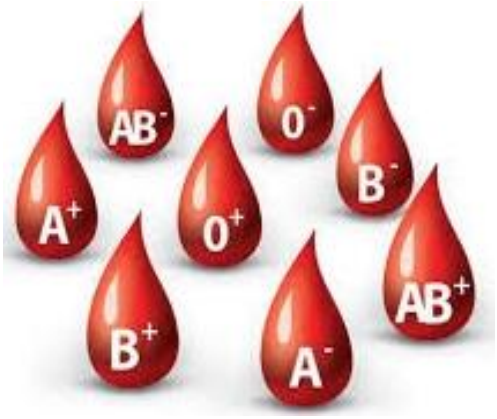
H.S.C (VOCATIONAL)

MEDICAL LABORATORY TECHNICIAN

STD: XII (PAPER-1)

Clinical Hematology, Clinical Pathology and Blood Banking

THEORY



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LESSON 1

Introduction to Hematology

Objectives- *At the end of the topic, students will be able to*

(a) Define Haematology

(b) Explain its importance in laboratory medicine.

1.1 - Definition (Hematology) –

The Branch of Science concerned with the study of blood, its components, coagulation, blood forming tissues and the disorders associated with them is called Haematology. (Haem = Blood, Logy = To Study). It includes

1. Analysis, Structure and functions of the cells and their precursors in the bone marrow.
2. Analysis of chemical constituents of plasma or serum intimately linked with the blood cell structure and its functions.
3. Study of functions of the platelets and proteins involved in blood coagulation.
4. Study of the changes in one or more components of blood due to haematological diseases or manifestation.

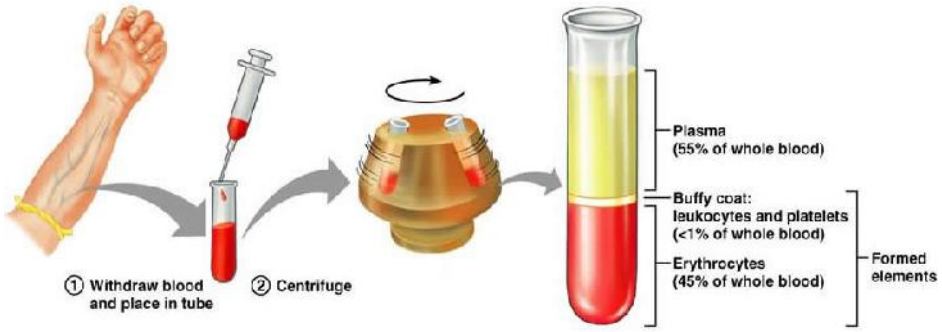


Figure 1.1 Collection of blood & separation of serum & plasma

The hematology laboratory deals with routine determination of total number of cells in circulation, Hemoglobin concentration, and differential count of leukocytes based on the study of stained smear which helps in detecting morphological abnormalities of various cells seen in *the* peripheral blood circulation

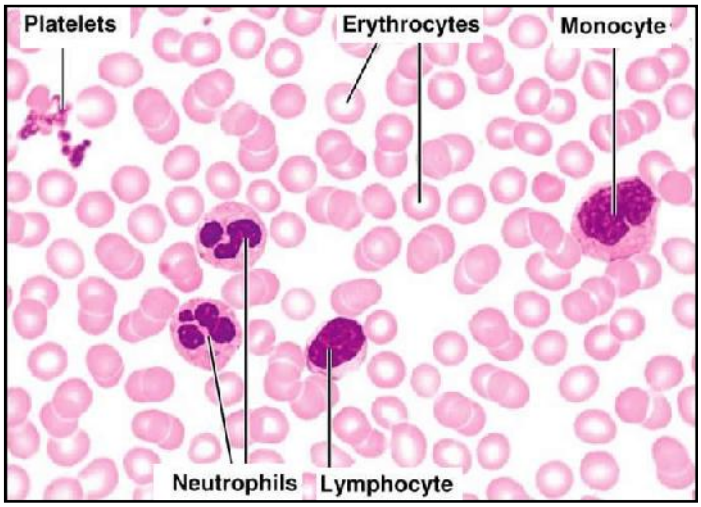


Figure 1.2 various types of blood cells in peripheral blood smear

1.2 Importance of haematology

1. To know the various components of the blood.
2. To know the normal and abnormal morphology of the blood cells.
3. It helps in diagnosis of various anaemias.
4. It helps to know about various bleeding disorders
5. It is necessary to diagnose various blood infections.
6. It helps in diagnosis of various blood diseases
7. It helps to determine blood parasitic infections
8. It helps in the treatment of various infectious diseases and blood related disorders.
9. It helps in medico-legal cases e.g. blood grouping, DNA testing, etc.

Review Questions:

1. What is haematology? What does it include?
2. Explain importance of haemotology.

LESSON 2

Collection of Blood - Use of Vacutainers

Objective- *Students shall learn use of vacutainers in collection of blood.*

2.1 Introduction

We have already studied collection of blood in XI standard. Nowadays advance techniques, instruments are there in field of pathology which have advantages of ease of collection of sample, ease of handling, less time consuming, providing accurate results, etc.

Accordingly in this chapter we will be studying how to use vacutainers for blood collection.

2.2 **Definition:**

Vacutainer is blood collection tube made of either plastic or glass with a closure that is evacuated to create a vacuum inside the tube facilitating the draw of a predetermined volume of blood.

2.3 Parts of vacutainer

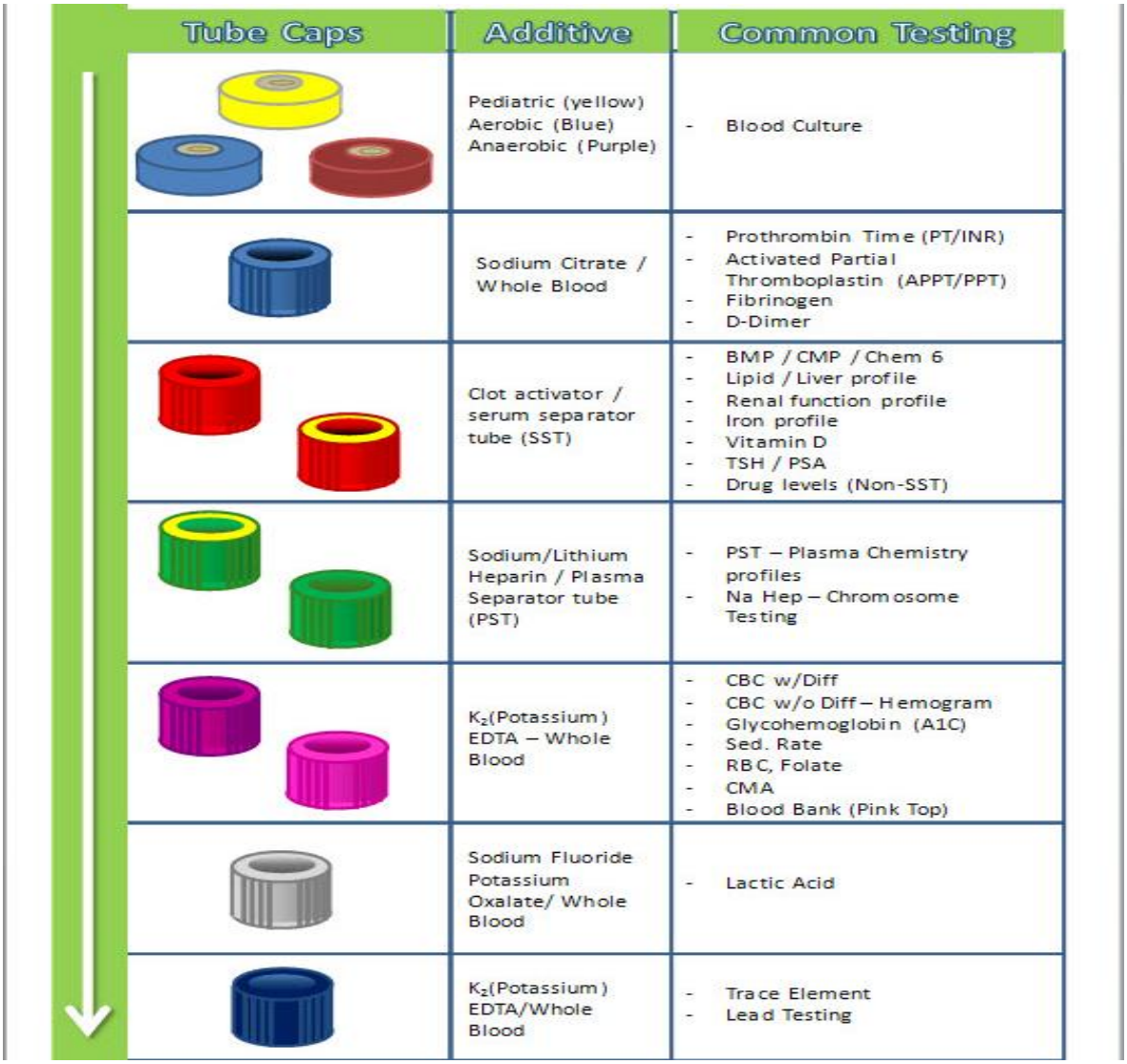
The vacutainer system consists of

1. Sterile single use blood collection needle.
2. Holder: It is used to secure needle during insertion into tube stopper and subsequent venipuncture.
3. Sterile vacutainer primary tube: It is an evacuated glass tube with rubber stopper containing pre-measured vacuum and anticoagulant for whole blood.
4. Vacutainer needles are specifically designed. The needle has two ends. The canula-tubing from which the needle is made, is sharpened at one end for puncture of the skin and vein. A plastic hub is at the center which screws into the holder. At the back end is a much shorter cannula. The rear canula has a rubber sleeve.

Fig. 2.1 Vacutainer with needle, holder and collection tube

2.4 Blood Collection by using Vacutainers

- During the blood collection process, the rear canula pushes through the rubber sleeve and punctures the rubber stopper, allowing the vacuum in the tube to draw blood from the vein. The pressure difference between the blood volume and the vacuum in the tube forces blood through the needle into the tube.
- When one tube is withdrawn from the back of the needle, to collect blood in another container, the sleeve slides back into position and keep the blood from flowing out through the rear end of the canula. When the last tube has been filled, the entire assembly is removed from the patient's arm and the needle is disposed off.
- The tube stoppers are color-coded so that the person using them can find out at a glance the type of additive (anticoagulant) used. The various additives used are EDTA, heparin, citrate, fluoride etc.
- The serum separating tubes contain clot activating material. A normal blood sample clots in 15 to 30 minutes. The tube is then centrifuged for 10-15 minutes, during which time its unique polyester gel forms a permanent barrier between the clot and the serum.



Tube Caps	Additive	Common Testing
	Pediatric (yellow) Aerobic (Blue) Anaerobic (Purple)	- Blood Culture
	Sodium Citrate / Whole Blood	- Prothrombin Time (PT/INR) - Activated Partial Thromboplastin (APPT/PPT) - Fibrinogen - D-Dimer
	Clot activator / serum separator tube (SST)	- BMP / CMP / Chem 6 - Lipid / Liver profile - Renal function profile - Iron profile - Vitamin D - TSH / PSA - Drug levels (Non-SST)
	Sodium/Lithium Heparin / Plasma Separator tube (PST)	- PST – Plasma Chemistry profiles - Na Hep – Chromosome Testing
	K_2(Potassium) EDTA – Whole Blood	- CBC w/Diff - CBC w/o Diff – Hemogram - Glycohemoglobin (A1C) - Sed. Rate - RBC, Folate - CMA - Blood Bank (Pink Top)
	Sodium Fluoride Potassium Oxalate/ Whole Blood	- Lactic Acid
	K_2(Potassium) EDTA/Whole Blood	- Trace Element - Lead Testing

2.5 Important precautions taken while using vacutainer systems.

While collecting blood by vacutainer systems, backflow from blood tubes into veins may occur due to a decrease in venous pressure. This can be prevented by proper positioning the hand & container while collecting sample.

2.6 Advantages of vacutainer system

- It eliminates the preparation of anticoagulant containing bulbs and tubes.
- There is no processing of containers.
- The system is closed and there is no possibility of blood spillage. The tubes are completely leak proof during transport.

- Each vacutainer tube is designed to draw exactly the right amount of blood. Correct blood to additive ratio is maintained. Hence chance of micro clot formation and hemolysis of blood are minimum.
- The interior of vacutainers is sterile, which prevents microbial growth.
- An inert polyester gel barrier facilitates rapid clotting and separates serum and cells during centrifugation. Many autoanalyzers are designed to sample directly from vacutainer tubes.
- The plasma separator tube is heparinized and contains an inert gel barrier. These tubes are mainly useful for quick separation of plasma and determination of electrolytes.
- The vacutainer blood culture systems offer early detection of microbial growth by ensuring minimum risk of contamination.
- Capillary blood collection tubes are designed for the collection; transport and processing of skin puncture blood from infants, children, and geriatric and critical care patients.
- Safety flow lancets are safe to use and less traumatic for the patient. The semiautomatic puncture mechanism eliminates the stabbing action required with many other lancets and allows a controlled and less traumatic finger puncture. After use, the blade retracts automatically, reducing risk of an accidental finger prick.

Review Questions:

1. What is vacutainer? Name the parts of vacutainer.
2. What are advantages of using vacutainers?
3. Mention different color codes of stopper used for blood collection.
4. Describe method of blood collection using vacutainer.

LESSON 3

Anticoagulant

Objectives- *Students shall understand the different types of anticoagulents, their mode of action and uses.*

3.1- Introduction

Anticoagulant is a substance, which is added to the blood to prevent it from clotting. Calcium is required for many steps involved in the clotting mechanism. Most of the anticoagulants act by binding with the calcium forming an insoluble salt. Such anticoagulants are called as "calcium chelators". Some anticoagulants like potassium oxalate act by precipitating ionic calcium in plasma, whereas anticoagulant like heparin acts as an antithrombin, thus preventing the action of thrombin on fibrinogen.

3.2- Classification-

Divided in to two groups

I. Calcium chelators-
   1. Oxalates-
      a. Potassium oxalate
      b. Ammonium oxalate
      c. Double oxalate
   2. EDTA
   3. Citrate-
      a. Trisodium citrate
      b. ACD
      c. CPD

II Heparin

3.3- The different Anticoagulants used are

1. <u>E.D.T.A.(Ethylene Diamine Tetra Acetic acid)</u>

- It is also called as Versene or Sequestrene and is the most commonly used anticoagulant in haematology.
- It is a crystalline acid with a molecular formula $C_{10}H_{16}N_2O_8$

- It is usually used as a dipotassium salt or a disodium salt of the acid as opposed to the free acid, which is not soluble in an aqueous media. Dipotassium salt is more preferred than disodium salt because the former is more soluble in water.
- Mechanism of action:
- EDTA acts by chelating the calcium ions and is the most powerful chelating agent.

Concentration:

- 2mg per ml of blood.
- A stock solution of EDTA is prepared by dissolving 10 gms of Dipotassium salt of EDTA in 100 ml of Dist. Water. Put 0.2 ml of this solution in bottle for 2ml of blood and 0.5ml of this solution in bottle for 5ml of blood. Evaporate to dryness in a hot air oven keeping it overnight.
- It is a very good anticoagulant because it preserves the morphology of the blood cells without causing any shrinkage or swelling of the cells.
- It is a commonly used anticoagulant for all types of blood counts and blood smears.

Disadvantages:

a. EDTA cannot be used for coagulation studies. (as it chelates calcium)
b. Platelets can sometimes see to satellite neutrophils.
c. It is more expensive than oxalates.

2 - Oxalates

There are two oxalate salts, which prevent the clotting of blood.

