhesion, Platelet release reaction and Platelet aggregation

**COAGULATION SYSTEM:** It serves to convert Plasma fibrinogen into solid mass of fibrin. The coagulation system is involved in haemostatic process as well as in thrombus formation .

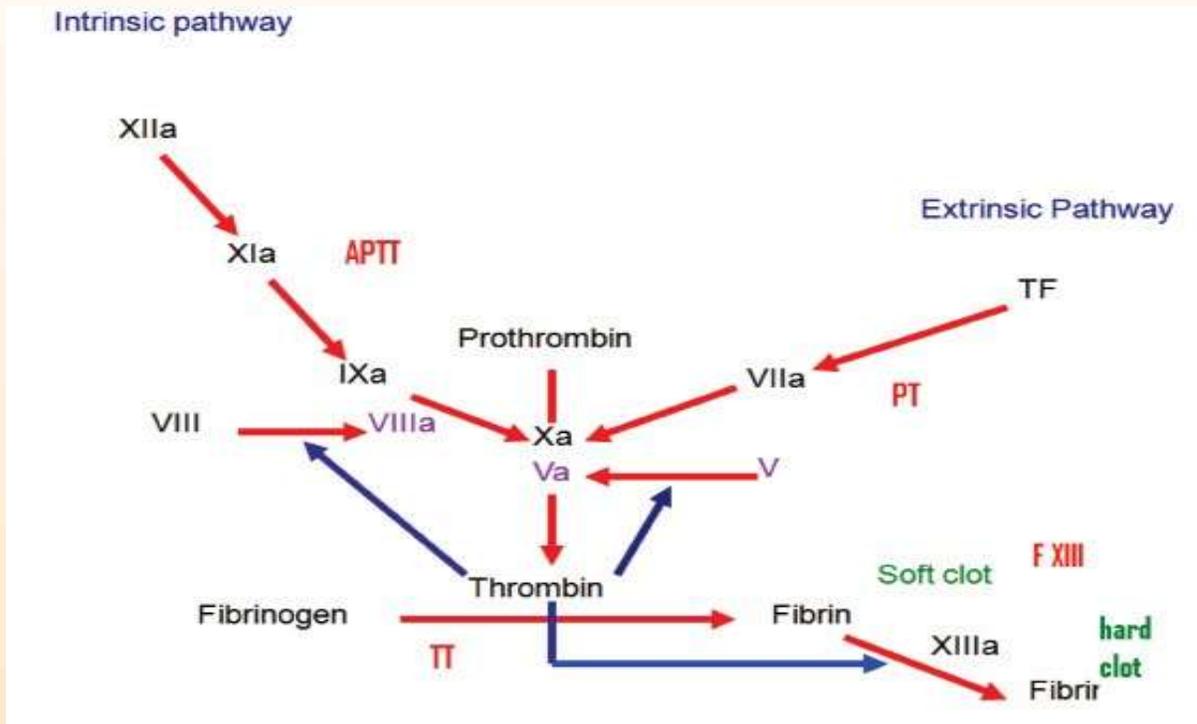
**The mechanisms are as follows :**

**Intrinsic pathway :** Contact with abnormal surface leads to activation of factor XII and the sequential interactions of factors XI, IX, VIII and finally factor X, alongwith calcium ions (factor IV) and platelet factor 3.

**Extrinsic pathway :** Damage to tissue results in the release of tissue factor or thromboplastin. Tissue factor on interaction with factor VII activates factor X.

**Common pathway:** It begins where both intrinsic and extrinsic pathways converge to activate factor X which forms a complex with factor Va and platelet factor 3, in the presence of calcium ions. This complex activates prothrombin to thrombin which then converts fibrinogen to fibrin. The monomeric fibrin so formed is polymerized to insoluble fibrin by activation of factor XIII.





## Sample required

32g/l tri sodium Citrate at a ratio of 9 parts blood to 1 part anticoagulant is used for all coagulation tests.

EDTA or heparin cannot be used for coagulation tests as EDTA irreversibly chelates Ca ions and heparin activates antithrombin which inhibits coagulation

## Tests for hemostasis

- Screening coagulation tests
- Confirmatory Tests

### (a) Screening tests

Peripheral smear are especially useful in (Bernards Soulier syndrome, leukemia, thrombocytopenia)

Complete Blood Count (BSS, Leukemias, thrombocytopenia)

Prothrombin Time (PT)

Activated Partial Thromboplastin Time (APTT / PTTK)

TT



## FXIII screening

**Prothrombin time** measures the deficiency of all vitamin K dependent coagulation factors and also the integrity of extrinsic pathway.

Normal values 10 to 12 seconds (when recombinant human thromboplastin is used for assay).

Prothrombin Time (PT) could be abnormal in congenital Factor VII deficiency and in certain Acquired conditions such as liver disease, malignancy, oral anticoagulant therapy and disseminated intravascular coagulation.

**APTT (Activated Partial Thromboplastin Time)** on the other hand measures the integrity of intrinsic pathway.

Normal values: 26 - 40 seconds.

A prolonged or abnormal APTT is seen in patients of liver disease, disseminated intravascular coagulation, on anticoagulant therapy and patients having deficiency of any coagulation factor except factor VII.

**(b) Confirmatory tests are based on results of screening tests and clinical findings in patients. Tests generally required are**

- Factor VII assay
- Factor IX assay
- Von. willebrand factor
- Mixing studies
- Platelet function tests

## 3.9.1. BLEEDING TIME

### Bleeding time by Ivy method

It is a Screening test to detect any vascular defect of the vessel wall or any abnormality of platelet number and function.

### Materials Required ;

- 1) Spirit Swab
- 2) Sterile disposable blood lancets
- 3) Sphygmomanometer
- 4) Filter paper - 1mm
- 5) Stopwatch



- BP cuff is placed on the patient's arm about 2 to 3 inches above the elbow joint. Pressure is increased to 40 mm Hg. This pressure is kept for the entire procedure.
- An area is selected on the volar surface of the forearm ( devoid of any superficial viens) and cleaned with spirit swab. The area is allowed to dry.
- 2 skin punctures, 5 - 10 cm apart 2.5 mm deep, 1mm wide are made and stop watch started.
- Blood is blotted from each puncture site on a piece of filter paper every 15seconds. The filter paper should not touch the wound. (as this may interfere with the process of platelet plug formation)
- When bleeding stops, the watch is stopped, time noted and BP cuff released.
- Bleeding times of the two puncture sites are noted and average of the two results are reported.

### Interpretation:

Prolonged Bleeding time if seen in following condition

1. Low Platelet Count- in conditions like ITP (Idiopathic Thrombocytopenic Purpura)
2. Platelet functional disorders like thrombasthenia, uraemia, and myeloproliferative disorders.
3. Vascular Abnormalities like Ehler-Danlos Syndrome.

### Reference Range:

2 to 7 minutes.

### Exercise

1. Explain the ivy method for the estimation of bleeding time ?
2. Enumerate few conditions where bleeding time may be raised.
3. Why one must take care not to touch the wound with the filter paper while blotting blood?

## 3.9.2. CLOTTING TIME BY LEE WHITE METHOD

It is a screening test to measure the efficiency of all stages of intrinsic pathway of coagulation.

### EQUIPMENT:

Water bath / Dry bath 37°C

Test tubes 15 x 125 mm

Stop watch



Disposable syringe with 21 to 22 G needle

Cotton & spirit

Method - Lee white

## **SAMPLE:**

Fresh whole blood 4 ml.

## **PROCEDURE:**

About 2 ml of blood is collected and dispensed in two test tubes (1ml each). The stopwatch is started immediately.

The test tubes are kept at 37°C after putting cotton plugs.

After about 3 minutes, take out one tube, tilt it gently by 45°C and observe whether blood has clotted (the test tube can be inverted without the blood spilling). Repeat the procedure every 30 sec till the blood clots and record the time.

Confirm the observation by checking the second tube.

(one can further observe the clotted blood for clot retraction and clot lysis time)

## **Sources of error:**

Volume of blood less than 1 ml.

Inaccurate temperature of water-bath.

Agitation of the specimen.

REFERENCE RANGE: 5-12 min.

## **INTERPRETATION:**

Prolonged Clotting time observed:

1. Patient on heparin
2. DIC
3. Severe Hemophiliacs

## **Decreased Clotting time:**

1. Hypercoagulable states

## **Exercise**

1. Briefly explain the Lee-White method for clotting time?



## 3.10. BONE MARROW ASPIRATION / BIOPSY

Bone marrow examination is an important test for correct diagnosis in many conditions. One may resort to marrow examination in cases of pyrexia of unknown origin, thrombocytopenia, leukemia, storage disease, Refractory anaemia, Paraproteinemias (rule out Myeloma), Leukaemia, staging of neoplasm including lymphoma.

The procedure is contraindicated if the patient has haemostatic failure.

We can do aspiration and / or biopsy for marrow examination.

Advantages Bone Marrow aspiration - Fine cytological details can be visualised, Cytochemical stains can be used, Microbiological culture, flow cytometry, cytogenetic and molecular studies can be performed.

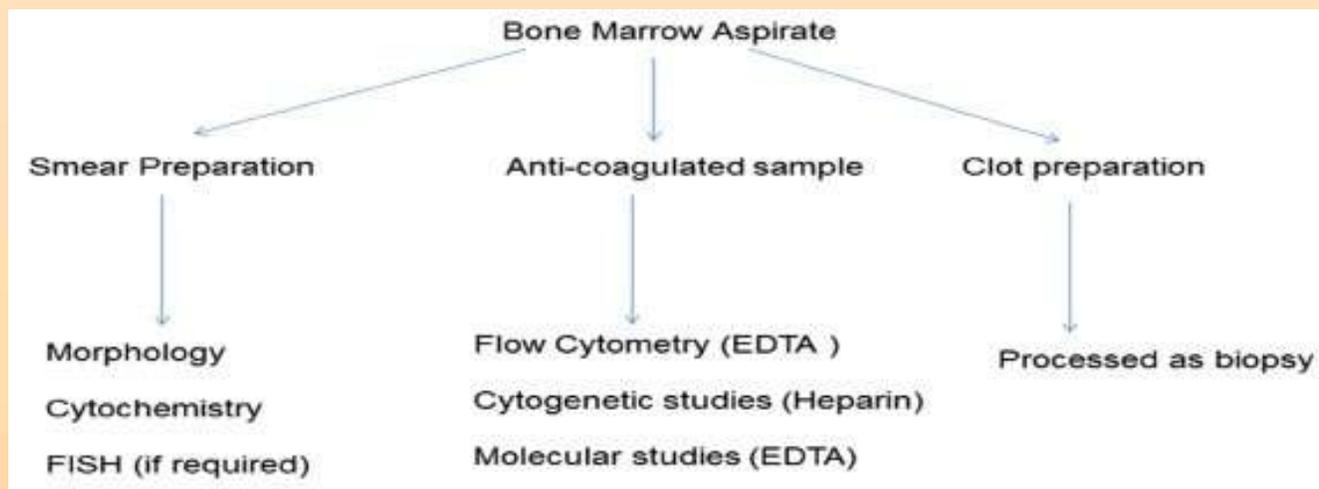
Advantages of Bone Marrow Biopsy - One can do complete assessment of cellularity and architecture. Sometimes focal lesions can be detected which can otherwise be missed. It is specially useful in cases of aplastic anemia, metastasis etc.