Potassium oxalate :It is used in the concentration of 2mg per ml of blood. It is used for chemical analysis, but it is not recommended for blood counts, ESR or PCV estimation, because it causes shrinkage of the cells.

Ammonium oxalate **:**It is also used in the concentration of 2mg per ml of blood. It is not preferred as an anticoagulant for routine haematological tests, because it causes swelling of the cells.

Double oxalate :

- It is a mixture of potassium oxalate and ammonium oxalate in the ratio of 2:3. Here the shrinkage effect of potassium oxalate is counteracted by the swelling effect of the ammonium oxalate.

- Mechanism of action : The oxalates combine with the calcium ions present in the blood to form an insoluble salt of calcium oxalate, which gets precipitated.
- Concentration: 2mg per ml of blood.
- A stock solution of double oxalate is prepared by dissolving 0.8 gm potassium oxalate and 1.2gm of ammonium oxalate in 100ml of Dist.water. Take 0.25 ml of this solution in bottle for 2.5ml of blood and 0.5ml of this solution in bottle for 5 ml of blood. Place the bulbs in suitable container and evaporate to dryness in an incubator at 37º C or in a hot air oven at 60º C.
- It is used mainly for ESR by Wintrobe's method and PCV estimation.

Disadvantages:

a. It does not preserve the morphology of the blood cells very well, particularly the WBCs, because WBCs phagocytose the precipitated calcium oxalate and hence this anticoagulant is not preferred for blood smears.
b. It does not prevent platelet aggregation as effectively as EDTA.
c. It is poisonous and should not be used for blood transfusion.

3- Citrate

Trisodium citrate

- Mechanism of action:
  Removes calcium ions by forming a soluble calcium citrate complex.
- 3.8% solution of trisodium citrate is isotonic with blood and is the anticoagulant of choice for determination of ESR by Westergren's method. Here, one part of trisodium citrate solution is diluted with 4 parts of venous blood.
- It is also used as an anticoagulant in estimation of Prothrombin time. Here, one part of citrate solution is diluted with 9 parts of venous blood.
- It can also be used for Reticulocyte count and Heinz body detection.

ACD Solution

Acid Citrate Dextrose Solution: This is anticoagulant which is used in blood bank.

As ACD is in a liquid form, the blood gets diluted and hence it is not suitable for haematological tests like blood counts and Hb estimation.

CPD Solution

Citrate Phosphate Dextrose Solution: This is also an anticoagulant used in blood banking.

4- Heparin

- Heparin is a natural anticoagulant present in small amounts (0.009mg/100ml of blood) in the body.
- Heparin is used for special tests like osmotic fragility test.
- It was first isolated from the liver and hence the name (In Greek 'Hepar' means Liver). It is also found in the extracts of other body organs. It is secreted by the mast cells of the connective tissue and basophils.
- Concentration: 0.1 to 0.2 mg per ml of blood.
- Mechanism of action: It acts as an anticoagulant by two ways -
    1. It acts as an inhibitor of thromboplastin formation and
    2. It acts as an anti-thrombin i.e. it inhibits the action of thrombin on fibrinogen.
- Heparin is rarely used as an anticoagulant because-
    a. It is very expensive.
    b. It is not stable in hot climate.
    c. It is not suitable for counting of blood cells and making blood smears because it gives bluish background to the smear.

5- Sodium fluoride and Potassium oxalate

- This anticoagulant is prepared by mixing 1.2% sodium fluoride with 6% potassium oxalate.
- Fluoride is an inhibitor of glycolytic enzymes. Thus, it prevents breakdown of glucose present in the blood. However, fluoride is not a strong anticoagulant and hence it is mixed with potassium oxalate.
- This anticoagulant is mainly used for estimation of blood sugar.

Color Codes

For proper identification of anticoagulents used in vacutainers
the colour codes of anticoagulants described in ISO/DIS 6710 are:

1. EDTA = lavender/red;
2. Citrate 9 + 1 = light blue/green;
3. Citrate 4 + 1 = black/mauve;
4. Heparinate = green/orange;
5. No additives (for serum) = red/white (86).

Review Questions:

1. Define an Anticoagulant?
2. Enlist commonly used anticoagulants in haematology laboratory.
3. Write a note on EDTA.
4. Write a note on Double Oxalate.
5. Why ACD is not used for counting blood cells?

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LESSON 4

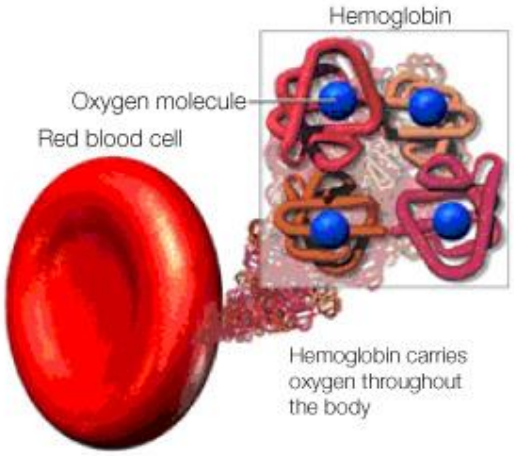
Hemoglobin

Objectives- *At the end of the topic the student shall understand functions of haemoglobin, methods of estimation and significance of Hb estimation.*

4.1- Introduction-

Hb is the red coloured pigment of RBC of red blood cells. It is large complex chromo protein consisting of two parts one specific simple protein Globin and other non specific prosthetic group called Haem. Four polypeptide globin chains linked together and an iron containing porphyrin called Haem is attached to each polypeptide chain. Iron remains in ferrous (fe^{++}) form. Globin helps haem to keep the iron in ferrous state and to combine loosely and reversibly with molecular oxygen.

Hb which constitutes about 33% of the cell (RBC) weight is responsible for red colour of blood.



4.2- Functions:

1. Haemoglobin carries oxygen from lungs to tissues of the body.
2. It plays important part in transport of CO_2 from tissues to lungs.
3. It constitutes one of the important buffers of the blood and helps to maintain acid base balance.
4. Hb forms various pigments bilirubin , biliverdin etc of Bile, stool and urine etc.

4.3- Types of Hb –

At least three distinct Hb types are found postnatal in normal individual

1. **Hb A** – HbA is major normal adult Hb it consists of the two types - polypeptide chains of the globin parts of Hb. Two identical alpha chains and two identical beta chains. Each chain is linked with haem group.
2. **Hb F** – It is the major haemoglobin of fetus and the new born infant consisting of two alpha chains and two gama chains each attached to haem part of the Hb.
3. **Hb A2** – It is present in 1.5 to 3.5 % of normal adult haemoglobin and it consist of two alpha chains and two delta chains each attached to haem part of Hb.

4.4- Abnormal haemoglobins

These are inherited abnormalities either in globin chain or synthesis of globin chain is reduced.

Abnormal globin Chain

Sickle cell anaemia (**Hb-S**) – There is alteration of amino acid on beta chain of globin. Valine is substitute for glutamic acid at sixth position of beta chain. Because of this alteration haemoglobin gets crystallised at low oxygen tension and RBC converts into sickle shape.

Other abnormal haemoglobins are **HbC**, **HbE**, Hb Bristol and HbD.

Reduced synthesis of globin chain –

This is also inherited disorder and common condition is Thalassaemia syndrome.

4.5- Haemoglobin Estimation

The object measuring Hb is to estimate the oxygen carrying capacity of blood in addition to providing as assessment of erythropoietic status.

1. Colorimetric method – Based on measuring the colour of Hb or derivatives of Hb. It is based on Beer's law which states that the optical density (depth of color0 of a coloured solution is directly proportional to the concentration of coloured material in the solution.

Commonly used colorimetric methods are:

a. Acid haematin or Sahli's method.
b. The cyanmethaemoglobin method.

Sr. No.	Acid haematin method	Cyanmethhaemoglobin method
1	Haemoglobin is converted in to	Haemoglobin is first converted in to

	acid haematin by the action of N/10 HCl. The brown colour compound is matched against standard brown glass comparator.	met haemoglobin and then cyanmethaemoglobin by the addition of K-cyanide and K-ferricyanide.
2	Color is matched visually.	Color is read by using colorimeter.
3	All haemoglbins i.e. methHb CarboxyHb sulphHb,etc are not converted in to acid haematin.	All haemoglobins are converted in to cyanmethhaemoglobin
4	Acid haematin is insoluble and it is present as colloidal suspension in the fluid. As light cannot pass through it, color cannot be read on colorimeter.	Cyanmeth Hb is optically clear solution. Light can pass through this solution. So color is read using colorimeter.
5	Not very accurate method. Error is there (5-20%).	Accurate method. No error.
6	Visual error is there.	No visual error as color is read on colorimeter.

2. Physical method or Specific gravity method- This is used in blood bank for screening of donor to determine whether the donor's Hb is above the specified level or not. .

Principle-

The specific gravity of a given blood sample depends on the concentration and weight of material present in it. Hb as it is large single constituent it affects the specific gravity of blood more than other substances. Hb measurement based on principle that the other substances remain constant and changes are due to Hb mainly.

- Here specific gravity of Hb is compared with specific gravity of copper sulphate.

  (Sp. gravity of $CuSO_4$ solution 1.047≈ 10.5gm% of Hb).
- The specific gravity of blood is measured by allowing a drop of blood to fall in to the solution of $CuSO_4$ of varying specific gravity.
- Note whether a drop of blood sinks or rises to the surface.

- If drop sinks then its specific gravity is more than that of $CuSO_4$ solution and if rises then sp. gravity is less than $CuSO_4$ solution.

3. Chemical method

   4 Gasometric method-

Chemical and gasometric methods are complex, difficult and time consuming but accurate. Good technical skill is required. So for routine screening these are not used. Other methods i.e. colorimetric and physical are used.

4.6- Normal Range

The average adult haemoglobin irrespective of sex is 14.5grms%

- Newborns: 14-22 gm/dl
- Children: 11-13 gm/dl
- Adult males: 14-18 gm/dl
- Adult women: 12-16 gm/dl
- Pregnant women: 11 to 12 gm/dl

Review Questions –

1. What is haemoglobin? Express its normal values.
2. Enumerate different methods of hemoglobin estimation.
3. Describe functions of haemoglobins.
4. Which are the normal; and abnormal haemoglobins?
5. Difference between Sahli's and Cyanmethaemoglobin method.

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LESSON 5

Study of blood cell counts

Objectives: *Students shall understand the techniques of manual counting of different blood cells, special counts like reticulocyte count & absolute eosinophil count and their significance.*

5.1- Introduction:

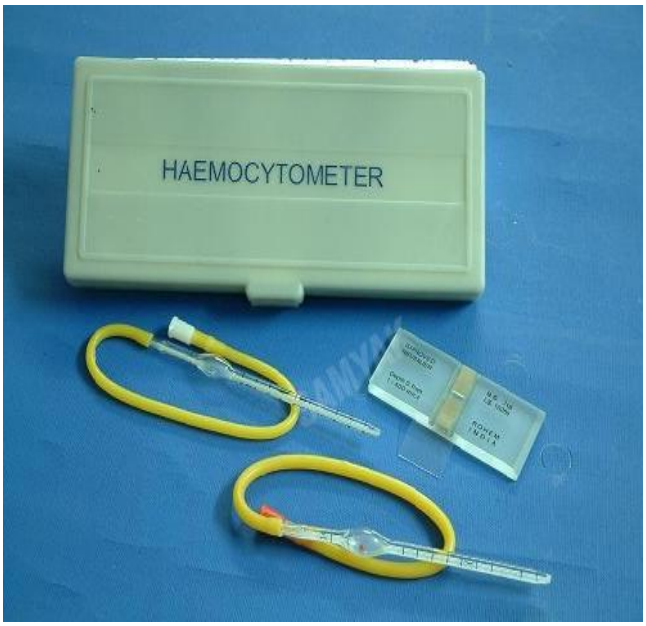
Commonly done blood cell counts are RBC count, total WBC counts, Differential leukocyte count & Platelet count, Absolute eosinophil count. Haemocytometer is used to carry out these blood cell counts manually. Hence it is also called as haemocytometry.

With this technique different cells cannot be identified. Despite of high-tech automatic cell counters, still the manual technique has got its own application.

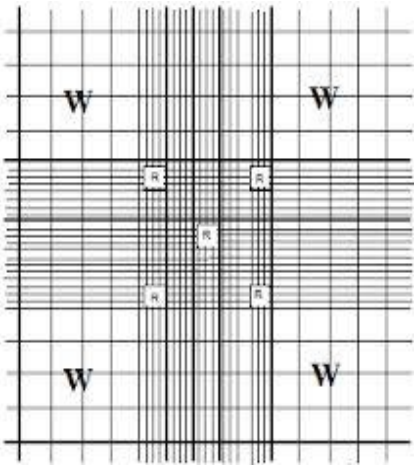
5.2- Haemocytometer: Haemocytometer consists of

1. **RBC pipette** - RBC pipette is used for RBC count. It has got large bulb containing red glass bead. The markings on RBC pipette are 0.5, 1.0 & 1.1.
2. **WBC pipette**- It is smaller than RBC pipette and it contains white glass bead. It has three marks 0.5, 1 & 11.
3. **Neubaur's chamber (Improved Neubaur's counting Chamber)**
   a. The counting chamber has an area of 9 sq. mm and depth of 0.1 mm.
   b. The 9 sq. mm area of counting chamber is divided into 9 squares.
   c. The four corner squares measure 1 sq mm area.
   d. The squares at the corner are divided into 16 small squares each and are used for counting white cells.
   e. The central square is divided into 25 squares and each of these squares is further divided into 16 small squares. Each square measures 1/400 sq. mm area.
   f. Five of the medium squares (i.e. 80 small squares) are used in counting red cells.

4. **Cover slips**- A special type of coverslip is used with a very smooth and even surface. The size of coverslip may be 16 x 22 mm or 22 x 23 mm. The thickness of coverslip may be 0.3mm, 0.4mm or 0.5mm.



Haemocytometer



Neubaur's counting chamber

5.3- Total WBC count

Total WBC count is one of parameter in CBC hence it is a very commonly and routinely done test

Requirements:

1. Microscope
2. Haemocytometer - Neubaur's chamber, WBC pipette

WBC pipette

3. WBC diluting fluid – It contains
    - Glacial acetic acid – 2 ml
    - 1% gentian violet – 1.0 ml
    - Distilled water – 97ml

Glacial acetic acid haemolyses RBCS & gentian violet lightly stains nuclei of WBCS. Blood specimen can be collected in EDTA bulb or double oxalate bulb or even by finger prick also WBC count can be done.

Procedure:

1. Draw blood up to 0.5 mark of a WBC pipette.
2. Draw diluting fluid up to the 11 mark carefully. Wipe excess blood or fluid outside the pipette using cotton.
3. Mix the contents of the pipette and wait for five minutes.
4. Discard few drops & let the contents of pipette flow in the Neubaur's counting chamber under the coverslip. Take care that there will be no air bubbles.
5. Wait for 2-3 minutes so that the cells will settle down.
6. Focus under low power and concentrate on the 4 corner squares. Each square will contain 16 small squares.
7. Count cells in all four squares.
8. WBC will appear as small dark blue round cells.

Calculations:

No. of white cell counted in all four squares= "N"

Dilution= 1 in 20

Area counted= 4 x 1sq.mm = 4sq.mm

Depth= 0.1 mm

Therefore, no. of white cells per cu.mm (µl) of whole blood

= N x dilution / area counted

= N x 20/ 4 x 0.1

= N x 50

Normal Values:

Adults: 4000 to 10,000/ cu mm (µl)

Children: 4,500 to 13,500/cu mm (µl)

Clinical significance:

1. **Leukocytosis:** Increase in total leukocyte count of more than 10,000/cu.mm (µl) is known as leukocytosis.

Causes of leucocytosis

Physiological causes:

- Age- At birth the total leukocyte count is about 18000/cumm it drops gradually to adult level.
- Pregnancy- At 'full term' the total count tends to be about 12000 to 15000 /cumm. It rises soon after delivery and then gradually returns to normal.
- High temperature
- Severe pain
- Muscular exercise

Pathological causes-

- It is for a transient period in infection.
- The degree of rise in leukocytes depends on the type & severity of infection& response of body.
- The infection may be bacterial, viral, protozoal, and parasitic.
- Leucocytosis is also observed in severe hemorrhage.
- Leukemia.

2. **Leucopenia:** The decrease in total leukocyte count less than 4000 is called leucopenia.

Causes of leucopenia:

Infections like typhoid, paratyphoid, tuberculosis can cause leucopenia.

In malaria total count can decrease.

Some cases of leukemia can cause leucopenia.

Aplastic anaemia or secondary bone marrow depression due to drugs, radiation can cause leucopenia.

- Anaemia (Iron deficiency anaemia, megaloblastic anaemia) can cause leukopenia.

Sources of error-

False high counts-

1. Inadequate wiping of pipette.
2. Improper pipetting of blood and also diluting fluid.
3. Improper mixing.
4. Uneven distribution in the counting chamber.
5. Dirt, clumped RBCs can be counted as white cells.
6. Errors in calculation.

False low counts-

1. Improper pipetting of blood and diluting fluid (When blood is drawn below the mark 0.5 and diluting fluid above the mark 11).
2. Improper mixing.
3. Dilution of the contents in the pipette by saliva.
4. Uneven distribution in the counting chamber.
5. Errors in calculation.

5.4- RBC Count

RBC count is very useful in diagnostic haematology. It is one of the tests in investigation of anaemia. RBC count is required for calculation of red cell indices.

Requirements:

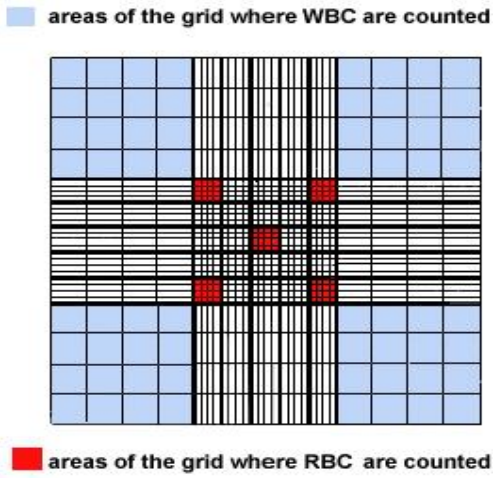
- Microscope
- Haemocytometer - RBC pipette and Neubaur's chamber.
- RBC diluting fluid:
  a) Sodium citrate 3.0 gr;
  b) Formalin 1.0 ml;
  c) Distilled water 100ml;

Sodium citrate acts as anticoagulant. Formalin preserves RBCS. The fluid is isotonic with blood hence there will be no haemolysis.

- RBC count can be done with EDTA blood specimen or double oxalate bulb specimen or even it can be done with capillary blood.

Procedure:

1. Mix the anticoagulated blood properly and draw blood up to the mark 0.5.
2. Draw diluting fluid up to the mark 101.
3. Wipe the excess blood outside the pipette using cotton.
4. If using finger prick technique, the prick must be deep & bold enough to get necessary quantity blood in RBC pipette without air bubbles.
5. Rotate the pipette rapidly to mix contents.
6. Wait for few minutes, discard 2.3 drops & charge the Neubaur's chamber with cover slip without air bubbles.
7. Allow the cells to settle and place the counting chamber over microscope stage.
8. Focus the chamber under low power & locate Central Square with 25 small squares.
9. Switch over to high power objective.
10. RBCS in the four corner squares & Central Square are counted. The squares are identified by triple lines. For the cells lying on border, marginal rule is applied. I.e. cells lying on left & lower border of square are counted in the respective square.



RBC pipette

Calculations:

Total red cells counted = N

Dilution = l in 200

Are a counted (16 x 5) = 80/400 = 1/5sq mm

RBC count = RBC / cu mm (µl)

= No. of cells counted x dilution / Area counted x depth

= $\frac{N \times 200}{1/5 \times 1/10}$

= N x 200 x 50

= N x 10,000

Normal Values:

Males: 4.5 to 6.0 million / cu mm (µl)

Female: 4.0 to 4.5 million / cu mm (µl)

Clinical significance-

An increase in RBC count is observed in condition such as –

1. Haemoconcentration due to burns, cholera.
2. Central cyanotic stages as seen in chronic heart disease.
3. Decreased lung function.
4. Polycythaemia.

Decrease in RBC count is observed in condition such as –

1. Old age
2. In pregnancy
3. Different anemias

Source of error-

False high counts-

1. Collection of blood from the area where there is hemoconcentration,
2. Inadequate wiping of pipette
3. In proper pipetting of blood
4. Improper mixing
5. Uneven distribution in the counting chamber
6. Errors in calculation

False low counts

1. Blood dilution with tissue fluid due to edema or squeezing.
2. Improper pipetting and dilution (When blood is drawn below the mark 0.5 and diluting fluid above the mark 101).
3. Dilution of the content in the pipette by saliva.
4. Errors in calculation.

5. Use of improperly standardized counting chamber and improper adjustment of cover slip.

5.5- Platelet Count

Platelets are the third formed elements in blood. Platelets are concerned with blood coagulation function. Hence platelet count is one of test useful in assessment of coagulation function of blood. Platelets are very tiny cells having size 2 to 3 microns. They are spindle shaped nonnucleated cells. Platelets are developed from a primitive cell i.e. megakaryocyte.

Requirements:

1. Haemocytometer with Neubaur's chamber and RBC pipette.
2. Platelet diluting fluid:
   - Tri-sodium citrate 3.8gm
   - Neutral formalin 0.2ml
   - Brilliant cresol blue 0.1gm
   - Distilled water 100ml
3. Microscope
4. Specimen
   - EDTA anticoagulated blood
   - Capillary blood

Procedure:

1. Mix the blood specimen and using RBC pipette & draw blood up to the mark 0.5.
2. Wipe out excess blood outside the pipette and draw diluting fluid up to the mark 101.
3. Mix the contents in bulb thoroughly.
4. Wait for five minutes and charge the counting chamber.
5. Place the charged counting chamber under petridish with wet filter paper. Let it stay for 15 min .before counting. This allows platelets to settle & prevents drying.
6. Place the counting chamber over the stage of microscope & first focus under low power, the red cell counting area. View corner square of red cell area & switchover to high power objective.
7. Keep the condenser down and reduce the light by adjusting diaphragm. Platelets will appear as highly refractile particles.

8. Count platelets in all 25 small squares. The area covered by 25 squares is 1 sq. um.

Calculations:

No. of platelets counted = N

Dilution = 1 in 200

Volume of fluid = 1 x 0.1 = 0.1 cu mm

Platelets count per cu mm (µl)

= No. of platelets counted x dilution/volume of fluid

= N x 200/0.1

= N x 2000

Note: Platelets are very tiny cells. Dust particles, bacteria can be mistaken for platelets. Hence filter the diluting fluid occasionally.

Platelet count should be carried out within 2 hours after blood collection. Delay can cause disintegration of platelets.

Normal Values: 1-4lakh/mm^3

Clinical significance:

1. Decrease in platelet count is called Thrombocytopenia and increase count is called Thrombocytosis.
2. Decrease count indicates aplastic anaemia, marrow depression etc.
3. Increase in platelet count indicates polycythemia vera, following spleenectomy and in chronic myeloid leukemia.

5.6- Absolute Eosinophil Count

Eosinophil is one of the granulocytes. It is a round cell with bilobed nucleus. In peripheral blood smear with Leishman's stain cytoplasm appears pink with coarse orange red granules,. Nucleus stains purple and it is bilobed. Eosinophils are related with allergic responses of the body hence absolute eosinophil count carries certain diagnostic importance amongst the laboratory tests.

Principle:

Blood is diluted with special diluting fluid, which removes red cells & stains eosinophils red. The cells are then counted under low power.

Requirements:

1. Improved Neubaur's chamber
2. Hb pipette (Sahali's haemoglobinometer)

3. Diluting fluid (Hingleman's solution):
    - Yellow eosin – 0.5g;
    - 95% phenol – 0.5ml;
    - Formalin – 0.5ml;
    - Distilled water – 99ml;

Procedure:

1. Pipette 0.36 ml of diluting fluid in a test tube
2. Add 0.04 ml of blood (use HB pipette twice)
3. Mix and keep for 10min.
4. Mix and charge the counting chamber.
5. Let it stand under moist petridish for about 2-3 min.
6. Count the cells under low power objective with reduced light. Count the cells in all 9 squares (WBC squares)

Calculations:

$$\text{Total Eosinophils/cumm} = \frac{\text{Number of cells counted x dilution factor}}{\text{Area counted x depth of fluid}}$$

Where

Dilution= 10

Area= $9mm^2$

Volume of fluid = Area counted x depth

= 9×0.1

= 0.9

$$\text{Total number of Eosinophil/cumm} = \frac{\text{Number of cells counted x 10}}{0.9}$$

$$= \frac{N \times 10}{0.9}$$

Normal values:

40 – 440/ cu mm (Nil)

Clinical Significance:

- Increased Eosinophil count is often associated with allergic reaction, parasitic infection, brucellosis & in certain leukemia.
- Decrease Eosinophil count is seen with hyperadrenalism (Increase in the adrenal function).

5.7 Reticulocyte Count

Introduction

Reticulocyte is a premature RBC. Erythropoiesis is a process of production of RBCS from primitive cell in bone marrow. During development of RBC from erythroblast number of stages occurs.

Proerythroblast → Early Narmoblast → Late normoblast → **Reticulocyte** → RBC

Reticulocyte is just one stage before fully mature RBC is formed. Hence number of reticulocytes in peripheral blood becomes the reflection of erythropoietic activity in bone marrow. In this way, reticulocyte count (Retic count) is an easily possible indicator of bone marrow activity.

Principle:

Supravital staining method is used for reticulocyte count. Blood is mixed with brilliant cresyl blue stain and the stain enters the cell in living condition. The RNA in the cells is precipitated by staining as dark blue network or reticulum.

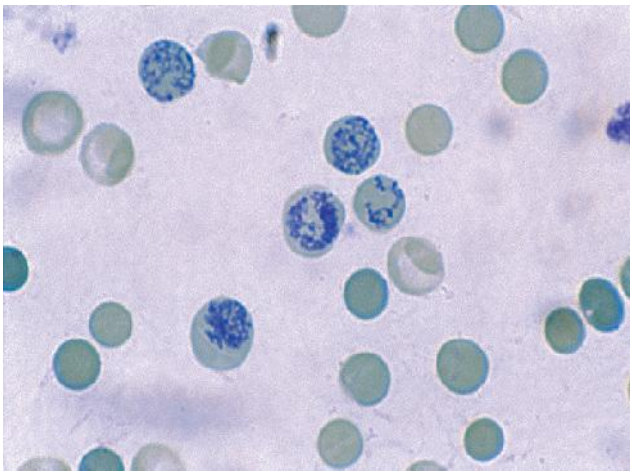
Requirements:

1. Clean glass slide
2. Test tube
3. Pasteur pipette with rubber teats
4. Capillary tube
5. Staining solution:
    - Brilliant cresyl blue 1.0gm;
    - Sodium citrate 0.4gm;
    - Sodium chloride 0.85gm;
    - Distilled water to 100ml

Procedure:

1. Filter 5ml of stain.
2. In a test tube add two drops of blood and two drops of stain (use separate Pasteur pipettes). Mix properly.

3. Cover the tubes with cotton plug & keep at 37^0C for 30 minutes.
4. Prepare a thin smear of stained blood specimen using spreader slide. Air dry the smear.
5. Examine the smear under low power objective and locate portion of smear where red cells are evenly distributed.
6. Change over to oil immersion lens.
7. Reticulocytes will appear as round cells having the size almost same as RBCs (7-8microns). Cells will appear pale blue in colour. Reticulocytes can be identified by fine deep blue filaments seen in them. These are the remnants of RBA.
8. Simultaneously count RBCS & reticulocytes. Screen around 10-15 fields.



Reticulocytes

Calculations:

Reticulocyte count = $\frac{\text{No. of reticulocytes counted x100}}{\text{No.of RBC counted}}$

e.g. No. of red cell counted = 100

No. of retialocytes = 50

Reticulocyte count = $\frac{50 \times 100}{1000}$

= 5%

Normal values:

- Adults: 0.2 to 2%
- Infants: 2 to 6%

Clinical Significance:

1. Reticulocyte count is a reflexion of bone marrow activity of RBC production (erythropiosis).
2. Increased reticulocyte count indicates increased marrow activity. This is observed in haemolytic anaemia or acute blood loss. This is called as reticulocytosis.
3. Decreased reticulocyte count indicates bone marrow suppression (aplastic anaemia).

Review Questions-

1. Describe Haemocytometer?
2. Explain the terms ‘Leucocytosis’ and “Leucopenia”.
3. Write about clinical significance of RBC count.
4. What are reticulocytes?
5. Describe clinical significance of reticulocyte count.
6. How will you do absolute eosinophil count?
7. Write normal value & importance of platelet count.

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LESSON 6

Differential Leucocyte Count

Objectives*:* *Students shall prepare peripheral smear and also perform DLC.*

Introduction-

Preparation & staining of smears: Romanowaski stains

- Blood smear means uniformly spread drop of blood over the slide. A thin layered blood film is prepared & stained which allows identification & counting of different WBCs. Smear can be prepared from EDTA blood specimen or even directly from sample obtained by finger prick.
- A well prepared & stained smear is very useful for differential count as well as lot of other information can be gained from good prepared & stained peripheral blood smear.

6.1- Preparation of smear:

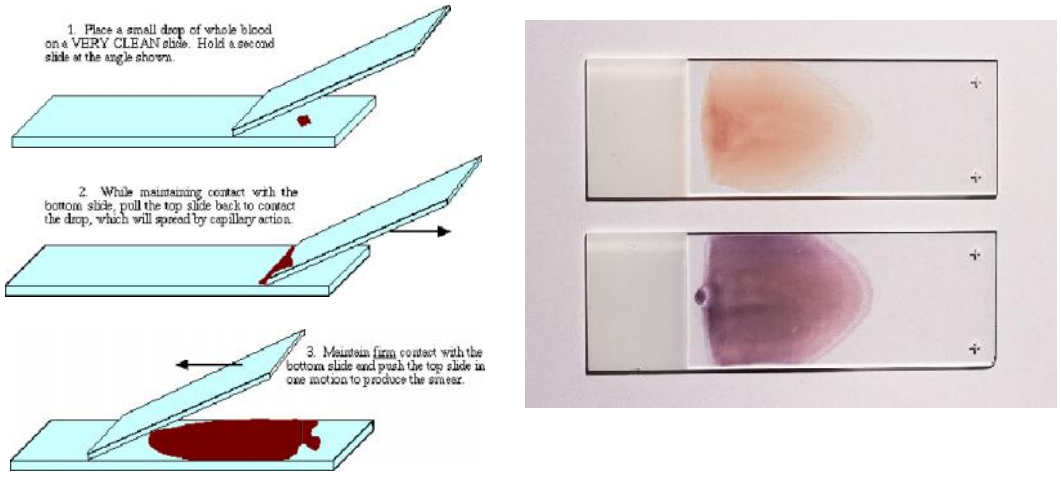
Requirements:

- Clean scratch free glass slide,
- spreader slide
- EDTA blood specimen or even directly from sample obtained by finger prick.