### Site for aspiration.

- Anterior superior iliac crest.
- Posterior superior iliac spine.
- Spinous process of the lumbar vertebrae.
- The sternum.
- The tibia is sampled only for infants younger than 1 year.

### Procedure

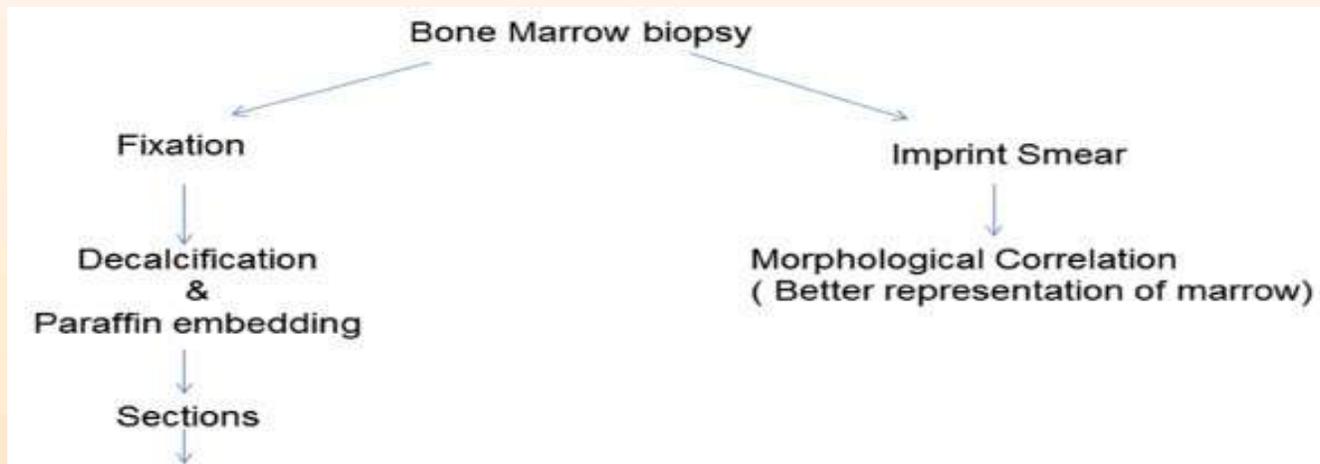
### Processing of BM aspirate



Once the bone marrow aspirate is collected.

Smears are prepared for morphology and cytochemistry and sample is collected in EDTA tube for flow cytometry and molecular studies. Sample is also collected in heparin tubes for cytogenetic studies clot, if present, is collected in formalin and processed as biopsy.

## Processing of BM Biopsy



Bone marrow biopsy is performed by Jamshidi needle and core of bone marrow obtained is kept for fixation in formalin and then for decalcification. Imprint smears are also prepared for morphological correlations.

Then it is processed as paraffin embedding followed by hematoxylin - eosin staining on thin sections.

## Decalcification

- 10% NITRIC ACID
- HYDROCHLORIC ACID (HCL)
- FORMIC ACID
- EDTA

## Embedding

- Paraffin

Now days disposable needle is performed for aspiration and biopsy.

## Exercise

1. List a few diseases in which bone marrow examination is indicated?
2. What are the various sites for bone marrow aspiration?

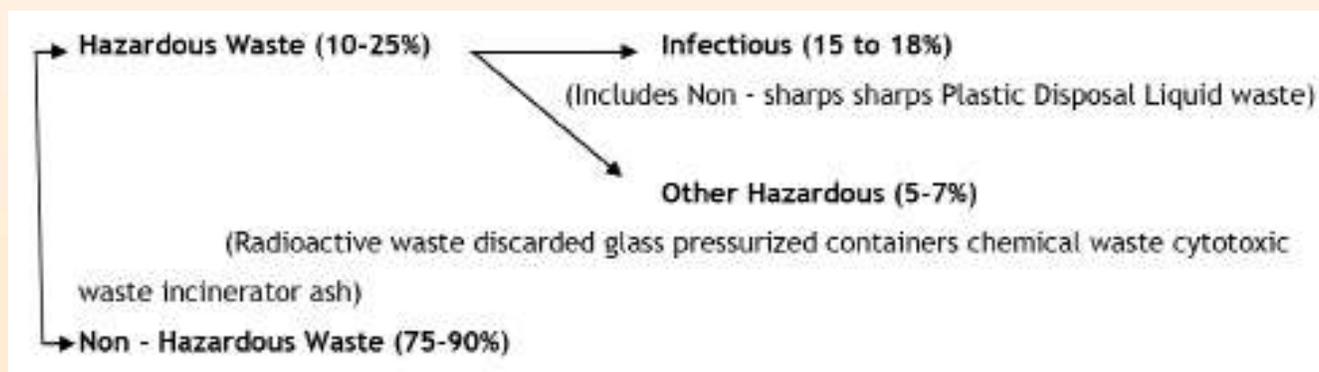


## 3.11 LAB SAFETY

### 3.11.1. BIOMEDICAL WASTE MANAGEMENT (BMWM)

Biomedical waste is waste generated by health care establishments and lab during diagnosis, treatment or research.

#### CLASSIFICATION OF HOSPITAL WASTE



#### IMPORTANCE OF BMWM

##### Extremely hazardous

- If it is not managed properly it can lead to serious health and environmental problems. Therefore it should be segregated, collected, stored, transported and disposed off properly to prevent the transmission of disease from patient to patient, from patient to health worker and vice versa and also to prevent injury to the health care workers and workers in support services, while handling biomedical waste. It is also required to prevent a general exposure to the harmful effects of the cytotoxic, genotoxic and chemical biomedical waste as much as possible. Disorganized management of hospital waste exposes community and surroundings to infection, toxic effects and injury. It is crucial that all medical waste materials are segregated at the point of generation, appropriately treated and disposed off safely.

##### People at risk are??

Healthcare staff

Patients

Visitors/attenders to healthcare setting

Bio medical waste Handlers

General public



- Waste should be collected and segregation at the site of generation itself and waste is separated into various specified categories as per its nature. Harmful and infected material shall be separated from harmless and non-contagious waste and specifically coloured waste containers and plastic bags are mandatory for this purpose.

## TRANSPORTATION OF BMW

### (A) Within the hospital

Waste routes must be designed to avoid the passage of waste through patient care areas. Separate time should be earmarked for transportation dedicated wheeled containers, trolleys or carts should be used trolleys or carts should be thoroughly cleaned and disinfected in the event of any spillage. The wheeled containers should be designed that the waste can be easily loaded, remains secured during transportation, does not have any sharp edges and easy to clean and disinfect

### (B) Outside the hospital

Untreated biomedical waste should be transported only in such vehicles as may be authorized for the purpose by the competent authority as specified by the Government under the Motor Vehicle Act, 1998.

The containers for transportation must be labeled with “Biomedical Waste” symbol on both sides and rear part

Personnel involved in BMW shall wear gloves, glasses, lab coat, boots and masks and also shall be immunized against tetanus & Hepatitis B.

## 3.11.1 PERSONNEL PROTECTION

It is the responsibility of every lab to establish and maintain a safe working environment. There shall be a named person ultimately responsible for the safety of personnel at work and others who may be affected by it. All hazards shall be identified and measures shall be taken to eliminate them or reduce to as low level as practicable by

Substitution,

Containment and

Use of personal protection equipments.

Safety is the primary consideration and cost should be of secondary importance.

### General Safety:

It Includes



Proper use of personal protective clothing/equipment (e.g., gloves, gowns, masks, eye protectors, etc.). Bio-medical wastes shall be disposed off properly along work. Proper handling of spillages (blood and other body fluids).

All occupational injuries or illnesses require medical treatment (except first aid)

## Fire Safety

Laboratory shall

(1) have an automatic fire extinguishing system at all required places.

In all cases, a fire-bell, public address system, or other alarm system must be audible in all sections.

## Electrical Safety

- All laboratory instruments and appliances shall be

Adequately grounded and

- Checked for current leakage (before initial use, after repair or modification, and when a problem is suspected)

This Task may be delegated to biomedical and electrical engineers

## Chemical Safety

- The lab shall have a comprehensive signage and labeling system in use.
- Material safety data sheets (MSDS) shall be on file for each hazardous chemical.
- Each laboratory shall have a written plan to reduce or eliminate mercury.
- Chemical fume hoods shall be checked annually for proper function.
- Piped eyewash fountains or the equivalent shall be present and should be checked weekly.

## Microbiological Hazards

- The laboratory should have policies and procedures for assessing the occupational risk associated with exposure to infectious agents handled in the microbiology laboratory.

Level II bio-safety cabinet should be used for working with infectious agents.

## Waste Disposal

- All solid and liquid waste must be disposed in compliance with applicable local, state, and national regulations.
- All sharps, especially those contaminated with potentially infectious materials, must be properly discarded in puncture-resistant containers with tightly fitted lids.



## Disaster Management

- The laboratory must have documented procedure on “Internal and External Disaster Preparedness.”  
It should be followed in the event of a catastrophe such as fire, flood, electrical outage, or spill of hazardous volatiles (internal disaster), or atornado, earthquake, or other mass-casualty situation (external disaster) and lab shall have
- Proper evacuation plan

## Ergonomics

- “Ergonomics is an applied science concerned with the design of workplaces, tools, and tasks that match the physiological, anatomical, and psychological characteristics and capabilities of the worker.”
- “The Goal of ergonomics is to ‘fit the job to the person,’ rather than making the person fit the job.” Ergotec

## PERSONNEL PROTECTION

Safety of personnel is of primary importance and both management as well as staff are responsible for it. There is international standards ISO 15190 for medical lab safety in which all management and personnel responsibilities are stipulated.

Personnel Protective equipment (PPE) : has been discussed previously

Infection Control Measures Like

- Cleaning, Hand Wash, Personnel Protection Equipments (PPE), Disinfection & Sterilization, Vaccination, Awareness & Training Programme shall be followed appropriately and according to documented procedure.

## Housekeeping

Housekeeping is defined as the provision of a clean, comfortable and safe environment for the patients and Laboratory staff.

## Purpose:

- The regular and routine cleaning of all surfaces and maintaining a high level of hygiene in the facility. Areas of the Laboratory shall be classified according to the varying need for cleaning.



## 3.11.2. HAND HYGIENE

One of the most effective and simple measure for preventing infections is proper hand washing. It prevents transmission of infection between Health care worker's (HCW's) and patients in the hospital and so plays a very important role in hospital infection control

Decontamination of hands is important - before having direct contact with patients, after contact with patient intact skin, after contact with body fluids or excretions, before donning sterile gloves, after removing gloves, after contact with inanimate objects, before eating and after using rest room.