Procedure:

1. Select clean scratch free glass slide.
2. Transfer a drop of blood specimen with applicator stick.
3. Place the drop approximately 1 cm from the end.
4. Hold the spreader slide in right hand, place over the slide just in front of drop of blood. (Spreader slide is narrower than the smear slide to avoid spreading of cells over the edge of the slide. To make the spreader, break the corners of a normal slide)
5. The approximate angle between spreader slide & the slide should be between 30^0 to 35^0.

6. Draw the spreader back so that it touches the drop. Blood will run along the edge of the spreader slide.
7. Push the spreader to the end of slide with a smooth quick movement.
8. Try to keep the angle of spreader constant throughout the process.
9. Label it promptly to maintain identity of patient.



Preparation of smear

Criteria for good smear-

- A good smear is thick at one end and thin at other end.
- It should occupy the central portion of the slide with clear margins on all sides.
- It should be uniformly spread without any hesitation intermittently.

Poor quality smear-

- Drop of blood is too large or too small.
- Spreader slide is pushed in jerky manner.
- The angle of spreader is not properly set at 30^0.

6.2- Staining of smear:

Peripheral blood smear is stained by one of the Romanowski stains. These are the stains universally accepted all over the world.

A Russian physician Romanowski in 1891developed technique of using mixtures of acidic (eosin Y) and basic (methylene blue) dyes to stain blood films. This resulted in

excellent differentiation in nuclear & cytoplasmic characters of W.B.C. Romanowski stains are as follows:

1. Leishman's stain
2. Wright's stain
3. Giemsa's stain
4. Field's stain

Leishman's stain:

This is a polychromatic stain which contains methylene blue & eosin in methanol.

Technique of staining:

Steps of staining of **Leishman's & Wright's** stain are almost similar and their composition is also not much different.

1. Cover the smear with Wright's or Leishman's stain, wait for one minute.
2. Add equal no. of drops of buffered water over the stain & wait for 5 to 10 minutes
3. A metallic shine or silvery scum is expected to form over the smear.
4. Wash the smear using tap water.
5. Air dry the slide.
6. Observe under oil immersion objective.

Field stain:

Field staining requires less time as compared to other staining techniques. Hence Field staining is preferred for rapid screening of blood smears. Field stain is also used for staining of thick smears for malarial parasites.

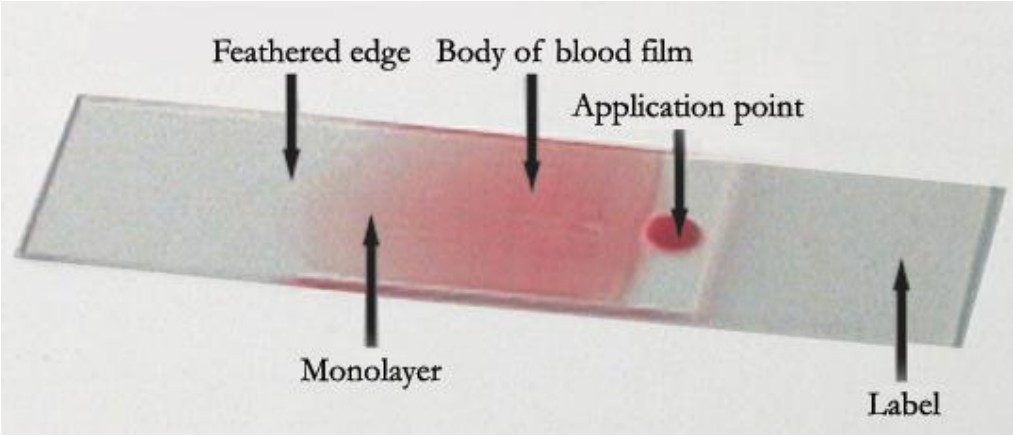
Technique of staining:

1. Fix the smear with methanol for 2 to 3 minutes.
2. Dip the fixed smear in Field stain "B" for 5 seconds. Wash with tap water.
3. Dip now in Field stain "A" for 5 seconds. Wash in tap water.
4. Drain the water.
5. Air dry the smear and examine under oil immersion lens.

Staining of smear

6.3- Counting methods

- Differential count is always done under oil immersion objective.
- The ideal smear will show three zones.
- Thick area or “head” of the smear from where the smear starts.
- “Body” which is in between area.
- “Tail” which is the thin part of smear
- For counting the WBCs portion of blood smear before the tail end is preferred.
- Put a drop of oil over this area of smear and focus under oil immersion objective.
- Identify various types of WBCs & count them. Total 100 WBCs are counted.
- To avoid double counting of same cell, zig-zag pattern can be followed.
- Count at least 100 leukocytes.



Ideal blood smear

Precautions in preparation of smear:

Dirty slide do not give an even smear. Use only clean grease free slide.

Use an appropriate size of blood drop.

After putting the drop on the slide make the smear immediately for even distribution of WBCs on slide.

The thickness of the smear depends on the angle of the spreader if the angle is less than 30^0 thinner smear is obtained

The film must be smooth at the end. There should be no lines extending across or down through the film and should not contain holes.

Precautions during staining:

Avoid formation of deposit of stain. They appear on the film as masses of

the little black spots. In that case rinse the slide twice with methanol and then restain using filtered stain for.

Poor staining makes the smear blue, pink or too dark.

Use neutral water. Acidic water produces red staining effects and alkaline

blue effects. If the film is too blue rinse it twice in 1% boric acid in 95%

Ethanol and examine under microscope after drying.

6.4- Morphology of W.B.C.

- Identify various types of WBCs on stained peripheral blood smear with the help of morphological characters.
- Their respective morphological characters are as follows.

WBCs are of two types:

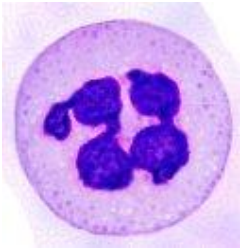
- Granulocytes;
- Agranulocytes

Granulocytes:

Granulocytes are those WBCs which contain granules in their cytoplasm.

Neutrophil:

Neutrophils are round cells having size around 10 to 12 microns. Cytoplasm stains pale pink with fine purple granules. Nucleus stains dark blue .the nucleus is characteristically multilobed having 3 to 4 lobes.

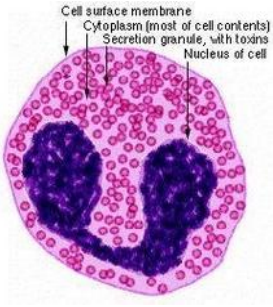


Neutrophil

Function: Neutrophil performs function of "phagocytosis" (To destroy bacteria). Neutrophils are increased in acute bacterial infection.

Eosinophil:

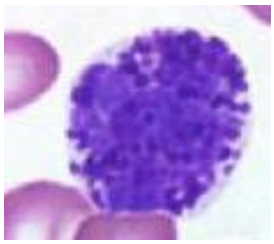
Eosinophils are also round cells having the size same as that of neutrophils. Cytoplasm appears pale pink. Being granulocyte, it shows coarse orange- red granules. Nucleus appears dark blue and it is bilobed.



Eosinophil

Function: Eosinophils are involved in allergic response of the body.Hence in allergic conditions like pulmonary eosinophilia, eosinophils are found to be increased. In parasitic infestations also eosinophils are increased.

Basophils: Basophils are circular cells having average size 10 to 12 microns. Cytoplasm is stained pale pink with large dark blue basophilic granules. Many a times granules almost overlap the nucleus.



Basophil

Function: Basophils are said to be precursors of histamine & heparin.

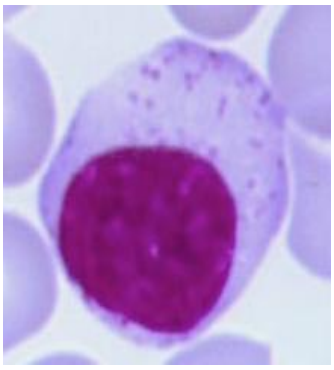
Agranulocytes:

Agranulocytes are those WBCs which do not have granules in the cytoplasm.They are of two types.

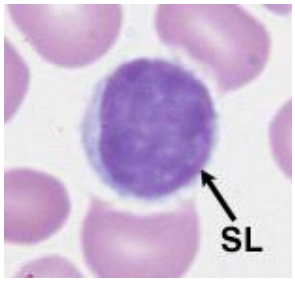
Lymphocytes:

Small lymphocyte is a round cell of the size 8 to 10 microns. Cytoplasm appears smooth & pale blue in colour.There are no granules in the cytoplasm. Nucleus is stained dark purple and occupies the whole cell so that cytoplasm is almost invisible.

Large lymphocyte is a round cell which is of the size 10 to 12 microns.(same as granulocyte).Cytoplasm stains pale blue ,smooth & visible around the nucleus.Nucleus is of purple colour & oval in shape.



Large lymphocyte

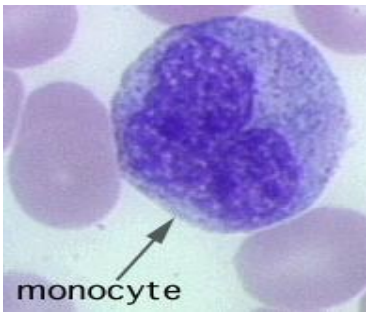


Small lymphocyte

Functions: Lymphocytes produce antibodies. Lymphocytes are increased in immunological response of the body.

Monocytes:

Monocyte is the largest of all WBCs. It's size is 14 to 16 microns. Cytoplasm without granules appears smooth & stains pale blue. Nucleus stains purple and it is kidney shaped.



Monocyte

Functions: Monocytes also perform phagocytic function.

Normal value-

Neutrophils-	40-70%
Eosinophils-	1-8%
Basophils-	0-1%
Lymphocytes-	20-40%
Monocytes-	2-10%

6.5- **Abnormalities in morphology of blood cells & related diseases**

Once a well prepared & equally well stained peripheral blood smear goes in hand of an expert, a lot of information can be gained by keen observation of peripheral blood smear. Peripheral blood smear can give following valuable information.

1. Differential WBC count
2. Platelet count (approximate judgement)
3. Abnormalities in RBC morphology
4. Abnormalities in WBC morphology
5. Parasites in blood
6. Haemoglobin concentration (approximate)
7. Peripheral blood smear can be used as cross verification test for CBC results.

Abnormalities in RBC morphology

Normal RBC: In blood film stained with Leishman's stain, RBCs appear as circular cells. They are stained light pink –red .RBCs are nonnucleated cells. Staining is characteristically darker at periphery & light in the centre. This is called as "central pallor".

Different abnormalities in RBCs are as follows:

1. Macrocyte: RBCs are larger in size than the normal. This is associated with megaloblastic anaemia due to vitamin B12 deficiency.
2. Microcyte: When RBCs are smaller in size than the normal then it is called as microcyte. Presence of microcytosis is suggestive of anaemia especially iron deficiency anaemia.
3. Anisocytosis: Anisocytosis means variation in size of RBCs.
4. Poikilocytosis: Poikilocytosis means variation in shape of RBCs.
5. Hypochromia: When the central pallor increases, it is called as hypochromia. Hypochromia indicates decreased haemoglobin concentration i.e. anaemia
6. Target cell: When RBC appears as if nucleated cell, then it is called as target cell. Target cell shows peripheral dark stained area, then lighter area and then again dark area in the centre. Target cell is a feature of thalassaemia.
7. Spherocyte: When the RBCs are spherical in shape instead of normal biconcave disc like shape they are called as spherocytes. Congenital spherocytosis is a known disorder which results in chronic haemolytic anaemia.
8. Acanthocytes: Acanthocytes are the RBCs showimg thorn like projections on outer edge.
9. Basophilic stippling: Presence of fine or coarse purple staining granules in RBC is called as basophilic stipling. This is observed in lead poisoning.

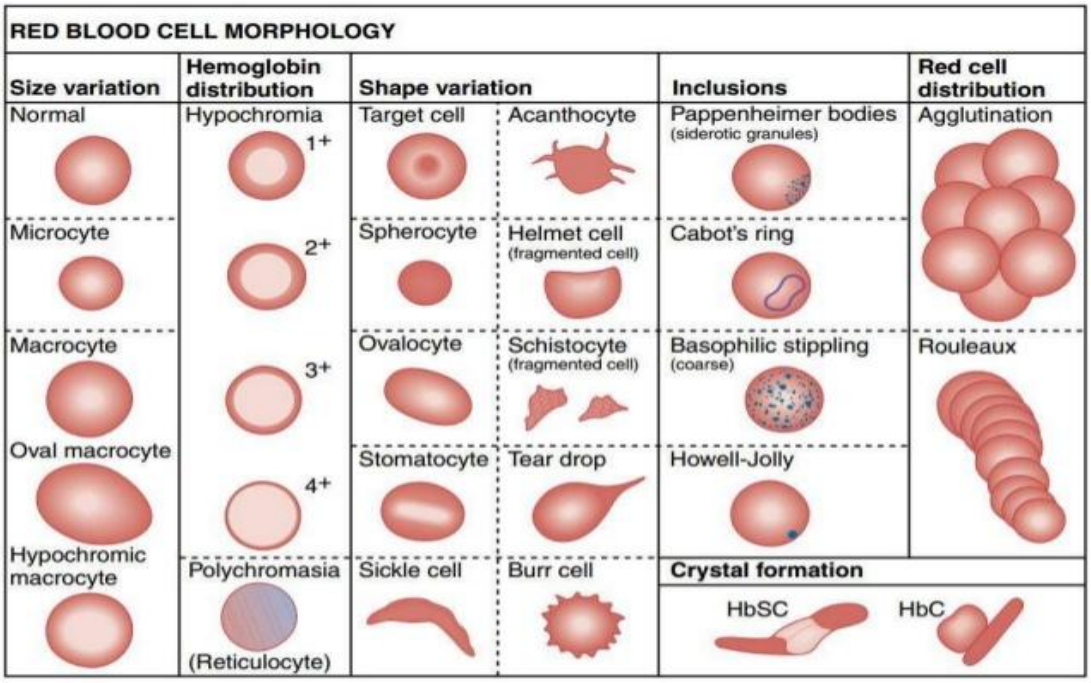


Fig 6.5.1 Abnormal RBCs

Abnormalities in WBC morphology

1. Toxic granules: In acute bacterial infection, neutrophils can show coarse, number of granules. Granules are seen more prominently. This is observed in severe acute bacterial infection.
2. Vacuoles: Vacuoles are seen in cytoplasm & nucleus. It is observed in severe infections, burns, malignancy, chemical poisoning.
3. Hyper segmentation: In neutrophils, nuclei show more than four segments. This can be inherited disorder.
4. Hypo segmentation: Neutrophil will show less than three lobes in the nucleus.it can occur in acute myeloid leukaemia, severe infections etc.

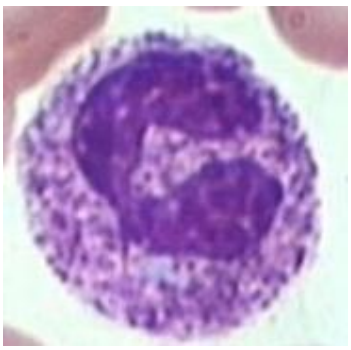


Fig. 6.5.2 Toxic granules

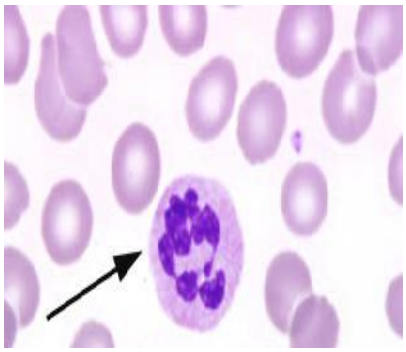


Fig.6.5.3 Hypersegmented Neutrophil

Questions:

1. Name different stains used for staining blood smear.
2. Describe morphology of each white blood cell.
3. Write about any six abnormalities in RBC morphology.
4. How will you differentiate between good smear and bad smear?
5. What are the various uses of stained blood smear?
6. How peripheral blood smear is prepared?
7. How will you do DLC?
8. What is the clinical significance of differential leukocyte count?