### Hand Hygiene Technique

When washing hands with soap and water.

- Wet hands first with water
- Apply soap solution to hands and rub hands together vigorously covering all surfaces of hands and fingers. After that rinse hands with water and dry thoroughly with a disposable towel

## 3.11.3. MANAGEMENT OF SPILLS

### Introduction

Spilled blood and body fluids/substances and infective agents may be encountered in many situations in Hospitals & Laboratories. These must be attended to immediately.

All body fluid spillage must be cleaned up effectively and immediately. Disposable gloves and aprons should be worn by the person handling the spill. It is advisable to cover wounds and lesions on exposed skin with a waterproof dressing. Mops are never used to clear up body fluid spillages. Hypochlorite solution must be prepared and used within 24 hours. One should ensure that there is adequate ventilation is when using Hypochlorite to clean areas.

### SPILL PROCEDURE

- Gloves and Appropriate Personal Protective Equipment (PPE) like - Protective Eyeware, Lab Coats, Masks and Face Shields where Splashing is Likely shall be used.
- Any broken glass or sharp objects from the spill shall be removed using mechanical means like forceps, hemostats, needle-nose pliers, broom and dust pan.
- Sharps / broken glass shall not be removed by hand.
- Spill is contained by covering with paper towels and carefully pouring appropriate disinfectant solution (1:10 to 1:100 dilution of Sodium hypochlorite) around and on the spill.



- Care shall be taken not to splash disinfectant solution or create aerosols while pouring.
- Paper towels are removed and process shall be repeated until all visual soilage is removed
- Cleaned area is re-wet with disinfectant and air dried
- All contaminated paper towels in a “red bag” or an autoclave bag for appropriate disposal (autoclaving, off site treatment, etc.)
- Then all PPE are removed and immediately hands washed
- A 1:100 dilution (500--615 ppm available chlorine) is used to decontaminate nonporous surfaces after cleaning a spill of either blood or body fluids in patient-care settings
- If a spill involves large amounts of blood or body fluids, or if a blood or culture spill occurs in the laboratory, a 1:10 dilution (5,000--6,150 ppm available chlorine) shall be used for the first application of germicide before cleaning

## Spills on the body

Splashes of body fluid or blood on to the skin must be washed off immediately with soap and water.

## Exercise

1. When all should one decontaminate hands?
2. Describe briefly the spill procedure?

## LIST OF REFERENCES

- (1) Practical Haematology (Tenth edition) - Dacie and Lewis
- (2) Textbook of Medical Laboratory Technology (Second Edition) - Praful B. Godkar & Darshan P. Godkar
- (3) Pathologic Basis of Disease (Seventh Edition) - Robbins and Cotran



## BLOOD BANK AND TRANSFUSION

### Unit overview:

- 1) This unit will provide the students information about the scope of blood bank and the various topics related to blood transfusion.
- 2) It helps to understand the relevant terms, procedure and working of equipment & personnel as relevant to a blood bank.

### Knowledge & Skill Outcomes

- 1) To understand the scope of blood banking.
- 2) To understand the organizational structure of a blood bank.
- 3) To know the relevant term, procedure and working of equipment and personnel as relevant to a blood bank.
- 4) The responsibility of personnel working in a blood bank.

### Learning Outcomes :-

After completing this unit the students should be able to demonstrate knowledge and shall be able to comprehend and apply the techniques related to :-

1. Safe Collection
2. Donor Selection
3. Grouping of Blood
4. Grouping associated problem solving
5. Transfusion related problems

### Material and equipment

#### 1. Equipment for haemoglobin estimation :

- a. Haemoglobinometer

#### HEMOCUE Hb 201 + ANALYSER :-

Used for measuring the hb of blood donors before they donate blood

#### 2. Equipment for blood collection



Blood donor couches

Blood collection monitor

Blood bags

Tube sealer

- a. **blood donor couch** : They are specialized couches so that the donor feels comfortable while blood donation. Generally there is a provision for giving a head low position when the donor is having giddiness.
- b. **blood collection bags** : either 350 ml or 450 ml bags, there are different varieties of bags they are single, double, triple or quadruple.  
**Single bag** : does not have any satellite bags double, triple and quadruple bags have one, two and three satellite bags respectively quadruple bags are also called buffy coat bags and are used for separating the buffy coat while preparing the blood components the bags contain the anticoagulant solution : CPD / PCD - A / SAGM  
Citrate, Dextrose, Phosphate, Saline, Adenine Glucose, Mannitol  
The anticoagulant in 350 ml bags is 49 ml and in 450 ml bags in 63 ml
- c. **blood collection monitor** : used for the actual collection of blood. It can collect the desired volume of blood from the donor which is either 350 or 450 ml. It has a visible volume display and flow rate also. There is a audible alarm if the flow of blood is slow. There is a provision for automatic clamping when the volume is reached. After collection this once again gives an audible alarm.
- d. **tube sealer** : used for clamping the segment of the blood bag after it is collected. Available in either fixed sealer or a mobile sealer. Mobile sealer can be taken anywhere for sealing and is used in blood collection camps.

### 3. equipment for blood component preparation

Electronic weighing machine

Refrigerated centrifuge

Plasma expressor

Cell separator

Sterile connecting device

- a. **electronic weighing machine** : for weighing the bags accurately.
- b. **refrigerated centrifuge** : bucket handle typed of centrifuge to hold the collected bags with a provision for a wide range of temperature is preferred.



The main unit is built on a sturdy metal frame resting on castors and enclosed by sheet metal, which has an electrical interlock. Rotor consists of 4 to 6 buckets.

## Features of cold centrifuge :

1. Digital speed indicator
  2. Stepless speed relulator with 'O' start switch
  3. Digital automatic timer
  4. Dynamic break
  5. Digital temperature indicator cum controller
  6. Unbalance cut out switch
  7. Brush wear indicator
  8. Hermatically Sealed refrigeration unit
  9. Temperature Range 0°C to 300°C
- c. plasma expressor : to manually express the plasma  
d. cell separator

Cell separator is a instrument used into separate what ever components required for the patients.

But in our blood bank we are using mainly for the whatever the large amount of components, platelets required.

Ex. Platelet's are required it is available to use this procedure. This procedure is called as "A-pheresis".

## Two types of process can be done by using cell separator.

1. Continuous flow process
2. Interrupted flow process

**Continuous flow** : It is a two arm procedure where in blood is drawn from one arm. The components are separated in a cart rid & the remaining cells & plasma flow back to the donor through the other area. Here the volume of blood which is outside the body is very small.

**Interrupted flow** : This is a one arm process. One line is connected to the donor the blood will be coming out after processing components will be separator, remaining required plasma & RBC's will be reinfused back to the donor with same line and this process will takes little longer time than the continuous flow.

**e. sterile connecting device** : used to connect ends of two different segments in asterile manner. Widely used for separation of small volumes of blood for paediatric transfusion, buffy coat pooling and lab side leukodepletion.



## 4. equipment used for storage of blood

Blood storage cabinets

Plasma storage cabinets

Ultra low freezers

Cryoprecipitate bath

Platelet agitator and incubator

Plasma thawing bath

### Blood storage cabinets 2-6 deg c+2

1. Blood storage cabinets are constructed of double walls with exterior made of powdered steel steel and the inner is made of stainless steel. It is provided with number of compartments having transparent Plexiglas doors.
2. The cabinets are provided with pull out type trays for proper storage of blood bags.
3. The control comprises of fully automatic digital temperature indications/ controls with off/on switches indicating lamps with protective fuse.
4. It also incorporates electronic safety circuit which gives audio visual alarm in case of power failure.
5. Temperature variation is +/-2C. In case the temperature increases more than 4C or if the door is open protecting. The precious blood bags inside the cabinets.
6. The alarm is set off thereby protecting the temperature maintenance.

### IMPORTANCE :

1. Uniform temperature between 2°C - 6°C (in AC room)
2. Stainless steel inner chamber
3. Digital temperature indicater cum controller with audio visual alarm
4. Inside acrylic door to avoid temperature loss
5. Full view glass doors for observation without disturbing the inside conditions

Plasma storage cabinets (-20 deg to -30 deg c)

The plasma storage cabinet is designed storing of plasma and related blood components at temperatures upto - 20°C.

All models feature integrated control panel with digital temperature indicator cum controller and temperature recorder. Constructed of double walls, the exterior is made of powder coated sheet steel finish while the inner chamber is made of stainless steel.



The unit is mounted on castor wheels for ease of mobility. Plasma Freezers are available in horizontal and vertical versions.

## **SPECIAL FEATURES :**

- Low temperature up to  $-35^{\circ}\text{C}$
- Stainless steel inner chamber
- Extremely efficient insulation to minimise heat loss
- Digital temperature indicator cum controller with audio visual alarm
- Inside acrylic door to avoid temperature loss

## **Ultra low freezers ( $-80\text{ deg c}$ )**

- Ultra low freezers are constructed of double walls, the exterior is made of sheet steel and finished with acrylic enamel paint. The inner chamber is prepared by stainless steel.
- The unit is mounted by on castor wheels for ease of mobility.
- The ultra low freezers are specially designed and suitable for blood bank research laboratories.
- The ultra low freezers are high-tech unite incorporating revolutionary cooling system giving advantage to the users of rapid pull down of temperature.

## **SPECIAL FEATURES :**

- Stainless steel inner chamber
- Extremely efficient insulation to minimize heat loss
- High tech solid state digital indicator com-controller
- Counter balanced door
- Inside acrylic doors to avoid temperature loss

## **Cryoprecipitate bath :**

Cryoprecipitate baths are used for quick thawing of Plasma at  $+4^{\circ}\text{C}$  /  $+37^{\circ}\text{C}$  and improves yields over conventional air thawing methods.

- Cryoprecipitate bath is fabricated out of sheet with a stainless steel inner chamber.
- Refrigeration is achieved by means of hermetically sealed compressor.
- The temperature is controlled by a digital indicator cum controller.

## **Special features :**

- Suitable for 15 plasma bags with rack holder.
- Direct digital temperature indicator cum controller.



- Stainless steel AISI 304 tank resistant to corrosion.
- Circulating pump to ensure uniform temperature within the bath.

**Platelet agitator and incubator** : 22 deg c specifically designed to maintain donor platelets in an even suspension throughout the blood plasma. Platelet Agitator agitates at the fixed speed of 70 to 80 strokes per minute.

**Plasma thawing bath** : It is provided with a metallic basket which can hold the plasma bags upright so that the ports are maintained above the water level. Side to side movement is maintained and the speed is adjustable. The temperature is maintained at 37°C with the help of a thermostat. The water bath is provided with a see through fiber glass lid.

## 5. Equipment and reagents for testing of blood :

**ABO grouping** : cross matching : either test tubes or gel cards or micrplates - depending on the protocol of the blood bank

**Infectitious disease testing** : ELISA reader and washer : either automatic or semi automatic  
Random access analyzer

Equipment for malaria and VDRL testing : respective reagents and strips

Centrifuges to centrifuge the blood samples

Records and Computer systems to enable documentation

### Other equipment :

#### a Autoclave :

An autoclave is used to sterilize various materials in the laboratory by steam sterilization method. Blood units that are sero positive or expired are autoclaved at 120 deg for 20 minutes.