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LESSON 7

Erythrocyte Sedimentation Rate (ESR)

Objectives:

1. *Comparative study of different methods of ESR estimation.*
2. *Importance & limitations of ESR.*

ESR is estimated by two methods-

- Westergren's method
- Wintrobe's Method

7.1- Introduction-

Definition: When an anticoagulted blood specimen is allowed to stand in vertical tube for certain time duration RBCs settle down at the bottom of tube. The speed or rate at which RBC's settle down is known as ESR.

It takes place in three stages-

- Stage of **aggregation** when the red cells form rouleaux.
- Stage of **sedimentation** in which the falling of red cells takes place. Larger the aggregates faster will be sedimentation.
- Stage of **packing** when individual cells or aggregates slow down due to crowding.

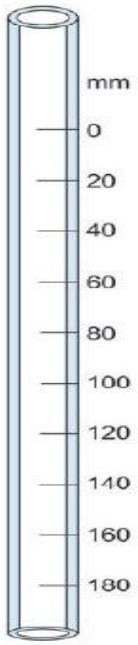
7.2- Westergren's method:

Westergren method is the most routinely used method of ESR estimation.

Requirements-

1. Westergren's pipette (**Westergren tube):** It is a long pipette like tube open at both ends. The markings are from "O" above to "200" down.
1. Westergren's stand

2. Timer or watch.
3. Specimen- Take 0.5ml 3.8% sodium citrate in a test tube add 1.5ml venous blood. Mix well.



Westergren pipette

Procedure:

1. Fill westergren's tube up to the "0" mark.
2. Allow the tube to be held in upright position in westergren stand and note the time.
3. Adjust the timer for one hour duration.
4. Exactly after one hour take the reading. Measure the distance traveled by RBC in westergren's tube.
5. If it comes to 12 then ESR will be noted as 12 mm at the end of one hour.

Advantages:

1. Westergren's method is very easy & quick method for ESR estimation.
2. When number of samples are more westergren's method is more convenient.

Normal value-

- Male- 3-5 mm at the end of 1hr
- Female- 4-7mm at the end of 1hr

Westergren method

7.3- Wintrobe's method:

Requirements-

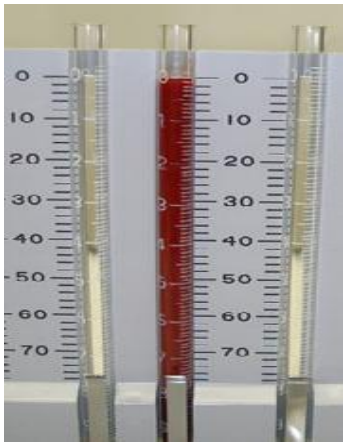
1. Wintrobe's tube
    - Wintrobe's tube is like a tube which is closed at one end. There are two types of scales on wintrobe's tube.
    - One scale indicates "0" mark down & "10" above.
    - On other side the scale shows "0" mark above & "10" mark down.
    - The first scale with "0" mark down is used for haematocrit (PCV) determination. The other scale with "0" mark up is used for ESR determination.
    - This is the advantage of wintrobe's method that simultaneously one can estimate ESR as well as PCV.

2. Wintrobe's stand
3. Pasteur pipette
4. Timer or watch
5. Specimen- EDTA blood

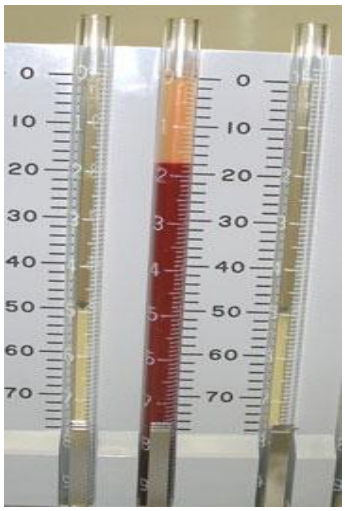
Wintrobe stand

Procedure:

1. Fill the wintrobe's tube with blood specimen up to the upper most "0" mark.
2. The tube is filled up with the help of Pasteur pipette without any air bubbles. This is little time consuming & tedious job.
3. Hold the tube exactly in vertical position with wintrobe's stand. Start the watch & set alarm for one hour.
4. Exactly after one hour take the reading. The scale with "0" above is used.



Wintrobe tube Filled with blood



RBCs settled down

Advantages:

- With wintrobe's method both ESR and PCV can be done with same quantity of blood specimen.
- However filling of wintrobe's tube with pasteur pipette is time consuming and hence for large number of samples westergren's method is practically used.

Normal value-

- Male – 0-9 mm at the end of 1st hour.
- Female – 0-20 mm at the end of 1st hour.

7.5- Landau Method:

In case of insufficient sample or in children when getting blood sample by vein puncture is difficult, Landau method can be used by microsedimentation technique.

The advantage is that even by finger prick technique ESR can be measured.

Requirements:

1. 0.5g/d1 sodium citrate solution as anticoagulant.

2. Landau pipette which resembles RBC pipette. It is having graduations from 0 to 50 mm.
3. Landau pipette stand.
4. Suction device for drawing blood into the pipette.

Procedure:

1. Attach landau pipette to the suction device.
2. Draw sodium citrate solution up to 1st line of stem.
3. Now draw blood by suction device up to second mark on the stem.
4. Wipe excess blood from external side of pipette.
5. Draw citrate solution & blood in to the bulb of pipette.
6. Force back the mixture into the stem of the pipette.
7. Set upper level of the mixture to the zero mm mark at the top.
8. Detach the suction device.
9. Place the pipette in vertical position in the stand, set timer for one hour.
10. Note the reading after one hour.

Normal value-

Male – 0-5 mm after 1st hour.

Female – 0-8 mm after 1st hour.

7.6- Factors affecting ESR

Physiological Factors:

a. Age: At the extremes of ages, ESR is less i.e. in children and in elderly people ESR is more.
b. Sex: In females ESR is higher as compared to males.
c. Pregnancy: During pregnancy ESR is more.

Laboratory Factors:

a. Time: The test should be done as early as possible after the collection of fasting specimen. As the time passes sedimentation decreases.
b. Length of ESR Tube: ESR is more with longer tube (westergren's tube) than with shorter (wintrobe's tube) tube.
c. Position of tube: The tube should be kept exactly vertical in position. Deviation of tubes can increase ESR.
d. Temperature: If the temperature is higher sedimentation is more i. e. with higher room temperature ESR will be more.

Pathological Factors:

a) Any condition where there is tissue break down will show higher ESR readings.
b) In many chronic infections, ESR is increased. e.g. tuberculosis, rheumatic fever etc.

7.7- Clinical significance of ESR:

- ESR estimation is simple and inexpensive laboratory test .Hence serial readings are possible.
- Raised ESR indicates inflammation but does not tell about any specific disease.
- On the contrary ESR is said to have prognostic importance.
- It helps physician to monitor condition of patient in diseases like Rheumatoid arthritis, SLE, Myocardial infarction, etc.
- In MI and ARF it is used to decide the moment when rest in bed may be discontinued. As long as ESR is raised bed rest is indicated.
- In many chronic conditions ESR increases e.g. tuberculosis, rheumatic heart disease, anemia, cancer etc. But this does not carry diagnostic significance.
- ESR is less in polycythaemia vera.

Review Questions:

1. Define ESR. Enumerate various methods of ESR estimation.
2. How will you determine ESR by micro sedimentation technique?
3. What are the various factors affecting ESR?
4. Write about clinical significance of ESR.
5. Describe the difference between wintrobe's and westergren's method of ESR estimation.

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LESSON 8

Haematocrit or Packed Cell Volume (PCV)

Objective- *students shall understand the technique of PCV estimation and its significance.*

8.1-Introduction-

The PCV or Haematocrit is a percentage of total volume of whole blood occupied by packed red blood cells. When known volume of whole blood is centrifuged at constant speed for a constant period of time, red blood cells are packed at the bottom of the tube. The volume of packed red blood cells is measured & expressed as haematocit or PCV(Packed cell volume)

Packed cell volume (Haematocrit) is carried out by two methods

- Wintrobe's (Macrohaematocrit) method
- Microhaematocrit Method

8.2- Macrohaematocrit method:

A large volume of blood is needed in this method and hence only venous blood can be used. This method can give the values of two diagnostic tests ESR and PCV (HCT).

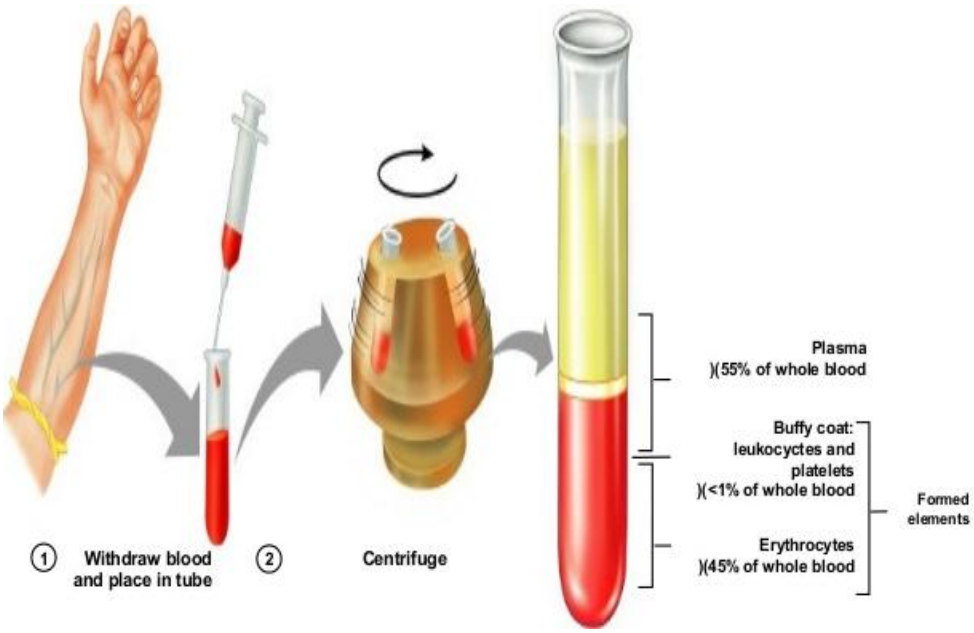
Principle: An anticoagulated blood is centrifuged to packed cells to maximum in a Wintrobe's tube at high speed. The volume of packed cells is determined.

Requirements: Wintrobe tube, transfer pipette, special syringe, centrifuge, blood sample, timer.

Procedure:

1. Carefully mix the blood specimen.

2. Label the required number of wintrobe tubes.
3. Fill the wintrobe tube with the help of the pasture pipette or special syringe to the 10 cm. mark which represents 100%. While filling the tube from the bottom gradually withdraw the pipette as the blood goes in. This will avoid trapping of air bubbles.
4. Place the wintrobe tube in a centrifuge cup. Use another specimen to balance.
5. Centrifuge the tubes for 30 minutes at 3000 RPM.
6. After 30 minutes switch off the centrifuge and allow it to stop by itself.
7. Take out the wintrobe tube after the centrifuge has stopped and read the PCV directly from the graduation given on the tube.



Technique of PCV

Normal value-

- Males- 40-54%
- Females- 36-47%

Sources of Error-

- Inadequate mixing of blood

- Irregularity of bore of needle.
- Incompleteness of packing.

Note-

When a column of blood is centrifuged it shows three layers.

1. A lowermost layer of packed cells-indicates PCV.
2. A Buffy coat- is a thin layer of WBCs and platelets which is just above the packed cells. It is grayish in color. It is 0.5 to 1mm thick. It gives approximate indication of number of WBCs and platelets..Buffy coat is used in demonstration of LE cell and in cases of sub-leukemic leukemia.
3. The uppermost layer is plasma. The color of plasma indicates certain conditions.
    - Pale yellow Normal
    - Yellow May be jaundice
    - Pink Haemolysis
    - Milky Lipemia
    - Brown Meth-haemoglobin
    - Cloudy May be multiple myeloma

Thus PCV is helpful in finding out information regarding RBCs, WBCs, platelets and plasma.

8.3- **Microhaematocrit method**:

Introduction

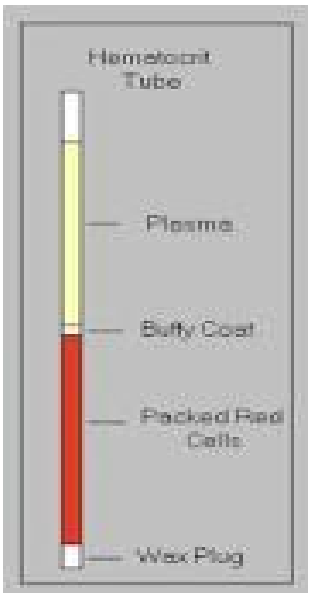
- This method requires only a small volume of specimen and hence it is ideal for skin puncture.
- It however requires disposable capillary tubes, a special centrifuge and reading device.
- The advantage of this method is it takes less time.

- The capillary tubes are easy to fill and a large number of specimens can be handled simultaneously.

Principle: Blood is centrifuged in a heparinized capillary tube and the volume of packed red cells and percentage of the whole blood are determined by a special haematocrit reader.

In case of anticoagulated venous blood obtained from vein puncture do not use heparinized capillary tubes.

Requirements: Microhaematocrit centrifuge, haematocrit reader, capillary tubes, moulding clay (this is used to seal the end of the haematocrit tubes), blood sample.



Microhaematocrit

Procedure:

1. Draw the blood sample into an appropriate capillary tube by capillary action. Use a plain tube for anticoagulated whole blood and heparinized tube for skin puncture. Fill the tube to about 3 to 4 cm length.
2. Seal both the ends of the tube with soft wax or moulding clay .It is plugged to a depth of about 1 cm.
3. Write the identification number on the tube by using a marking pencil.

4. Place the tube with another similar tube in the radial groove of the centrifuge head; exactly opposite to each other (empty capillary tube can also be used).
5. Close the centrifuge cover and centrifuge the tubes at high speed (13,000 rpm ± 200 rpm for 5 minutes).
6. Remove the capillary tubes. It will show three layers. Clean plasma at the top, whitish buffy coat at the middle and column of red cell at the bottom.
7. Use the haeamatocrit reader for finding out the value and haematocrit for each sample. Note the value and immediately write in the register.

Sources of Error-

- Hemolysed sample will give false low values.
- Inadequate mixing and incompleteness of packing may lead to errors in result.

Advantages-

- Useful when there is difficulty in drawing sufficient amount of blood.
- Requires a small volume of sample, so capillary puncture is preferred.
- Useful in pediatric patients.
- Takes less time.
- Capillaries are easy to fill.
- Large number of samples can be handled.

Disadvantages-

- Requires special centrifuge and disposable capillary tubes.
- Doesn't give ESR.

Review Questions:

1. What is Haematocrit? Give its clinical significance.
2. Describe the wintrobe's method of measuring Haematocrit.
3. Describe the Microhaematocrit method of measuring haematocrit.

LESSON 9

Erythrocyte Indices (Red cell indices)

Objective:

At the end of the topic the student shall be able to calculate the various erythrocyte indices from the given PCV, RBC count and Hb value and study their importance in diagnosis of anemia.

9.1- Introduction

Red cell indices are also known as wintrobe's constant. They depend upon Hb gm%, RBC count in million and PCV in percentage (%). This gives quantitative information about RBCs. Initially they were known as 'Absolute values' but not now because tests on which they are dependent, those values are not absolute but have some error. So they are called as indices. Red cell indices help in morphological classification of anaemia.

9.2- **Blood cell indices**

1. MCV – Mean cell volume
2. MCH – Mean cell Haemoglobin
3. MCHC – Mean cell Haemoglobin concentration
4. CI- Color Index

1. MCV – It is the volume of average red cell, i.e. it is indicative of size of the cell.

$$MCV = \frac{PCV \times 10}{RBC\ count}$$

Normal Range – 78-94 cubic microns

Significance- Increases in macrocytes and decreases in microcytes.

2. **MCH** = Hb x 10
RBC count

Normal Range - 27 to 32 picograms.

Significance- Decreases in hypochromia.

3. MCHC- It is sensitive indicator of Hb saturation with reference to its volume.

MCHC = Hb x 100
PCV

Normal Range – 30-38%

Significance- Decreases in hypochromic anaemia.

4. CI- Estimates average amount of Hb in red cells in comparison to normal.

CI = Hb% (of normal)
RBC% (of normal)

Percentage of haemoglobin is calculated by taking 14.5gm% as 100%.

Percentage of RBC is calculated by taking 5millions as 100%.

Normal Range- 0.85- 1.15

Significance-

- CI less than normal seen in hypochromic anaemias and shows deficiency of Hb is more than deficiency of RBCs.
- In megaloblastic anaemia RBC count decreases to a greater degree than Hb, so the CI will be more.

Note-

- CI doesn't have much value as it does not take in to consideration the size of red cells and concentration of Hb.
- In case of MCV, MCH, MCHC; MCHC is more accurate because it depends on PCV and Hb which are more accurate than RBC count which is less accurate.

Review Questions:

1. Which are erythrocytes Indices? Give their clinical Significance.
2. What is MCV? How is it calculated? Give normal value.
3. What is MCH? How is it calculated? Give normal value.
4. What is MCHC? How is it calculated? Give normal value.
5. Write short note on color index.

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LESSON 10

Sickle Cells

Objective:

At the end of the topic the students shall understand the screening for the sickle cell anemia in the population and its importance.

10.1 - Introduction

Sickle cells are abnormal red cells. They are narrow crescent shape/ half-moon shape/sickle shape cells with defective membrane. They contain abnormal haemoglobin (HB-S) which is inherited. Haemoglobins-S (HB-S) is insoluble when oxygen tension is lowered and this makes the red cells susceptible to sickling. The sickle cells tend to clump together and cannot freely flow in the circulation. These cells are easily haemolysed because of their abnormal shape and cause a chronic hemolytic anemia.

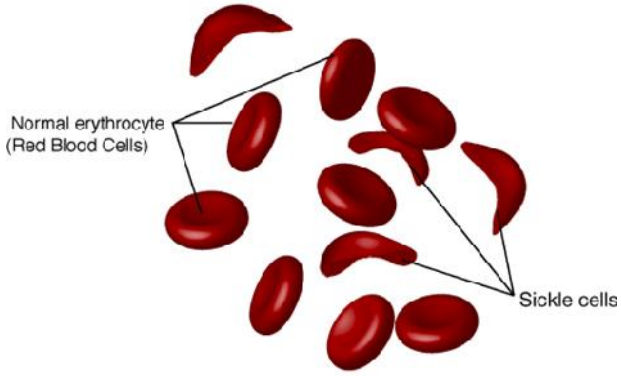

Figure 10.1 Sickle cells

10.2 Laboratory diagnosis of sickle cell anemia

It is based on three observations-

1) Sickling of red cells under deoxygenated conditions.
2) Precipitation of HB-S in a special medium.

3) Haemoglobin electrophoresis.

Specimen:

EDTA anticoagulated blood/ capillary blood can be used directly.

10.2- Sickling under reduced oxygen tension

This test is convenient but does not differentiate sickle cells trait from sickle cell aneamia (Sickle cell disease).

Principle :

When whole blood is mixed with sodium metabisulphite, a strong reducing agent that deoxygenates haemoglobin. If cells contain haemoglobin – S (HB-S) they become sickle shaped or half moon shaped. This test does not distinguish between sickle cell trait and sickle cell aneamia.

Reagent-

Sodium metabisulphite or sodium bisulphate (Prepare fresh reagent).

Requirements:

Dropper, Pasture pipette, Petri dish, glass slide, cover slips, petroleum jelly /wax. Blood sample etc.

Procedure :

1. Place a drop of blood to be tested on a slide.
2. Add an equal drop of the sodium metabisulphite solution by means of a pasture pipette.
3. Mix carefully and cover with the cover slip making sure that no air bubbles form under the cover slip.
4. Then carefully seal the cover slip with petroleum jelly. If petroleum jelly is not available, keep the preparation in a moist Petri dish & incubate for 15 min. at 37^0 C.
5. Examine the preparation under high dry objective of the microscope.
6. Sickling is visible almost immediately in cases of sickle cell disease

7. In case of positive result the red cell become sickle shaped or with spikes.

10.3- Solubility test for sickle cell

- This test is more sensitive than sodium metabisulphite test.
- It is observed visually & does not require microscope.
- Useful for mass screening.

Principle: HB-S in reduced state is less soluble than Hb-A (normal). Dithionite in phosphate buffer reduces HB-S, thus it forms a turbid suspension. These crystals prevent reading of lines on a paper card.Saponin is used to lyse the RBCs.

Reagent- Dithionite solution.

Requirements –

Test tube , test tubes rack with a background lined reader scale, Sahli's pipette, blood sample etc.

Procedure -

1. Pipette 2ml of reagent in a test tube.
2. Add 20 micro-liter /0.02 ml of blood specimen.
3. Mix and wait for 10 min.
4. Place the tube in a test tube rack with a background lined reader scale.
5. Read the turbidity and note the result.
6. A positive test is indicated by very turbid solution; the black lines on the background reader scale cannot be seen, through the solution.

Errors

- False positive results may arise from polycythaemia, more blood added to reagent and hyperlipemia.
- Outdated reagents may cause false negative results.

- Use best quality saponin. Impure saponin may give false positive result.

Clinical significance

1. Sickle cell aneamia is due to a homozygous inheritance of the abnormal haemoglobin (HB-S-S).
2. In a heterozygous condition HB-S –A, the disease is called as sickle cell trait. In trait morphology of red cells is normal or slightly abnormal. But they sickle under decreased O_2 supply. Degree of sickling depends on concentration of Hb-S in RBCs.

Review Questions:

1. What are Sickle cells? How the sickle cells differ from normal RBCs?
2. What is Sickle cell preparation?
3. What are the advantages of solubility tests for sickle cell anaemia?
4. What is principle of sickling test?

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LESSON 12

Coagulation of Blood

Objectives : *After reading this topic, students will understand about how blood clots after injury, Factors of coagulation, determination of bleeding time & clotting time, Prothrombin time with reference to its significance, procedure, normal range & precautions.*

12.1 Introduction: Bleeding occurs when a blood vessel is injured, and bleeding stops by a process called haemostasis . Abnormal bleeding occurs spontaneously or following trauma, due to the derangement of haemostasis which warrants investigations. Patients prepared for surgical procedures must be routinely tested to ensure normal haemostasis and appropriate care must be taken in case of any abnormality.

Haemostasis- Haemostasis (Haem means blood and stasis means to stop) is a complex process of stoppage of bleeding that involves three major steps in sequence –vascular response, platelet plug formation, and coagulation or clot formation.

1. **Vascular response:** Vasoconstriction is the immediate response of the blood vessels to the injury. This constriction reduces the blood flow which assists in the process of platelets plug formation. Muscular contraction and release of serotonin (and other chemical mediators) by the damaged platelets are contributory factors in the formation of the plug. The vascular response cannot be clearly separated from the platelet response, and hence, the basic screening tests – capillary fragility test and bleeding time – measure both.

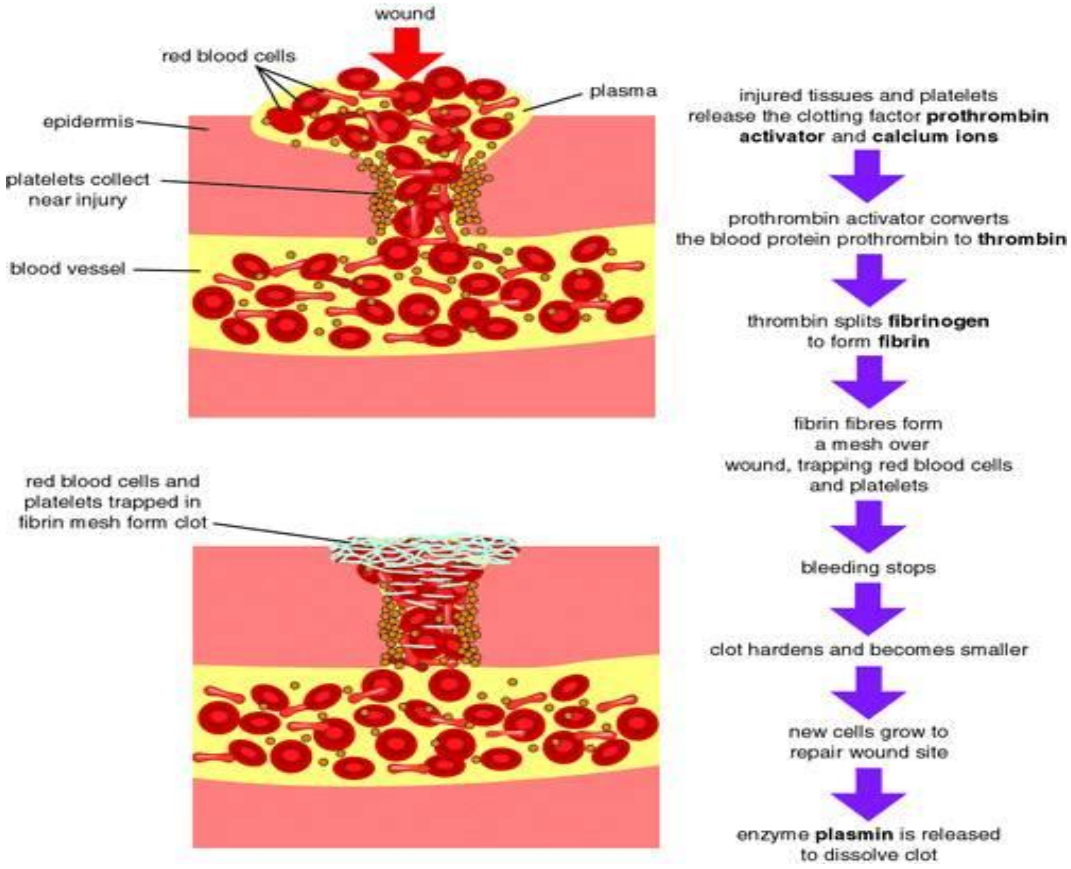


Fig.12.1 Events taking place at the site of injury

Platelet plug formation: This occurs when the platelets adhere to the vessels wall at the site of vessel injury. The steps of plug formation include aggregation of platelets followed by deposition of fibrin around the platelet aggregate. The fibrin binds the platelets together and anchors the platelet plug to the vessel wall as an impermeable seal over the injury site in order to arrest bleeding. The effectiveness of platelets in haemostasis and platelet related defects can be assessed routinely by bleeding time determination, platelets count and clot retraction test.

Coagulation: The circulating plasma and tissues surrounding the blood vessels contain a total of twelve coagulation factors. (Note: Although the coagulation factors are numbered I to XIII, factor VI is missing which was later found out to be the same as factors V). These coagulation factors are various organic compounds, like proteins and co – enzymes, and the inorganic element calcium. This coagulation factors are present in the circulation under normal conditions, in an inactive form. whenever there is a bleeding episode, these coagulation factors get activated in order to form the blood clot.

11.2 Coagulation Factors:

Factors	Synonym
I	Fibrinogen
II	Prothrombin
III	Tissue Thromboplastin
IV	Calcium
V	Labile factor
VI	Combined with V(does not exist)
VII	stable factor
VIII	Antihaemophilic factor
IX	Christmas factor
X	Stuart factor
XI	Plasma thromboplastin antecedent(PTA)
XII	Hageman factor
XIII	Fibrin stabilizing factor

Platelets: These are not included in the list of coagulation factors. Platelets, however, release several factors that help in the clotting process. Normal fresh plasma contains all the factors listed in the above table except factor IV (calcium) which is removed by the anticoagulant. Thus, addition of calcium will make normal plasma clot within a few seconds.

11.3 Mechanism of Blood Coagulation: The classic theory of coagulation was described by **Paul Morawitz** in 1905.Blood coagulation occurs in three stages

1. Generation of plasma thromboplastin ,
2. Formation of thrombin from prothrombin and
3. Formation of fibrin clot from fibrinogen.

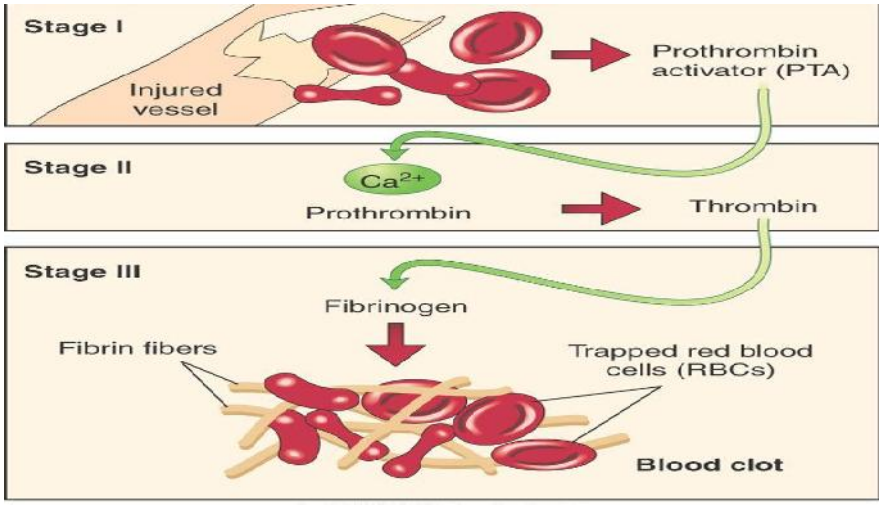


Fig. 11.3 Blood clotting steps

Stage 1: Generation of Plasma Thromboplastin:

The tissue extract or tissue thromboplastine (factor III) enters the blood vessel through the site of injury, and combine with factor VII and calcium (factor IV). This is designated as the **extrinsic system** that originates from outside the blood vessel and includes such factors as III, VII and IV (calcium). The extrinsic system then joins the **intrinsic system**. The factors

that participate in the intrinsic system are all present in the circulation (XII, XI, IX, VIII and calcium). The extrinsic and intrinsic systems after joining together within the blood vessel follow a common path involving factors X and V. Finally, the end product of stage 1- plasma thromboplastin, is formed. The thromboplastin then triggers the stage 2, which otherwise remain dormant in the blood with the inactive prothrombin (factor II). Any defect in the intrinsic system of stage 1 is recognized by activated partial thromboplastin time (APTT).