#### b microscope

**Introduction** : A microscope magnifies the image of an object. The modern compound microscope is the most important apparatus in a laboratory. It is a precision instrument and needs careful handling.

The light microscope use white light, either the external sunlight or the internal tungsten filament lamp, as the source of illumination. As viewed under the microscope, objects look dark or coloured, contrasted against a lighted background. In case of the dark field microscope, a special dark field condenser is used, that lights up the object, like stars against a dark sky. This is limited use in the microbiology laboratory for observing spirochetes. The fluorescent microscope uses a special ultraviolet lamp as the source of illumination. A fluorescent dye is attached to the object through laboratory procedures, which glows when exposed to the ultraviolet radiation. Fluorescent antibody testing requires this kind of microscope.



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## Parts of the microscope are :

1. The support system consists of the base or foot rest which also holds the light source
2. The tube or the arm holds the optical system and also the coarse/fine adjustments.
3. The objectives are at the lower end of the tube and the eye pieces are at the upper end.
4. The objectives are attached to the revolving nose piece and can be shifted. The objectives are 10x (low power) 40x (high power) and 100x (oil immersion).
5. Below the objectives is the stage which holds the object/slide. The stage has a central hole through which the light passes. The stage may be fixed or movable. Below the stage is the iris/diaphragm which focuses the light.
6. The eye pieces are also of varying magnification; the commonly used one is 10 xs. The eye pieces are situated in the binocular tube.

## Principles of microscope:

The magnification system is the optical component of the microscope. That magnifies the object placed on the stage. It is done by the combination of two series of lenses. The objective and the eye piece. The objective stays near the object of the tube. The low power objective (10x) is smallest in size, usually with a green ring on the objective for easy identification and when the image is in focus, the low power objective has the largest working distance (5 to 6mm). The working distance is the distance between the front lens and the objective slide. The working distance decreases with increasing magnification and is 0.5 to 1.5mm in case of the high power and 0.15 to 0.20mm in case of the oil-immersion objective. The eye piece magnifies put on a screen.

The total magnification of the object is the multiplication value of the magnifying powers of the objective and the eye piece. Thus with 10x magnification of the eye piece, the low power objective gives  $10 \times 10 = 100x$  total magnification. The high power  $10 \times 40 = 400x$  magnification and for the oil-immersion lens,  $10 \times 100 = 1000x$  magnification. The cost of the lens increases with higher magnification.

When using oil immersion a drop of cedar wood oil is applied directly on the smear.

## c.incubator

Incubators are temperature controlled chamber's which are well insulated. Necessary for various investigations where body temperature.  $30^{\circ}\text{C}$  incubation is required.

## USE :

These are mainly used for

1. Determination of enzyme's in the specimen by end point reaction methods.
2. Determination of glucose, urea, uric acid etc., by enzymatic methods.

3. Growing microorganisms on various culture media.
4. Right temperature for immune antigen reaction.

## d. hot air oven

use :

Hot air oven is mainly used for the following purposes.

1. Dry sterilization of syringes & needles.
2. Preparation of anticoagulated bulbs.
3. Drying of glass ware.
4. Heating of chemicals used for the preparation of primary standards.

## Principle :

When electricity is passed through the heating coils, electrical energy is converted to heat energy.

## Components :

1. Double walled heavy gauge aluminum chamber.
2. Thermal insulation by thick glass-wool between the working chamber and the outer mild steel casting.
3. Thermostat.
4. Adjustable ventilator.
5. Perforated metal shelves.
6. Thermometer.

Temperature Range :-40 °C to 300 °C

The main unit is built on a sturdy metal frame resting on castors and enclosed by sheet metal, which has an electrical interlock. Rotor consists of 4 to 6 bucket's which can add blood bags i.e., either single, double (or) Triple.

## e. Clean air cabinet : (laminar flow cabinet)

70% of the air is recirculated through filters. So that the working area is bathed in clean (almost sterile) air. The air flow carries along any aerosols/particles produced in the course of the work and these are removed by the filters. Some of the air (30%) is exhausted to atmosphere and is replaced by a curtain of room air which enters at the working face. This cabinet is fitted with a UV lamp. The filters need to be cleaned periodically and swabbed every day with disinfectant.



## Records in blood bank

1. **Blood donor record** : it shall indicate the foll :  
Serial number, date of bleeding, name, address and signature of donor, age weight, hb, blood grouping, blood pressure, medical examination, bag number, patient's details incase of replacement donor, category of donation (voluntary / replacement), deferral records, sign of medical officer.
2. **Master record for blood and components** :  
Bag serial number, date of collection, date of expiry, quantity in ml, ABO / Rh, results of testing for HIV, HBsAg, HCV, VDRL, malarial parasite, irregular antibodies if any, name and address of the donor, utilization issue number, components prepared or discarded, sign of the medical officer.
3. **Issue register** : serial number, date and time of issue, bag serial number, ABO, Rh, total quantity in ml, name and address of the recipient, group of recipient, unit/institution, details of crossmatching report, indication for transfusion, components issued, quantity issued, signature of the issuing person.
4. **Records of the ACD, CPD, CPD-A, SAGM bags** having details of the manufacturer, batch number, date of supply, results of testing.
5. **Register for diagnostic kits and reagents used** : name of the kits, reagents, batch number, date of expiry and date of use.
6. **Transfusion adverse reaction reports** :the reports and the investigations for all transfusion adverse reactions are maintained.
7. **Records of purchase, use and stock in hand of disposable needles, syringes, blood bags,** all the records must be maintained for a minimum of 5 years.

## Laws of genetics

Genetics is the study of inheritance - the transmission of characteristics from parents to offspring. In 1865, Gregor Mendel did some experiments with garden peas thereby bringing to light the science of genetics. His studies led to the basic understanding of how genetic traits are passed to each generation.

Humans have 46 chromosomes, in which 22 pairs are autosomes and one set of sex chromosomes (XX and YY).

As a cell divides it reproduces the chromosomes so that all the daughter cells are identical to the parent cell. This process is known as mitosis.

In one set of experiments Mendel cultivated sweet peas until they bred off spring with flowers of all one color (e.g., red or white only). He then cross-bred these two plants and obtained a



first filial generation that were all red flowered. When plants from this generation were bred with each other, they produced red and white flower in a ratio of 3:1. When the traits are co dominant, both are expressed equally. This type of codominant expression is important in blood group genetics such as inheritance of AB group. This is the law of inheritance.

The next law of Mendel is the law of independent assortment. This means that factors for different characteristics are inherited independently from each other (if they reside on different chromosomes).

## Deoxyribonucleic acid (DNA)

Human chromosomes are composed of linear strands of deoxyribonucleic acid (DNA) wound around proteins called histones. This complex of DNA and proteins is called chromatin. It is this wrapping and condensing of DNA that allows so much genetic material to be stored in a small piece of the chromosome.

DNA is composed of four nitrogenous bases, a molecule of deoxyribose, and one phosphate group. The four nitrogen-containing bases are the purines adenine (A) and guanine (G) and the pyrimidines thymine (T) and cytosine (C). Each base can bind to a deoxyribose sugar to form a nucleoside; the addition of the phosphate group makes the compound a nucleotide.

## Ribonucleic acid (RNA)

Composed of nucleotides AND usually exists as a single strand. Other differences include the substitution of ribose for deoxyribose and the nitrogenous base uracil for thymine. Ribose differs from deoxyribose by the presence of a hydroxyl group at the 1 carbon. Uracil differs from thymine by its lack of a methyl group.

## Basics of Immunology

Blood groups are inherited characters which give rise to antigen-antibody systems. Blood group antigens are chemically either glycolipids or glycoproteins and are immunogenic expressions of the components of the red cell membrane. They have a polysaccharide component responsible for the specificity and an amino acid fraction that determines the antigenicity.

## Antigen :

An antigen is any substance, either protein or non-protein but when introduced into an animal causes the production of another specific substance called antibody. The antibody reacts specifically with the antigen.

In blood banking red cell antigens are of importance.



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## Antibody:

Antibodies are serum proteins, more specifically immunoglobulins. Antibodies are recognized by their interaction with antigens. In blood group serology this interaction is usually detected by agglutination of red blood cells.

## Naturally occurring antibodies :

The presence of antibody in serum is not always because of exposure to antigenic stimulus by red cells. Some foreign substances like bacteria and plants (containing red cell like substances) are capable of causing antibody production and these antibodies are called naturally occurring antibodies. These are commonly of IgM type, occur in serum without any specific antigenic stimulus, e.g. anti-A, anti-B, and anti- Wra. These antibodies are present in individuals who lack that antigen.

## Immune antibodies:

Immune antibodies are IgG. They develop due to immunization following pregnancy, previous transfusion or deliberate injection of immunogenic material. In some instances immunogenic event is unknown.

## The antigen-antibody reactions In-vitro

1. **Agglutination** :is defined as clumping of particles that have antigen on their surface and is brought about by anti-bodies. This forms the basis of blood grouping tests.
2. **Hemolysis** :Rupture of red cells with release of intracellular haemoglobin can occur if the Antibody has the property of hemolysin. It requires presence of complement.
3. **Precipitation** : is the formation of visible insoluble complex when soluble antibody reacts with soluble antigen.
4. **Enzyme linked immunosorbent assay (ELISA)** :Here an enzyme label is used and a colour reaction that takes place in presence of a substrate denotes the presence of antigen/ antibody as the case may be. This is the principle sued in Transfusion transmitted disease tests.

## Factors affection agglutination reaction:

1. **The antigen (Ag)** -The red cells should be properly stored to achieve optimum reactivity. If the cells are frozen the reactivity is better preserved. The reactivity is better in fresh cells. Thus with dilute cell suspension, increase in sensitivity is achieved, as concentration of antibody in solution does not fall even when all cells are saturated with antibody. One drop (2-5%) of cell suspension is therefore found ideal to be added to two drops of serum.

## Need to wash the cells :

1. Washing improves reactivity.
  2. Removes plasma that contains fibrinogen and forms clot when mixed with serum giving false positive.
  3. Plasma can cause rouleaux formation.
  4. Anticoagulant present in plasma is anticomplementary and inhibits complement binding reactions.
  5. Plasma contains blood group substances that can neutralize that reaction.
- II. **The antibody (Ab)** from one individual may react differently at different times and sera from different source with same type of antibody react differently. The stored sera lose reactivity and hence the importance of the batch numbers and expiry dates. The speed with which a particular antibody combines with the antigen forming a stable complex is called avidity, which is variable. The reactivity of Ag/Ab depends also on titre. The titre of the antibody is the highest dilution of serum with a positive reaction. Very high titre sera are likely to give prozone effect. The prozone is the inhibition of the reaction in presence of excess antibody.
- III. **Factors depending on reaction condition :**
1. **Incubation time** :Upto 60 minutes is adequate for all blood groups.
  2. **Incubation temperature** :depends on type of antibody.
  3. **Centrifugation** : should be adequate to produce a cell button with a clear supernatant but without packing cells tightly so that they are difficult to dislodge. Over centrifugation leads to false positive.
  4. **Ionic strength** :low ionic strength i.e. low concentration of dissolved salts increases the amount of body binding to cell.
  5. **pH**: Normal blood pH is about 7.42.
  6. **antigen-antibody ratio** : The ratio of antigen and antibody in the mixture should be optimum.
  7. **Zeta potential** :Erythrocytes have a genitive electrical charge. The electrical activity of this ionic cloud is called Zeta potential.