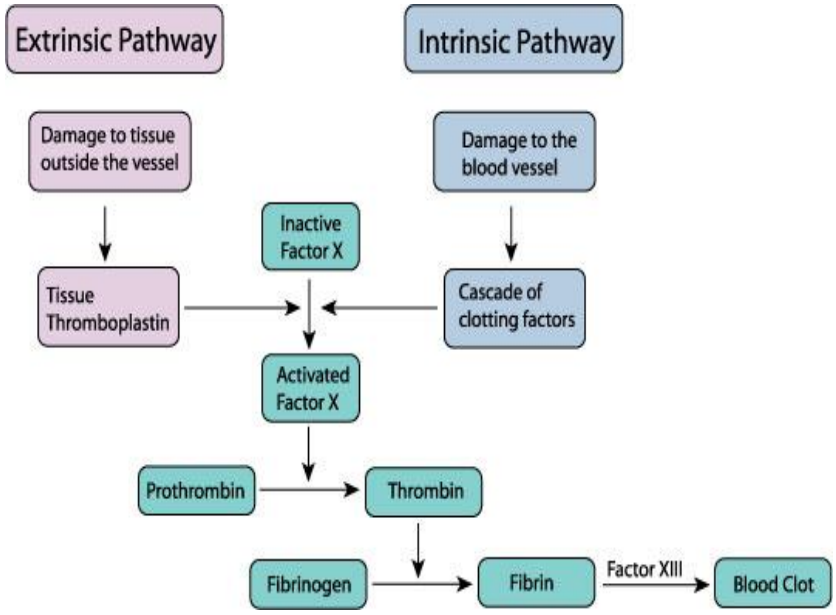


Fig. 11.3 Schematic presentation of clotting process

Stage 2: Formation of Thrombin from Prothrombin:

In stage 2 prothrombin (factor II) is activated by the thromboplastin-the end product of stage 1. This results in the formation of thrombin. Any defect in stage 2 (factor II deficiency) will be recognized by prolonged prothrombin time (PT).APTT will also be prolonged as a secondary effect. The

cascade system of coagulation steps further continues to stage 3.

Stage 3: Formation of Fibrin from Fibrinogen:

This is the final stage of the coagulation process. Inactive fibrinogen (factor I), is activated by the end product of stage 2- thrombin. The fibrinogen is first converted into soluble fibrin (fibrin monomers). Soluble fibrin polymerizes with the help of activated factor XIII to form an insoluble and mechanically strong fibrin clot.

11.4- Laboratory investigations of bleeding disorders

Laboratory investigations of bleeding disorders are set up for patients who have a history of spontaneous bleeding or excessive bleeding after trauma or surgery.

Important screening tests are:

1. Bleeding Time (BT),
2. Clotting Time (CT)
3. Prothrombin time (PT).

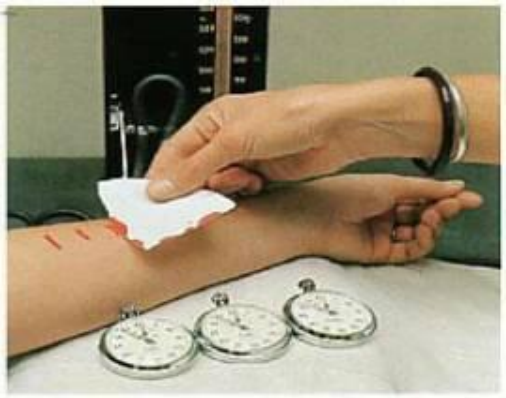
Determination of bleeding time

Bleeding time determined by:

A) Duke's method B) IVY's method

Principle- A standard incision is made on the skin of the patient. The length of time required for bleeding to stop is recorded.

Duke's method	**IVY method**
Site of puncture- Ear lobe or finger tip	An area of about 3 finger width below the bend in the elbow by applying the blood pressure cuff on the patient's arm above the elbow. Inflate the cuff to 40 mm of

	mercury and hold this exact pressure for the entire period.
Single puncture	Two punctures 5-10cm apart
Normal range: 1-5 minutes	**Normal Range:** 5-11 minutes
Easy to perform and requires minimal equipments or laboratory skill.	More reliable than duke method but requires additional equipment and skill to use Blood pressure apparatus.
	

Clinical significance:-

1. Determination of bleeding time recognizes vascular defect & platelet disorder.
2. Bleeding time is prolonged when the level of platelets is decreased (platelet count <50,000 /cu.mm) or when platelets are qualitatively abnormal, as in
   a. Thrombocytopenia
   b. Platelet dysfunction syndromes.
   c. Abnormality in walls of small blood vessels- vascular defects.
   d. Severe liver diseases
   e. Leukemia
   f. Aplastic anaemia

g. DIC (Disseminated intravascular coagulation)

3. A single prolonged bleeding time does not prove the existence of haemorrhagic disease because a larger vessel may have been punctured. The puncture should be done twice and the average of the bleeding can be taken.

11.5 COAGULATION TIME (Clotting time)

Defination : It is the time taken by whole blood drawn from a vein & placed in a container or in vitro to clot.

Clinical significance:

a. Severe deficiencies of any of the coagulation factors must be present before the coagulation time will be prolonged..
b. When prothrombin is diminished to a level of 30% of normal, there will be a small change in coagulation time.

Capillary tube method of Wright

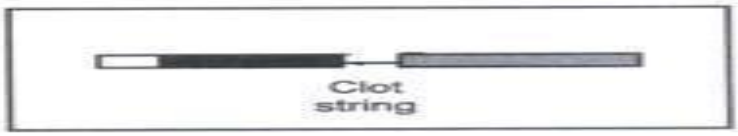


Fig Capillary tube

Procedure

Blood is collected in a capillary tubes (in a single or number of capillaries) from a finger prick made after aseptic precautions. The tubes are sealed with plasticine and immersed in water bath at 37˚C. After 3 minutes remove the first tube from the bath and expel the blood in it with one end immersed in dish containing water. Repeat this every 30

seconds with the other tubes till the blood is expelled in form of clot and note the time.

An alternative way of determining the end point is to break the capillary tubes every 30 seconds until the clot is seen between the two broken ends. By this method the normal clotting time is 5 to 10 minutes and the longer if performed at room temperature. This test should be avoided as tissue thromboplastin contaminates the oozing blood and hence false report may be obtained.

2. Lee & White method

Principle :- Whole blood, when removed from the vascular system and exposed to the foreign surface, will form a solid clot. Within limits the time required for the formation of the solid clot is a measure of the coagulation system.

This is more reliable & sensitive than the capillary method.

Requirement

1. Stop watch
2. Equipment for collection of blood
3. Clean, Dry glass test tube(10 x75mm)
4. Water bath (37˚C)

Lee & White method

Procedure:

1. Make a clean venipuncture with a little trauma to the connective tissue between skin and vein as possible.
2. Timing is begun when the blood first enters the syringe.
3. Draw 3-5 ml of blood and withdraw the syringe and needle. Disconnect the needle. Place approximately 1 ml of blood in each of three test tubes.
4. Place the tubes in a stand so that they remain upright and undisturbed, for 3 minutes in a water bath at 37˚C.
5. After 3 minutes take the first of the tubes and gently tip it every 30 seconds to test clotting. If the blood is not clotted, return the tube to the water bath & examine at every 30 seconds interval to see if it is clotted.
6. When the first tube is clotted, record the time and start the tilting the second tube every 30 seconds until it also is found to be clotted. Then do the same with the third tube.
7. The time recorded for the clotting of the third tube is taken as a clotting time.

Normal range

Normal time depends on temperature used. Normal range at 37˚C is usually 4-10 minutes

Precautions

The venipucture must be without trauma to avoid contamination with tissue thromboplastin. Vigorous agitation of the tubes will significantly shorten the coagulation time. So tilting should be very gentle just to see if the blood has clotted.

11.6 PROTHROMBIN TIME (QUICK ONE STAGE METHOD)

Definition: Prothrombin time is the time required for clotting of citrated or oxalated plasma in a glass tube after the addition of calcium and tissue thromboplastin.

Procedure

1. Place 1.8 ml. of patient's blood obtained by a clean venipuncture into a tube containing 0.2 ml. of 0.1 M sodium oxalate or 0.2 ml. of 3.8% sodium citrate. Mix blood well, centrifuge at 2,000-3,000 rpm for 2 - 3 minutes and remove the plasma.
2. Obtain one or more control plasma in the same way.
3. Place 0.1 ml. of saline thromboplastin suspension and 0.1 ml of 0.15 M $CaCl_2$ into a 10X75 mm test tube. The tubes are agitated slightly to assure mixing of the solution and they are placed in 37˚C water bath.
4. After one minute, 0.1 ml of the plasma, a small quantity of which has been brought to 37˚C in the water bath is blown out of a 0.1 ml pipette into the tube containing the $Cacl_2$-Thromboplastin mixture. After 5-7 seconds of gentle mixing at 37˚C the tube is gently tipped under constant observation, the stop watch having been started simultaneously with the addition of the plasma.
5. The stop watch is stopped the moment the appearance of clot is observed and the time is recorded.

Normal range: 12 to 18 seconds.

Interpretation

The commonest causes of prothrombin time prolongation are liver disorders, and treatment with one of the dicoumarol

drugs (oral anticoagulants) though congenital deficiencies of the involved substrates may occur .Vitamin K is essential for the formation of prothrombin in the liver and lack of vitamin K therefore causes prolongation of the prothrombin time. This occurs most commonly in the newborn.

Clinical significance

Conditions accompanied by an increased prothrombin time (PT) include

-- Prothrombin deficiency

-- Vitamin K deficiency

-- Haemorrhagic disease of the newborn

-- Liver disease (e.g. alcoholic hepatitis)

-- Anticoagulant therapy

-- DIC

Note: Prothrombine time is also interpretated in terms of INR.

INR : International normalized ratio.

ISI : International sensitivity index

1. INR = (PT test / PT normal control) x ISI
2. Normal range of INR for healthy persons is 0.9 – 1.3.
4. For patient on warfarin therapy, INR values range from 2.0 - 3.0.
5. A high INR level (i.e. INR > 5 indicates that there is a high chance of bleeding. Similarly, if the INR < 0.5 indicates that there is a high chance of having a clot.

Review Questions:

1. What are the factors of coagulation?
2. Define bleeding time, clotting time & prothrombin time.
3. Enumerate any three factors affecting coagulation. Give the stages of mechanism of coagulation.
4. Define clotting time. Describe it’s determination of capillarytube method.
5. Describe the method for determination of bleeding time by any one method.

LESSON 12

Examination of bone marrow

Objective*: Students will understand about clinical significance of bone marrow examination, methods of collection, preparation of smear & staining of smears, detection of iron in bone marrow.*

12.1 Introduction

Bone marrow is present in the cavities of bone in postnatal life. It is cellulovascular tissue occupying medullary cavities and the cancellous spaces of bones.

Bone marrow is composed of

- Reticulum cells and fibers
- Blood vessels and nerves
- Developing blood cells and mature cells which they produce
- Fat cells

12.2 Types of bone marrow

a. **Red bone marrow-** It is blood forming and it owes its color to the Hb in developing and mature red cells and to the blood in bone marrow. Blood cells are actively manufactured here. It is active marrow. From birth to 4^{th} year all the bone contain red marrow. But with advancement of age, the red marrow is only located in the upper end of humerus, femur, bones of skull, thorax, vertebrae & pelvis.

b. **Yellow bone marrow** – It is made up of mainly fat cells with small no of capillaries and reticulum cells in between. It is inactive marrow. Here blood cells are usually not manufactured.Reticulum cells of yellow marrow are haemopoietic and can form blood cells whenever required.

12.3 Clinical Significance

- The red blood cells, white blood cells & platelets are manufactured in the bone marrow .If the manufacturing process is abnormal, it may indicate disease.
- Bone marrow examination helps in diagnosis of wide variety of disorders affecting haemopoietic system.
- It helps in confirming diagnosis suspected on clinical examination or on the blood film. E.g. Anaemia,Leukamia etc.
- Bone marrow may be obtained from one person for transplantation to another.

A peripheral blood smear examination must be done before bone marrow examination.

Indications for bone marrow examination

1. Megaloblastic macrocytic anaemia
2. Multiple myeloma
3. Leukemias of all types
4. Idiopathic thrombocytopenic purpura
5. Storage disorders (Gaucher's disease, Niemann Pick disease)
6. Myelofibrosis
7. Secondary deposits (metastasis) in bone marrow
8. Leishmaniasis
9. Aleukemic leukemia
10. Staging of lymphoma
11. Haemolytic anaemia

12.4 Bone marrow biopsy:

A. Aspiration or Needle biopsy-

- It is a simple, safe method & can be repeated number of times.

- It is performed by using a specially designed needle such as Klima needle.
- Bone marrow is aspirated through wide bore needle. It is done under all aseptic precautions. 0.2ml of marrow fluid is aspirated by gentle suction. Aspirate consists of marrow elements diluted with blood.
- Smears are prepared from this aspirate and stained with Romanowsky stains.

Dry tap- With proper method at different sites when there is no aspirate it is called dry tap. In such cases trephine biopsy is performed.

Fig. 12.4.1 Klima needle

B. Trephine Biopsy-

A modified Vim Silverman needle is used. It is used to obtain a biopsy specimen. From this histological sections as well as smears are prepared.

Indications-

a. Aplasia of marrow where repeated needle biopsy at different sites fail to give satisfactory sample.

b. Myelosclerosis

c. Osteosclerosis

d. Metastatic deposits

Contraindications for bone marrow biopsy-

- Hemophilia and other major coagulation disorders.

12.5 Sites for obtaining bone marrow are:

In adults:

1. Sternal aspiration

2. Anterior iliac crest

3. Vertebral spinous process

4. Posterior superior iliac spine

In Children

1. Tibia, superior medial surface of the tibia,

2. Inferior to the medial condyle & medial to the tibial tuberosity

3. Posterior iliac crest

4. Calcaneum

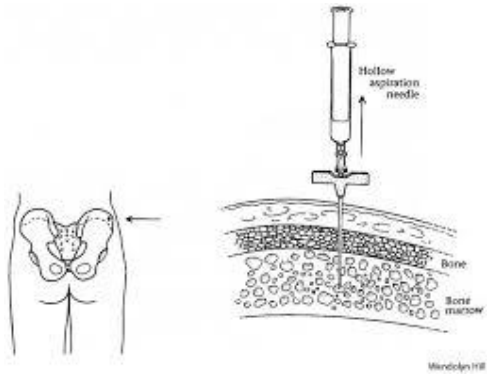


Fig 12.5

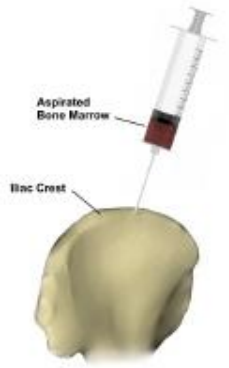


Fig 12.5

12.6 Preparation of marrow films

1. Direct film- Delay in marrow film preparation should not be there. A single drop of marrow fluid is delivered on one end of glass slides and extra blood is sucked with Pasteur pipette. Marrow fragments are left behind. Make a smear as for peripheral blood. The smear should be 3-

5 cm long & should not be more than 2 cm wide. The particles should be dragged behind but not squashed. A trail of cells is left behind. Criteria of a good preparation are presence of both particles & free marrow cells in the smear. Prepare 4 to 5 smears.

2. Imprints- Another way is imprint method. The marrow particle is picked and transferred immediately to a slide and made to stick to it by a gentle smearing motion. The slide is air dried rapidly by waving.
3. Crush preparation- Marrow particles in a small drop of aspirate are placed on a slide near one end. Another slide is carefully placed over the first. Slight pressure is exerted to crush the particles & the slides are separated by pulling them apart in a direction parallel to their surfaces.

   Dry the smears immediately. The appearance of fat (irregular holes) on the smear implies that the marrow has been obtained. It takes 10 - 12 minutes for drying.

   Along with bone marrow take peripheral blood smears by finger Prick method.

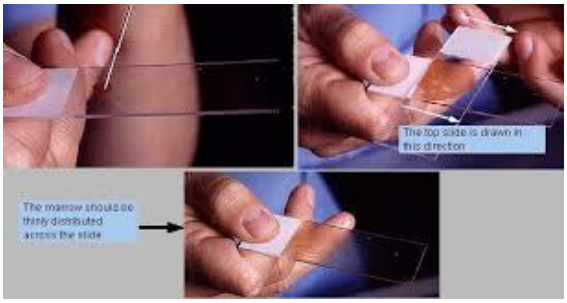


Fig.12.6 Bone marrow smears by crush preparation

12.7 Staining of marrow films

Romanowsky stains: Leishman, Geimsa, and Wright stains are commonly used.