Agglutination is the endstage of all grouping reactions and this can be carried out on slides, tiles with well microtitre plates, capillaries or tubes. The tubes are most advantageous.

**Macroscopically** : After centrifugation, the cells should be gently dispersed to observe for agglutination.



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**There are many causes for false positive reaction such as :**

1. Autoagglutination
2. Bacterial contamination
3. Polyagglutination
4. Chemicals e.g. detergents
5. Cell mixtures
6. Leukocytosis ie wbc count
7. Rouleaux formation-caused by:
  - (i) Presence of large molecular weight substances e.g. fibrinogen, dextran, Polyvinylpyrrolidone (PVP)
  - (ii) Abnormal proteins-e.g. multiple myeloma.

**Causes of false negative :**

1. Deterioration of antigen on storage
2. Chemical contamination
3. Inhibition due to improper washing of cells
4. Absence of complement
5. Thick cell suspension
6. Agammaglobulinemia
7. Poor technique of reading

## **ABO Blood group system**

Landsteiner discovered the ABO group antigens in 1900 and since then this is one of the most important discoveries in the field of medicine.

**The importance of blood grouping :**

1. Safe blood transfusion
2. Organ transplant especially liver, heart and kidney
3. Medicolegal and forensic, paternity disputes
4. Immunology and genetics

Landsteiner's law

The reciprocal relation between ABO antigens and antibodies is called Landsteiner's law

Cell grouping			serum grouping		blood group
anti-A	anti-B	anti-AB	A cells	B cells	
+	-	+	-	+	A
-	+	+	+	-	B
-	-	-	+	+	O
+	+	+	-	-	AB

## The incidence of ABO groups in India

A 27%

B 31%

O 34%

AB 8%

Inheritance of ABO blood groups : A B antigens are inherited as codominant fashion. Each individual inherits two ABO genes from each parent and they determine the ABO antigen present on their red cells. O is an amorph and does not produce A or B substance. The serological typing reveals the phenotype and the family studies help to reveal the genotype.

Phenotype genotype

A	AA
	AO
B	BB
	BO
AB	AB
O	OO

## Biochemistry of ABO system

The A,B antigens are glycoproteins. Each molecule of a precursor is made up of a peptide and a sugar. The blood group specificity is determined by the terminal sugar

Terminal sugar	antigen
L Fucose	H
N Acetylgalactosamine	A
D Galactose	B



**Transferases** :enzymes which assemble individual sugars into chains forming the antigen As each sugar is added on a new structure is formed which acts a substrate for another transferase. The A gene specified transferase adds N acetyl galactosamine, the B gene transferase adds N acetyl galactose to the H gene. The H gene transferase is the L focolysyl transferease. In the AB individual 2 different sugars are added to different chains of the same redcells.

**Secretor states:** The A, B and H substances on erythrocyte surfaces are lipopolyccharides, not water soluble but they can be dissolved in fat solvents, such as alcohol and chloroform. They are most clearly detectable in saliva and also identified in some other body fluids. These people are referred to as secretors. The secretor characteristic is inherited in the classic fashion by a dominant gene designated se. Itsallele se has no effect. Hence noon secretors are sese while SeSe and Sese are secretors. The gene is independent of ABO and Hh.

**Concept of H:** H substance is the precursor substance from which A and B substances are formed by action of the genes A and B. All red cells have some H. but the amount varies according to the blood group. O cell have the maximum and it diminishes in the following order. A2, A1B.

In 1952 Bhende, Bhatia and Deshpande identified a new blood group called Bombay group. The red cells appear to be group O, but their serum contains a powerful antibody reacting with all other red cells except those of the same group. The cells do not contain H antigen

## CONCEPT OF H

A GENE > A AND H ANTIGENS

H SUBSTANCE-B GENE—B AND H

O GENE>H

PRECURSOR\_\_\_\_\_

SUBSTANCE

## PRECURSORABO GENE

### SUBSTANCE INEFFECTIVE Oh

The red cells and secretions of such a person lack H substance and the serum has anti-H Antibody. Since His absent, there is no substrate on which A or B genes can act, even if present, hence, genetically A or B group can result, but the expression of these genes and their secretor status is prevented by the absence of H. these ABO genes when present, even though not expressed, are inherited and transmitted normally. It is suggested that an inhibitor gene is involved to suppress A and B antigens. The presence of this h gene in homozygous



condition result in suppression of all A, B and H antigens on the red cell and saliva. The ABO and secretor genes are however fully expressed in individuals, heterozygous for the H-h gene. The H-h genes are not located on the same chromosome carrying ABO genes. Thus this phenotype is called Oh. Family studies are helpful in confirming the Oh phenotype. In India this is more common compared to other countries. The presence in India is more amongst Marathas of south west district of Maharashtra. The frequency in India is 1:13000.

The phenotype is easily detected by its failure to react with anti-H or positive reaction with all Oh group are no secretors of ABH antigens, Le (a+b-) and rarely Le (a-b-).

Due to the presence of anti H, the patient cannot be transfused with any other blood except Bombay blood.

Recently blood with weak A or weak B, in individuals with antibodies of H antigen is reported and this is explained on the basis of partial suppression and is called Ah-Bh phenotype or Para Bombay type.

## ABO antibodies :

A and B antibodies are naturally occurring antibodies present in the serum of persons who lack the corresponding antigen. At birth these antibodies are not developed. A and B like substances are widely distributed in nature, in plant and animal tissues and in bacteria. Every one is exposed at the time of birth to these substances and by second or third month develop the antibodies. Hence the test on the serum of a new born or an infant upto 6 months are not reliable. In old age again the level of anti-A and anti-B is significantly reduced.

## Sub groups in ABO System

### Subgroups of A:

The cells which are agglutinated by anti-A but not by anti-A1 are A2 antigen and those agglutinated by both are A1 antigen cells. Anti-A1 occurs in the serum of approximate 1 to 8% of A2 and 22-35% of A2B individuals. The weaker subgroup of A are based on

- The reactivity of red cells with anti-A, anti-AB, anti-H and anti-A1.
- The presence or absence of anti-A1 in the serum and
- The secretion of A and H substance by the secretors.

The few important ones are as follows:

	Anti-A	Anti-AB	Anti-A1	Anti-H	Substance in saliva
A int	++++	++++	++	+++	A & H



A 3	++	++	0	++++	A & H
A X (A4/A5/ A6/A0A2)	Mixed field 0/+w	+/>++	0	++++	H
Am	+W	+W	0	++++	A & H

These subgroup are at times difficult to detect, and they pose a problem in case of the donor grouping. A donor unit of weak A may be grouped as O and if issued to an O group individual could cause a transfusion reaction. For recipient's grouping if it is missed, it does not matter, as the recipient can receive O group blood without any ill effects.

## Subgroups of B

The B group antigen also has similar weaker variants which are much less common (B2, B int, B3 Bx, Bm).

It has been noticed that the strength of the ABO antigens changes in the disease state. The weakening of A antigen has been reported in cases of leukemia, the depression is related to the course of the disease and thus during remission the reactivity appears to be normal. Report of suppression of A antigen in aplastic anemia, Hodgkin's disease and in osteogenic sarcoma have been reported.

The other fascinating phenomenon noticed is the acquired B antigen. Many cases have been reported, associated with carcinoma of colon and rectum, appendicitis, peritonitis, bacteremia and urinary tract infections. It is presumed that the change is caused by the pathogenic bacteria which modify the red cell membrane or the bacterial lipopolysaccharides which are similar to B group antigen (especially of *E. coli* O86) get passively adsorbed on the red cells.

Modifications of ABH antigens during the course of pregnancy have also been reported. The loss of antigenicity is more towards the delivery. Women on contraceptives for over a period of 9 months also show a similar loss of antigens. The loss is apparently 20% for A and B and 35% for A1 and H antigens. The L-D-galactosyl transferase enzymes in sera of B group women are found to decrease in pregnant women reaching their lowest at term. The changes are also observed during the menstrual cycle which relate to the hormonal levels.

## PRACTICAL IMPORTANCE OF OTHER BLOOD GROUPS

### I blood group system

Discovered in 1956

All adult red cells have I antigen and cord blood has I antigen

By the time the infant is 18 months old the red cells have I antigens



## Clinical significance :

Anti-I antibody is usually a cold antibody, occasionally reacts at 37 deg to cause hemolytic disease of newborn or hemolytic reaction

## Duffy system

Duffy system contains two antigens Fya and Fyb

Plasmodium vivax infection does not affect red cells lacking Fya and Fyb

antiFya and Fyb antibodies are IgG and react at 37 deg c and cause hemolytic reaction and hemolytic disease of the newborn (HTR and HDN)

## duffy antigens are classified as

Fy (a+b-)

Fy (a+b+)

Fy (a-b+)

Fy (a-b-)

## Kidd blood group system

Two antigens Jka and Jkb

The antibodies may be either IgG or IgM and may cause HTR or HDN

## P system

Three antigens P1, P2 Pk are present for most of the population

Few case of HDN have been attributed to anti-P1

Hydatid cysts contain P1 substance

The donath Landsteiner autoantibody found in cases of PCH (Paroxysmal Cold Hemoglobinuria) shows anti-P1 specificity

## MNSs system

Anti-M and anti-N are cold antibodies rarely they may react at 37 deg and cause HTR or HDN

S and also may react at 37 deg and cause HTR

## LEWIS system

Two main antigens are Le a, and Le b and the phenotypes are

Le (a+b+), Le (a-b+), Le (a+b-), Le (a-b-)



Secretors are either Le a+b+ or Le a-b+

Non secretors do not have the Le b antigen

## **KELL SYSTEM**

The Kell system was discovered by Coombs and Mourant in 1946. The Kell antigen is strongly immunogenic. It is known to cause many transfusion reactions and Haemolytic disease of the newborn. Incidence of Kell antigen in India is reported as 0.3 to 0.4%

Antibodies of this Kell system anti K and anti c are always immune and are best detected by indirect antiglobulin test.

## **LUTHERAN SYSTEM**

The original antigens described were Lua and Lub.

Anti Lub has been reported to be mainly IgA. Anti Lua has not been incriminated as a cause of hemolytic disease of the newborn or transfusion reaction.

## **Rh NULL**

Rh Null is a clinically significant member of Rh system which is responsible for Haemolytic anemia in individuals who possess them.

Rh null people are usually observed when Rh positive blood is transfused. In some cases of pregnancy.

**-D-**

Only -D- antigen is found and these cells lack Cc and Ee.

For transfusion the same phenotype only will be compatible. Transfusion of other phenotypes where C/c and E/e will result in antibody formation and hemolysis.

## **Rhesus blood group system**

Land Steiner and Wiener discovered this system in 1940 after Levine and Stetson in 1939 reported an irregular antibody in a mother which was later shown to be anti-D.

Rh antigens are found only on red cells. The Rh antibodies usually develop only in absence of Rh antigen, by a known stimulus, e.g. transfusion or pregnancy.

Rho (D) is the most immunogenic factor and therefore unless specified Rh positive and negative denote D positive or D negative. The incidence of Rh negative amongst India's is approximately 5%.

Rhesus system is comparatively more complex. Wiener postulated that there are multiple allelic genes.



There are five main antigens reported and the various gene combinations are as follows:

The Rh genes are found to reside on chromosomes 1; and they behave as autosomal dominant characters

Fisher	Wiener
CDE	R1
Cde	R2
cDe	R0
CDE	Rz
Cde	R
Cde	r'
cdE	r''
Cde	rY

For determining genotypes five different antisera D, C, c, E and e should be used. In case of D negative it is easy to denote genotype but in case of D positive it is relatively difficult to differentiate homozygous and heterozygous without help of tables of gene frequencies, as serologically anti-d is non existent.

D is the weak expression of D antigen. The cells which are not immediately agglutinated by anti-D sera cannot be easily classified as D Negative because some of these agglutinate after addition of antiglobulin sera. This weak reactivity is termed as Du. The genetically transmissible Du is more common in blacks and is transmitted in Mendelian dominant pattern of inheritance. The gene in this case appears to be Ro (cDe) and this is referred as low grade Du represents CDe gene which is due to the position effect and is commonly seen in whites, this is referred to as high grade Du.

The Du positive cells are likely to elicit an immune response in D negative individuals and the Du cells could be destroyed if the recipient is already immunized. Therefore Du positive donor is treated as D positive, and a recipient is treated as D negative. Hemolytic disease of the newborn has also been reported in a D negative mother with D antibodies due to earlier Du positive baby.

## Rh antibody titres

An Rh negative mother with an Rh positive foetus may get alloimmunised with anti-D and cause hemolytic disease of the new born. Rh antibody titres are done in the antenatal period to rule out such a condition.



## PRINCIPLE :

Titration is a semi-quantitative technique of measuring the concentration of an antibody in a serum. The titre of an antibody is usually determined by testing two fold serial dilution of the serum against selected red cells.

## SAMPLE :

4 to 5 ml clotted blood.

REAGENTS : SALINE

AGH

Pooled Cells.

## METHOD :

Label a row of tubes according to serum dilution 1 to 10 (1 : 2 to 1 : 5 12)

Place 1 volume (0.1 ml) or 1 drop of saline in all tubes except the first.

Add 1 volume (0.1ml) or 1 drop of serum to tubes 1 and 2 so that the first tube contains neat serum (1:1) and 2nd tube has 1 volume of serum in volume of saline (1:2). Using a clean pipette mix the contents of tube 2 (1 : 2 dilution) without forming any bubbles and transfer one volume of mixture to tube 3 (1:4).

Continue the same process through all dilutions, Remove 1 volume from last tube and save for use if further dilutions are required.

Add 1 volume of 2-5% saline suspended appropriate red cells to each tube. (For Rh antibody titration use 'O' positive pooled cells).

Mix well and incubate at RT for 60 minutes (IgM antibodies) and centrifuge all tubes at 1000 rpm for 1 minute.

Gently dislodge the cell button and record results using grades of agglutination reaction.

The last tube showing positive reaction is considered as the titre of the antibody.

For detection of IgG antibodies : arrange a 2nd row of tubes with the same serial dilution.

Incubate at 37 C. Centrifuge and remove supernatant, incubate at 37C for 45 minutes. Wash with saline thrice.

Arrange fresh tubes and add 1 drop of AHG and add the corresponding washed cells. Incubate at room temperature for 5 minutes, spin at 1000 rpm for 1 minute and look for clumping.



## INTERPRETATION :

If there is clumping in first row of test tubes, it indicates the presence of saline antibodies or IgM.

If there is clumping in the second row of test tubes in indicates the presence of IgG antibodies.

The tube which shows minimum clumping shows the titration value; for eg: if third tube shows clumping then the titration value is 1 in 8 dilution positive.

## RESULT REPORTING :

-----  
Titre 1:1 1:2 1:4 1:8 1:16 1:32 1:64 1:128 1:256 1:512  
-----

Reaction 4+ 4+ 4+ 3+ 2+ 2+ 1+ - - -  
-----

## Question :-

Q1. Why Rh antibody titres done in pregnant ladies?







## CYTOPATHOLOGY

### OVERVIEW AND DESCRIPTION

#### OVERVIEW

This unit will provide the student information about the scope of cytopathology and the organizational structure of a cytopathology laboratory. It will help to understand the relevant terms. Procedures and working of equipments pertaining to cytopathology.

#### Organization of Cytopathology Laboratory:

The personnel needs of a laboratory depends on overall work load and the different types of cytology materials to be processed.

The chief of the laboratory should be a cytopathologist / pathologist or a gynecologist / medical officer trained in cancer related cytology.

The chief of the laboratory should be a cytopathologist / pathologist or a gynecologist / medical officer trained in cancer related cytology.

The Cytotechnologist is a well trained and certified person who should have undergone one year cytology training from a recognized, accredited centre or should have passed National Examination for cytotechnologists conducted by Indian Academy of Cytologists (IAC), after graduation/post-graduation in any of the life science subjects. The duties of the cytotechnologist include preparation of stains and maintenance of its quality, processing of cytopathology material, screening of smears and formulation of preliminary diagnosis. They are also responsible for supervising the record keeping, analysis of data and slide filing system.

Cytotechnicians should have a diploma in medical laboratory technology from a recognized institution and must have undergone 6 months training course for cytotechnicians from an accredited laboratory or passed the National Examination for cytotechnicians conducted by IAC. They are responsible for specimen collection, cytology preparation and staining. Support Staff include clerical and secretarial workers in the laboratory. Physical Infrastructure of the laboratory must be well designed and conveniently located to enable the professional and support personnel to perform their duties effectively. It must contain four definitely separated areas:



- Reception
- Specimen collection room
- Processing and staining area
- Reporting room

## Receiving of specimens

- Ensure that the specimen is properly labeled and submitted along with the specific requisition form which has detailed patient history including the previous Cytology / Histopathology reports, if any.
- Verify the patient's name, age, registration details, history and site of specimen collection. Mismatches, if any, are to be reported to the referring doctor.
- Give a unique accession number to the specimen.
- The number of slides received from each site should be mentioned in the requisition form.
- Nature and method of sample collection are to be mentioned in the requisition form. (cytobrush / spatula / swab / for gynaecological smears and plain / guided FNAC for aspiration smears).
- Check whether the fixation is proper (mention type of fixation: alcohol / spray fixative / prefixed / air dried).
- Enter relevant patient details (name, age, sex, address, brief clinical details, and name of referring hospital / doctor) in the register.

## KNOWLEDGE AND SKILL OUTCOMES

- i) Understand the scope of cytopathology.
- ii) Know the organizational structure of a cytology laboratory.
- iii) Know the relevant terms, procedures and working of equipments pertaining to cytopathology.

## RESOURCE MATERIALS

- George L. Wied, Catherine M. Keebler, Koss L.G, Regan JW, Compendium on Diagnostic Cytology, Eighth Edition, Tutorials of Cytology. Chicago, Illinois, USA, 1997.
- Svantle R. Orell, Gregory F. Sterrett, Max N-I. Walters: Manual and Atlas of Fine Needle Aspiration Cytology. 4th ed. Churchill Livingstone; Elsevier; Churchill Livingstone, Edinburg, London, Madrid, Melbourne, New York and Tokyo 2005.
- Koss LG, Melamed MR. editors. Koss' diagnostic cytology and its histopathologic basis. 5th ed Vol 2. Walnut street, Philadelphia: Lippincot 2006.



## DURATION

### LEARNING OUTCOMES AND OBJECTIVES

After completing this unit the students should be able to

1. Demonstrate knowledge, comprehension, and application of general techniques in the areas of cytopathology including:
  - Specimen accessioning
  - Fixation of specimen
  - Processing of specimen
  - Staining, cover slipping and labeling of slides
  - Storage and archiving of slides
2. Set-up, operate and maintain routine cytology equipments.
3. Solve basic problems associated with reagents and methods relevant to general cytology techniques.
4. Apply principles of lab safety in completing all laboratory work.
5. Ensure quality control while performing general cytology procedures.

### 6.1 INTRODUCTION TO CYTOPATHOLOGY

Diagnostic cytopathology involves the interpretation of cells that spontaneously exfoliate or are removed from tissues by abrasion or fine needle aspiration, such as specimens from the cervix (Pap test), breast, thyroid, lymph node, liver, etc. Two broad categories of samples are received in the cytology laboratory:

1. Exfoliative cytology: It is the study of cells that have been shed or removed from the epithelial or mesothelial linings. Normal cells are cohesive in nature, but malignancy and infection increase exfoliation. Malignant cells show reduced intercellular adhesion due to defective desmosomes. These cells can be recovered either from natural secretions. Such as urine, sputum, vaginal, and prostatic fluids, or by artificial means, such as paracentesis or lavage of fluids like pleural, pericardial, cerebrospinal, synovial, ascetic, CSF, cyst fluid, bronchial washings etc.
2. Fine needle aspiration cytology includes aspiration done by the pathologist or the clinician as well as guided aspiration done by the radiologists and aspirations. It is a diagnostic procedure used to investigate pathological lesions in organs that do not shed cells spontaneously. In this technique, a thin, hollow needle is inserted into the lesion (usually a lump or a swelling) to obtain cells and tissue fragments, which, after being stained, are examined under a microscope.



Note: Consent form available in local language and in English should be signed by the patient prior to the procedure. Procedure details are explained to the patient by the consultant pathologist/radiologist.

## 6.2 EXFOLIATIVE CYTOLOGY

Common sites for exfoliative cytology

1. Body Fluids
  - (a) Pleural
  - (b) Pericardial
  - (c) Peritoneal
  - (d) Synovial
  - (e) Cerebrospinal
2. Surface Epithelia
  - (a) Female genital tract
  - (b) Respiratory tract
  - (c) Nasopharynx
  - (d) Larynx
  - (e) Gastrointestinal tract
  - (f) Urinary tract
  - (g) Nipple discharge
3. Buccal Smear

### Female Genital Tract (FGT)

The cytological specimens collected from FGT include cervical smear, vaginal smear, aspiration from posterior fornix of vagina (vaginal pool smear) and endometrial smear.

Cervical smear: Cancer of the uterine cervix is the commonest cancer in the FGT. Almost all invasive cancers of the cervix are preceded by a phase of preinvasive disease, which demonstrates microscopically a continuing spectrum of events progressing from cervical intraepithelial neoplasia (CIN) grade I to III including carcinoma in-situ before progressing to squamous cell carcinoma. This progressive course takes about 10 to 20 years. Early detection even at the preinvasive stage is possible by doing cervical smear (Pap Smear Test). This can identify patients who are likely to develop cancer and appropriate interventions may be carried out.



## Advantages of Pap Smear:

- It is painless and simple
- Does not cause bleeding
- Does not need anesthesia
- Can detect cancer and precancer
- Can identify non-specific and specific inflammations
- Can be carried out as an outpatient procedure

Sampling Devices: the collection device may play an important role in sample adequacy. The shape, surface, texture and material of the device may determine how much of the scraped material is deposited on to the glass slide and is available for screening and analysis. Several methods of obtaining cytologic material from the uterine cervix are available. However, use of cotton swab for collection of cervical smear is to be discouraged, in view of the drying artifacts and loss of cells, which are caused by this method. Smears obtained with original Ayre's spatula are often easier to screen. Wooden spatula is preferable to plastic spatula, because of its mildly rough surface that can collect more material. The disadvantages are that the method may occasionally be traumatic to the patient, and the tip of spatula that does not fit the external os may fail to remove some of the valuable material from the squamo-columnar junction.

Endo-cervical brush is a small bottlebrush like device with one end having fine bristles made up of nylons. This device is strictly for taking materials from endocervix. Gently insert the brush in endocervix and rotate one turn pressing in the upper and lower wall (Figure 3). The cytobrush is similar to that of endocervical brush except that the projected tip is without bristles. This can be used for obtaining cells from the whole cervix. Single sampling devices and methods have their limitations in obtaining adequate smears from the cervix. A combination of two devices, usually spatula and endocervical brush, give better results. Triple smear or the vaginal-cervical-endocervical (VCE) technique can provide the best results. However, feasibility and cost factor need to be taken into consideration.

In postmenopausal women. The squamo-columnar junction recedes making it difficult to obtain good amount of endocervical cells and cells from TZ.

Hence a combination of two devices, spatula plus endocervical brush is preferred.

Preparation of Smear: After smear collection, the cellular sample is evenly smeared on to the centre of the non-frosted area of the glass slide, by rotating both sides of the scrape end of the spatula in multiple clockwise swirls in contact with the slide and fixing it immediately. Excessively thin or thick smears can result in false-negative reports. The smear should be visually inspected after fixation. If it does not appear satisfactory, repeat it during the same examination and submit both slides for cytological examination. Some studies have shown



that two-slide cervical smears detect more abnormalities than a one-slide smear. Two smears do increase screening costs over a single-slide smear, but those costs are not double that of a single-slide examination. A two-instrument collection on a single slide increases screening time only minimally over a single instrument.

**Vaginal smear:** Introduce an unlubricated speculum, scrape the lateral vaginal wall at the level of cervix with a spatula. The broad and flat end of Ayre's spatula is used for this purpose. The cellular material is rapidly but gently smeared on a clean glass slide and the smears are fixed immediately. If no spatula is available a cotton swab dipped in normal saline can be used.

**Vaginal pool Smear:** The aspiration can be performed after the introduction of unlubricated speculum. The technique allows collection of cells under direct vision from posterior fornix pool. When a speculum is not employed the pipette is gently introduced in to the vagina until resistance is encountered. It is important to compress the suction bulb during the introduction of the pipette to avoid collecting the cellular material of the lower vaginal organ. The cellular material is spread on a clean glass slide and fixed immediately.

**Endometria aspiration smear:** After preliminary visualization and cleaning of cervix a sterile cannula is introduced into the uterine cavity and aspiration is then carried out with a syringe. The specimen is squirted on a clean glass slide, gently spread and rapidly fixed.

## Respiratory Tract

Respiratory tract malignancies can be detected mainly by sputum cytology or by bronchoscopic material.

**Sputum Cytology:** Sputum specimen can be obtained from the patient either spontaneously or by aerosol - induced method. Morning specimen resulting from overnight accumulation of secretion yields best results. Three to five consecutive days' sputum samples should be examined to ensure maximum diagnostic accuracy. Fresh unfixed specimens are better than prefixed specimens in 70% ethyl alcohol or coating fixative such as carbowax or saccomano fixative. (Fixation of slides is discussed in a separate chapter). The sputum must be carefully inspected by pouring the specimen into a petri dish and examining on a dark background. Select any bloody, discolored or solid particles, if present, place a small portion of each particle on a micro slide, spread evenly and fix it immediately. Prefixed specimens should be smeared on albumen or polylysine coated slides.

**Bronchoscopic Specimens:** Specimens that are obtained by bronchoscopy are secretions (bronchio-alveolar lavage), direct needle aspirate from suspicious area and bronchial brushing and washings. Post bronchoscopic sputum is one of the most valuable specimens for the detection of pulmonary lesions.



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## Other Sites

- Oral lesions: Scrape the lesion with a tongue depressor, spread material on a clean slide and fix immediately.
- Nasopharynx: Cotton tipped applicator is used to obtain material for cytological examination.
- Larynx: A cotton swab smear of larynx may be a useful adjunct to clinical diagnosis if biopsy is not contemplated.

## Fluid for cytology

- On receiving, the sample is checked for labels and details to match it with the requisition.
- Samples are processed immediately on receipt and must be refrigerated at 2°C to 4°C if there is a delay.
- Physical properties of the fluid are recorded on the requisition from i.e., volume, colour, turbidity, presence of clot, etc.
- All fluids except CSF receive in small quantity (<2ml) are processed on the regular centrifuge.
- If the fluid received is a large volume, the top portion is decanted into a second container and the bottom portion is processed.
- 5 to 10 ml of the fluid (depending on the volume received) is taken in a test tube and centrifuged at 3000 rpm for 10 minutes.
- The supernatant is emptied into the main container.
- The sediment is removed with the aid of a plastic pipette, and a drop each is placed on a minimum of three slides and a thin smear made. A drop of sediment is placed towards one end of the slide. A second glass slide is held at an angle of 45 degree to the first slide and with gentle pressure, the second slide is drawn out to make a tongue shaped smear.
- Smear is numbered using a diamond pencil.
- At least one smear is immediately wet fixed in 95% alcohol. The remaining smears are air dried.
- The wet smears are stained with Pap stain and H&E, and the air dried smears are stained with Giemsa. Special stains are put up if required.
- Cell block is made from sediment left behind in all cases.

## Procedure for hemorrhagic fluids

- Frankly hemorrhagic fluids are centrifuged like all fluids and fish tailed smears made from the sediment of the centrifuged deposit.

- Alternatively, if very hemorrhagic, smears can be air dried and then flooded with normal saline for 30 seconds. This causes lyses of red cells, smears are then air dried or wet fixed and stained by pap and Giemsa stains respectively.

## 6.3 FNAC

### Equipment required

- Aspiration is done using disposable needles of 21 gauge (external diameter approximately 0.6-1.0mm) attached to a 20ml syringe.
- The FNAC needles are available in a variety of lengths. Lengths of a to 1/2 inches are found to be adequate for most palpable masses.
- The 3 1/2 inches 22 gauge disposable needle is used for deep seated soft-tissue masses.
- Ultrasound or computerized tomography (CT) guidance can be utilized, whenever indicated.
- Multiple aspirates should be undertaken from different areas, in case of a large tumor, to improve the yield and overcome the problem of tumor heterogeneity.

### Technique

- Taking all aseptic precautions, the lump is palpated and localized, and the site of puncture determined.
- The lump is then immobilized with the left hand in a position favorable for needle aspiration and holding the syringe by the barrel in the right hand; the needle is pushed into predetermined site of the lump until needle tip penetrates the center of the lump.
- The plunger of the needle is then retracted backward to create a negative pressure inside the syringe and needle bore; and without withdrawing the needle through the skin, the syringe is rotated and moved in and out through the lump whilst negative pressure sucks cells into the lumen of the needle.
- In order to obtain sufficient material, particularly from fibrotic lesions, the needle is moved back and forth three or more times and directed into different areas of the tumor.
- Throughout this manipulation, negative pressure is maintained in the syringe by keeping the piston retracted.
- After completion of the aspiration, the pressure in the syringe is allowed to equalize before the needle is withdrawn from the lesion.
- This is achieved by releasing the piston of the syringe.



- After the needle had been withdrawn, the syringe is disconnected from the needle, filled with air and reconnected.
- The material in the needle is expelled onto a glass slide, care being taken to deposit it as a single drop at one end of the slide.
- The needle tip is then brought into light contact with the slide and the aspirate carefully expressed from it.

## Preparation of smears

- The aspirated material (tissue fragments, semisolid or fluid) is collected at the edge of the slide and gently crushed by pressure with another slide/cover slip and spread.
- Excess blood or tissue fluid in the aspirate is dealt separately with a centrifuge preparation; if required. A “hematologic-type” smear is first prepared by touching the droplet with the edge of a cover-slip.
- A minimum of six smears should be prepared in all cases. Half the smears should be fixed in 95% ethanol and half air-dried.
- Air-dried smears are stained with May-Grunwald-Giemsa (MGG) stain.
- Alcohol-fixed smears are stained with Haematoxylin and Eosin (H&E) and Papanicolaou stain.
- A cytology number is marked on all the slides received using a diamond pencil.
- The smears are evaluated for detailed cytological features.
- If fluid is aspirated, the physical details (volume, color and turbidity) are recorded and the fluid is processed as described in section on fluids.
- If solid particles are present, they are placed in 10% neutral buffered formalin and processed as a small biopsy sample/cell blocks.
- During the FNAC procedure, smears are checked, if required, by the concerned consultant for adequacy by using the field stain.

## Procedure the preparation of cell blocks

- Cell blocks are made from all fluid aspirates received where sediment is present.
- Fluid received is centrifuged at 3000 rpm for 10 minutes.
- Smears are made and stained.
- To the sediment, approximately double the volume of Bouin’s fluid is added followed by one drop of egg albumin.
- This is then centrifuged at 200 rpm for 10 minutes.



- Supernatant is poured off and the button is transferred with forceps to formalin for 4 to 6 hours, after which it is taken for processing.

## 6.4 Cytological Fixatives

- It is critical to fix cytology specimens immediately after collection for proper preservation of the cellular components. It is important that no air-drying occurs prior to fixation. If a smear is already air-dried it should not be put in an alcohol fixative. Please note on the requisition if the slide (s) being submitted are fixed or air-dried.

### Properties of a good cytological fixative:

- It should not excessively shrink or swell cells.
- It should not distort or dissolve cellular components.
- It should help preserve nuclear details.
- It should improve optical differentiation and enhance staining properties of the tissues and cell components.

### Cytological fixatives are classified into

#### A. Routine Fixatives

Freshly prepared smears can be immediately submerged in a liquid fixative. This is called wet fixation and is the ideal method for fixing all gynecological and non-gynecological smears. Any of the following alcohols can be used:

1. 95% Ethyl Alcohol (Ethanol): The ideal fixative recommended in most of the laboratories for cytological specimens is 95% ethanol alone. It produces optimal nuclear details but some amount of cell shrinkage. Absolute (100%) ethanol produces a similar effect on cells. But is much more expensive.
2. Ether alcohol mixture: This fixative was originally recommended by Papanicolaou. It consists of equal parts of ether and 95% ethyl alcohol. It is an excellent fixative, but ether is not used in most of the laboratories because of its safety hazards, odour and hygroscopic nature.
3. 100% Methanol: 100% methanol is an acceptable substitute for 95% ethanol. Methanol produces less shrinkage than ethanol, but it is more expensive than ethanol.
4. 80% Propanol and Isopropanol: Propanol and Isopropanol cause slightly more cell shrinkage than ether-ethanol or methanol. By using lower percentage of these alcohols the shrinkage is balanced by the swelling effect of water on cells.
5. Denatured alcohol: It is ethanol that has been changed by the addition of additives in order to render it unsuitable for human consumption. This can be used at a concentration



of 95% or 100%. One formula is 90 parts of 95% ethanol + 5 parts of 100% methanol + 5 parts of 100% isopropanol.

**Time of Fixation: Minimum 15 minutes fixation Fixation prior to staining is essential.**

## B. Coating Fixatives

- Coating fixatives are either aerosols applied by spraying or a liquid base, which is poured onto the slide. They are composed of an alcohol base, which fixes the cells and wax like substance, which forms a thin protective coating over the cells e.g. Carbowax (Polyethylene Glycol) fixative. Diaphine fixative Spray coating fixative (Hairspray) with high alcohol content and a minimum of lanolin or oil is also an effective fixative.
- 10 to 12 inches is the optimum distance recommended for aerosol fixative.
- Aerosol sprays are not recommended for bloody smears, because they cause clumping of erythrocytes.
- Prior to staining, the slides have to be kept overnight in 95% alcohol for removal of the coating fixative.

## C. Special Purpose Fixatives

- Carnoy's fixative: This is a special purpose fixative for haemorrhagic samples. The acetic acid in the fixative haemolyses the red blood cells. It is an excellent nuclear fixative as well as preservative for glycogen but results in considerable shrinkage of cells. Carnoy's fixative must be prepared fresh when needed and discarded after each use. It loses its effectiveness on long standing, and chloroform can react with acetic acid to form hydrochloric acid.
- AAF Fixative: This is the ideal fixative used for cellblock preparation of fluid specimens.
- Saccomanno collection fluid: A green colored fixative of the collection of sputum.
- Cytolyt solution: This is a clear water based buffered fixative for the collection of fluid specimens. A 50:50 ratio of specimen to fixative is appropriate (if this unavailable use 50% alcohol).
- 50% Alcohol: This is a clear fixative for the collection of fluid specimens. A 50:50 ratio of specimen to fixative is considered appropriate.

## 6.5 Cytospin

- Used for CSF, small volume of fluid samples and occasionally for larger volumes.
- Two drops of fluid or of re-suspended sediment (for larger volume fluids after regular centrifugation as above) are placed in the special tubes and capped.
- A minimum of four tubes are taken for each sample.



- Filter paper cards placed between the slide and tube and clipped.
- Tubes balanced in the cytopspin.
- The basin is closed with the cover, the main lid shut and instrument programmed for 1000 rpm for 10 minutes.
- A bleep sounded indicates the end of the procedure.
- The main lid and the basin cover are opened and the tubes removed.
- Two slides are immediately wet fixed in 95% alcohol (for Pap stain) and two are air dried.
- Cytology numbers are assigned to the slides using a diamond pencil.
- The fluid remaining after the processing is retained till the reporting is over.



Figure 13: Cytospin (courtesy Thermoshandon)

### Procedure for disinfection of plastic reusable cytopsin cuvettes

- Cuvettes are immersed in 4% hypochlorite solution for 1 hour.
- Latter washed with soap water.
- After washing, dried and reused.

## 6.6 STAINING PROCEDURES IN CYTOLOGY

Papanicolaus stain: Routine stain in cytology used for all cytology samples.

Solutions used:



- a. Orange G
- b. EA 50
- c. Harris's Haematoxylin

Haematoxylin	5g
100% isopropyl alcohol	50ml
Ammonium/potassium alum	100g
Distilled water	1000ml
Mercuric oxide, red	2.5g

Dissolve the haematoxylin in the alcohol and the alum in the water with the aid of heat. Remove from heat and mix the two solutions. Bring to a boil as rapidly as possible (limit the heat to less than one minute and stir often). Remove from heat and add the mercuric oxide slowly. Reheat to a simmer until it becomes dark purple. Remove from heat and plunge the vessels into a basin of cold water until it cools. Addition of 2-4 ml of glacial acetic acid per 100ml of solution increases the precision of the nuclear stain. Filter before use.

### Procedure (manual):

Smears are fixed for a minimum of half an hour in 95% alcohol before staining.

Agitation is important during staining.

- |                                 |            |
|---------------------------------|------------|
| - 95% alcohol                   | - 10 dips  |
| - 80% alcohol                   | - 10 dips  |
| - 70% alcohol                   | - 10 dips  |
| - 50% alcohol                   | - 10 dips  |
| - Wash in running tap water     | - 1 min    |
| - Harris's Hematoxylin          | - 5 min    |
| - Wash in running tap water     | - 2 min    |
| - Decolorize in 1% acid alcohol |            |
| - Wash in running tap water     | - 2 min    |
| - 1% Liquid ammonia             | - Few dips |
| - Wash in running tap water     | - 2 min    |
| - 50% alcohol                   | - 10 dips  |



- 70% alcohol
  - 80% alcohol
  - 95% alcohol
  - OG - 6 Solution
  - 95% alcohol
  - 95% alcohol
  - EA - 50 solution
  - 95% alcohol
  - 95% alcohol
  - Xylene - I
  - Xylene - II
  - Slides mounted with DPX/Entellan and labeled.
- 10 dips
  - 10 dips
  - 10 dips
  - 5 min
  - 10 dips
  - 10 dips
  - 5 min
  - 10 dips
  - 10 dips
  - 10 dips - 2 min
  - 10 dips - 2 min

## Results:

Nuclei	:	Blue/Black
Cytoplasm		
Non-keratinizing squamous cells	:	Blue/Green
Keratinizing cells	:	Pink/Orange

## Solution changes:

- 350 ml glass staining dishes are used.
- All solutions changed every 7 days; change recorded in register.
- Solutions can be topped up if level drops.

## Haematoxylin and Eosin stain (manual):

Solutions: Refer to procedure in histopathology.

## Procedure:

- Fix the smears in 95% alcohol for 30 min
- Wash in running tap water
- Stain in haematoxylin for 5 min
- Wash in running tap water



- Decolorize in acid alcohol
- Wash in running tap water
- Ammonia water - 1 dip
- Wash in tap water
- Stain in eosin for 2 min
- Dip in 100% alcohol for 2 min
- Dip in 100% alcohol for 2 min
- Dip in 100% alcohol for 2 min
- Dip in acetone for 2 min
- Dehydrate and mount with DPX

## Results:

Nuclei : Blue/Black  
Cytoplasm : Varying shades of pink

Giemsa

## Solution:

- Readymade Giemsa - 500ml
- Working solution (prepared fresh every day): 5 ml of Giemsa solution diluted with 45 ml of distilled water.

## Procedure:

- This stain is performed on air dried smears.
- The smears are appropriately assigned a cytology number using a diamond pencil.
- Air - dried smears are fixed in methanol for 10 min.
- Smears are placed on the staining rack and flooded with the working solution for 25 min.
- Wash in running tap water
- Allow to dry at room temperature

## Results:

Nuclei : Blue  
Cytoplasm : Pale blue/Grey; pink if metachromatic



## 6.7 MAINTENANCE OF STAINS AND SOLUTIONS AND GENERAL PRECAUTIONS

- Stains keep longer if they are stored in dark colored, stoppered bottles.
- All solutions and other stains should be filtered daily after use, to keep them free of sediment.
- Avoid contamination from one smear to another.
- Keep stains and solutions covered when not in use.
- All dishes should be washed daily.
- OG and EA stains lose strength more rapidly than hematoxylin and should be replaced each week or as soon as the cells appear without crisp staining colors.
- Bluing solution and HCl should be replaced at least once daily.
- Water rinses should be changed after each use.
- Alcohol used for the process of dehydration prior to the cytoplasmic stains may be replaced weekly.
- The absolute alcohols should be changed weekly and can be kept water free by adding silica gel pellets.
- Xylene should be changed as soon as it becomes tinted with any of the cytoplasmic stains. Xylene becomes slightly milky if water is present in it and if so the clearing process may be disturbed.

## 6.8 STORAGE AND ARCHIVING OF SPECIMENS

Maintenance of cytopathology records is an extremely important aspect of pathology services. This includes filing and storage of cytology slides usually in order of accession number.

### General guidelines for storage and archival in a pathology laboratory

- Report duplicates/records/diagrams and copies of any representative images prepared should be kept for at least 20 years.
- All laboratories must retain cytology slides for a minimum of five years.
- Currently, some patients with cancer survive for more than 10 years, and review of previous pathology material may be required for comparison with a recurrent tumor or for enrollment in clinical trials. Therefore it is recommended that once the regulated length of time for storage is met, institutions may continue to store cytology slides based on the room they have for storage.
- The entire archive should be professionally stored in a climate-controlled environment, and its index should be available, preferable in digital format.
- Paper records should be stored as bound volumes. All documents, relevant radiographs, and additional material available should be photographed and scanned to be digitally archived as well.



## 6.9 SAFETY IN THE LABORATORY

- Treat all biological materials used in the laboratory as potentially infectious and pathogenic to humans.
- Laboratory coats must be worn by laboratory personnel at all times.
- All open cuts on hands and other exposed skin surfaces must be covered by gloves.
- Long hair should be tied back neatly, away from the shoulders.
- The lab should be well-ventilated and should strictly follow the regulations governing the acceptable limits of the reagents used.
- If solvents are used during practical sessions, the exhaust fan must be switched on.
- Whenever doing staining procedures ensure that protective gowns, gloves and safety glasses are worn.
- Inspect centrifuge tubes for cracks.
- Never pipette samples with mouth.
- A safety data sheet should be maintained for every chemical compound used and it should specify the nature, toxicity, and safety precautions to be taken while handling the compound.
- Proper disposal of hazardous wastes is a must.
- Every instrument used in the laboratory should meet electrical safety specifications and have written instructions regarding its use.
- It is advisable that flammable materials are stored with utmost care in appropriate storage cabinets that are designed for this purpose.
- Fire safety procedures are to be strictly adhered to. Safety equipment including first aid kits, fire extinguishers, fire blankets, and fire alarms should be within easy access.

### Self-Assessment

1. Enumerate the broad categories of samples are received in the cytology laboratory.
2. Enlist the indications of FNAC. Outline its methodology.
3. Write briefly on
  - a) Cytological fixatives
  - b) Exfoliative cytology
  - c) Fluid cytology
  - d) Cell block preparation
  - e) Cytospin preparation
  - f) Papanicolaou staining
  - g) Giemsa staining in cytology





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