1. May-Grunwald Giemsa stain-

Reagent

May-Grunwald stain- 0.3gms

Methyl alcohol- 100ml

Dissolve powder in methyl alcohol and place it in water bath at 56^0c for 24hours. Filter after 24 hours. Before use dilute it with equal parts of buffer water.

2. Giemsa stain

Giemsa- 1gm

Glycerine- 66ml

Methyl alcohol- 66ml

Method-

1. Fix the slide.

2. Immerse in May Grunwald stain – 5minutes

3. Remove and without washing immerse in Giemsa stain- 15minutes.

4. Wash in buffer water- 5-7 times

5. Dry it and examine it under microscope. (Microscopic examination is done by pathologist only).

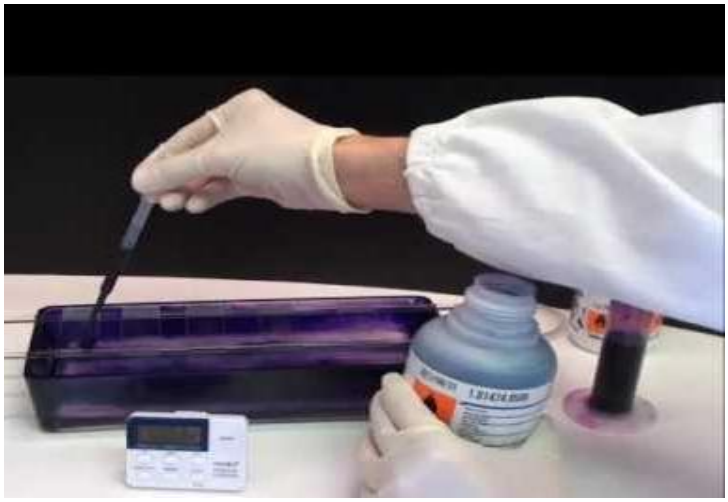


Fig. 12.7

12.8 Examination of marrow film-

Select a cellular area (usually in tail portion of the film around particles), study the cytological details by high power 40 x & oil immersion 100x objectives. Following features are to be seen.

1. Cellularity of bone marrow- normocellular, hypocellular or hypercellular
1. Type and activity of erythropoiesis.
2. Look for maturity of leukopoietic cells & the M:E ratio. Generally ratio is 3-4:1
3. Look for megakaryocytes , their number, size, nuclear lobulations.
4. Presence of foreign or tumor cells, increased number of normal & abnormal (myeloma) plasma cells.
5. Presence of parasite or organism. E.g. L.D bodies of Kala azar, in malaria,etc.
6. Cells containing unduly large amount of lipid, carbohydrates etc-as seen in storage diseases.
7. Iron content of marrow.

Clinical Significance-

1. In Iron deficiency anemia the iron stores are completely depleted
2. In sideroblastic anemia prominent increase in the number of iron granules is observed.

Review Questions

1. What is bone marrow?
2. What are different sites of bone marrow collection?
3. What are indications for trephine biopsy?
4. What are advantages of needle biopsy?
5. Give clinical significance of bone marrow examination?

LESSON 13

URINE ANALYSIS

Objective: *After reading this topic, students will understand the importance of urine examination, in detail about its processing including physical, chemical and microscopic examination and preparation of urine report.*

19.1 Introduction: Urine analysis is having diagnostic value. It helps in diagnosis of Diabetes, Jaundice, Urinary tract infection, Haematuria, diseases of kidney like nephrotic syndrome, glomerulonephritis etc. It also helps in diagnosis of inborn errors of metabolism, Multiple myeloma, pregnancy, acidosis & diagnosis of patients or carriers of enteric fever. It helps in diagnosis of drug over dosages, Parasitic infestation of Schistosoma haematobium.

19.2 Collection of Urine: Sample should be fresh & should be examined within two hours. It is best to collect an early morning midstream specimen, as it is most concentrated & because of low pH preserves the formed elements well. The concentration of the urine varies throughout day. It depends upon water intake & activities of the patient. The random urine specimen may be diluted & may not be suitable for detection of certain substances present in low concentration.

For determination of presence of sugar & protein, specimen is collected 2 hours after a meal, is preferable. For any quantitative test & for detection of tubercle bacilli in urine, 24 hours urine sample is collected.

Instructions to the patient(for urine culture)

Patient is asked to void out initial portion, collect middle portion of urine directly into a sterile container and remaining portion again

outside. This is known as midstream urine sample (MSU).Specimens for bacteriological examination and culture have to be collected with utmost care to prevent contamination. Prior to collection, patient is asked to clean genitals.

Specimens from infants and young children are collected in disposable plastic bags. For collection of 24 hours urine sample, the early morning sample is discarded and all the urine during the next 24hours including the early morning urine on next day is collected. Collection is done in a large bottle of suitable size with addition of preservative. The whole quantity is mixed, its volume measured and about 150 to 200ml is submitted for examination.

If it is not possible to obtain voided specimen, then it is collected by catheterization. Specimen from urine bag is usually not accepted for examination but in difficult situations, freshly passed urine in the bag is accepted.

19.3 Preservation of specimen: Urinary decomposition occurs quickly at warm temperature. If delay is unavoidable, it is refrigerated. Preservatives used are Toluene, Formalin, Thymol, Chloroform, Boric acid etc.

Containers : For routine examination, clean, dry, wide mouth glass or plastic bottle with screw cap tops with capacity of 200-250ml.is used. For bacteriological examination, sterile containers are used. These containers are commercially available or can be prepared in the laboratory.

19.4 Normal constituents of urine:

Composition of normal urine: A normal adult discharges approximately 1500 ml of urine in a 24hour period. Normal urine is clear and pale yellow in colour with a characteristics aromatic odour. The yellow colour is due to urochrome.

Composition: Water- 95%, Soluble waste products- urea, uric acid, creatinine, excess electrolytes (Sodium, potassium, chloride, calcium, phosphate & others), hormones, enzymes, vitamins, fatty acids, cholesterol, trace amounts of metals.

The chemical composition of urine varies widely depending upon the food & fluid intake. When chemical composition of urine deviates from the normal, it can be helpful in the diagnosis of metabolic disorders.

Routine examination of urine:

1. Physical examination
2. Chemical examination
3. Microscopic examination

19.5 Physical Examination:

Physical examination includes volume of urine submitted, colour, odour appearance, pH and specific gravity.

a)Volume: Measurement of 24hours urine volume is required in clearance and quantitative tests. Volume is measured by use of measuring cylinder. Many times collecting bottle is having graduations. The 24hours urine discharged by a healthy adult is about 1200-1500 ml. Children (6 to 12 year) about 1000 ml. Infants up to 600 ml.

Polyuria: Increased urine output (>2000 ml / 24hours) is called polyuria. It occurs physiologically in cold weather and after high intake of fluids. Urine output is also increased with diuretic drugs, caffeine and alcohol. It is seen in diabetic mellitus and diabetes insipidus.

Oliguria: Oliguria is the term used for a decreased in the quantity of urine discharged (<400 ml / 24hours). It occurs physiologically in hot weather and after reduced fluid intake. In pathological circumstances it occurs in disease of kidney, dehydration from any cause, fever and congestive cardiac failure etc.

Anuria: Complete absence of urine for 12hours and less than 100 ml volume is seen in mismatched blood transfusion, acute glomerulonephritis, surgical shock etc.

b) Colour : Normal freshly voided urine varies from nearly colorless to dark yellow or straw-colored. These are the degraded products of urobilinogen after the latter is exposed to air. Some of the diagnostic colors of abnormal urine are referred here:

- **Pale:** Diabetes insipidus, chronic renal failure, due to very dilute urine.
- **Reddish:** Presence of blood in urine (Heamoglobinuria and haematuria) porphyria and trauma, certain drugs and food pigments.
- **Brownish yellow or green with yellow foam**: Presence of bile pigments, associated with jaundice.
- **Brown- Black**: Associated with poisoning (Lead, mercury, phenol) , Hemorrhages, and melanotic tumours, alkaptonuria.
- **Milky white**: Presence of fat globules (chyluria) due to filariasis.

Odour : Freshly passed normal urine has a characteristic aromatic odour due to volatile organic acids. A foul or a putrid smell of urine indicates urinary tract infection. A fruity smell is often associated with diabetic ketosis. The urine of phenylketonuria gives musty odour.

Appearance: Normal urine is clear in appearance but may be turbid on cooling due to the precipitation of mucin from the urinary tract or precipitation of calcium phosphate. These are non-pathological characters; the turbidity may disappear with the addition of dilute acetic acid to neutral or alkaline urine, or heating of acidic urine. Presence pus cells, bacteria & mucous produces cloudiness of urine and a mucoid, whitish sediment. Fat, chyle causes turbidity. Presence of blood gives red smoky appearance.

c) Reaction &pH : The freshly voided normal urine is usually slightly acidic. In health normal pH vary from 5 to 8, with average around 6.0.

Acidic urine: Diet high in meat protein, respiratory acidosis (Retention of carbondioxide), metabolic acidosis (uremia, renal tubular acidosis, diabetic ketoacidosis, starvation, severe diarrhoea)

Alkaline urine : Diet rich in vegetables, citrus fruits like lemon, post prandial urine, respiratory alkalosis (Hyperventilation), metabolic alkalosis (severe vomiting), urinary infections caused by Proteus and Pseudomonas.

Measurement of urinary pH : It is usually done by strip method, pH meter can be used for this purpose. Litmus paper can be used to know the reaction of urine.

d)Specific gravity:

Specific gravity is the density of a substance (weight per unit volume) as compared to that of the density of water which is 1.000. Specific gravity of a solution largely depends on the amount of solute present and also on the temperature of the solution.

The specific gravity of normal urine (24 hour collection) ranges from 1.015-1.025. The specific gravity of urine is subjected to wide variation. For example, excessive water intake will dilute the urine and the specific gravity will be less than 1.015. On the other hand, profuse perspiration and poor water intake will raise the specific gravity above 1.025. If the kidney is functioning properly, the concentrated first morning specimen of urine should have a specific gravity greater than 1.020.Presence of protein and sugar causes increase in specific gravity.

Methods to detect specific gravity :

1. Reagent strip method
2. Urinometer
3. Refractometer

Procedure:

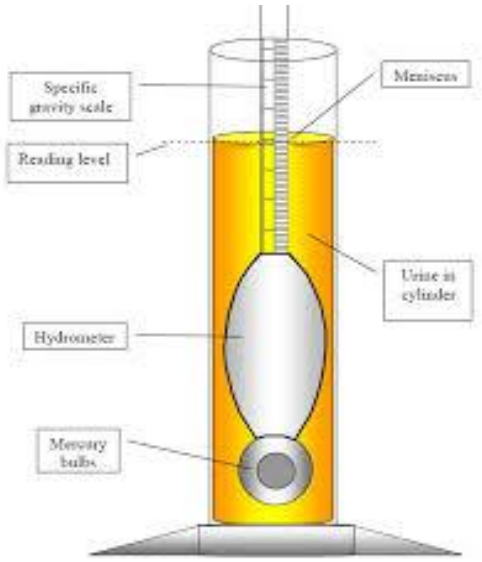
1) Mix the urine and pour into the cylinder.

2) Carefully float the urinometer by grasping the stem of the urinometer at the top and inserting slowly into the urine slowly.

3) Read the specific gravity as soon as the urinometer comes to rest.

4) For accurate result do calibration and temperature correction.

5) Urinometers are usually calibrated for 20˚c. So the observed figure has to be corrected for the difference between this temperature and room temperature in the proportion of 0.001 to every 3˚c.



Urinometer

Refractometer method:

This is a dependable optical instrument that can conveniently determine the specific gravity with a drop of the urine specimen. Hence, it is ideal for pediatric patients. It is, however, expensive and needs delicate handling.

19.6 Chemical examination of urine:

The routine urine analysis includes chemical testing for protein, glucose, ketone bodies, occult blood, bile pigments, bile salts.

Use of reagent strips: Nowadays strips are available commercially which are used for detection of single, two or many substances in the urine. The strips are firm plastic strips to which are affixed various separate reagent areas. Careless improper storing of strips make them ineffective, hence strictly follow the manufacturer's instructions. As these strips are costly usually chemical tests are performed.

a)Detection of protein in urine :

One of the important tests in routine urine analysis is to detect the presence of protein. Normal urine contains only traces of protein (Albumin) and increased discharge of albumin in urine (referred to as 'proteinuria' instead of albuminuria) signals renal disorder.

Causes of proteinuria

Disease of kidney : Inflammation of kidney (nephritis), urinary tract infection, degenerative diseases of kidney(nephrosis), chemical poisoning, some cardiovascular diseases, obstruction due to stone or tumor, eclampsia etc.

Heat Test for Protein

Principle Proteins are coagulated and denatured by heat thus rendering the solution turbid (Turbid metric method). Acetic acid is added to the urine specimen to make it slightly acidic. This facilitates the precipitation of protein, and in addition, false positive results due to the precipitation of phosphates which are a normal constituent of urine. If the turbidity persists after the addition of acid, or develops after the addition of acid, it is considered to be positive.

Procedure:

1. Centrifuge the urine specimen. Take the sediment for microscopic study & use the supernatant fluid for the testing of protein.
2. Hold the test tube with a test tube holder and start heating the upper half inch or inch of the column of urine, on a Bunsen burner (keep the open end of the test tube away from you, do not heat the bottom of the tube). Watch for the appearance of any cloudiness at the top.
3. If no cloudiness appears, report as negative. If cloudiness appears, remove the test tube from the flame and add 2 – 3 drops of 3% acetic acid solution. Do not add acid in excess. Heat the tube again and watch the effect of the acid on the cloudiness. If the cloudiness disappears, report as negative (The cloudiness was due to phosphate or carbonates). If it persists, even after reaching boiling point, report as protein positive with the possibility of albumin discharge

Sulphosalicylic acid test

Procedure

1. Take 2 ml of centrifuged (clear) urine specimen in a test tube. Add equal amount of sulphosalicylic acid reagent.
2. Shake the test tube gently and let it stand for 10 minutes.
3. Note the degree of turbidity by looking at the illuminated tube against a dark background.
4. Grade and report the results as follows:

Negative: No turbidity or no increase in turbidity

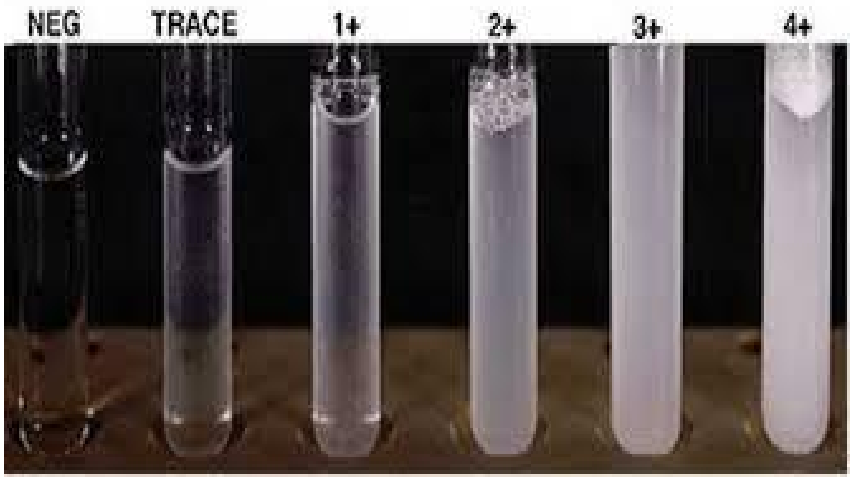
Trace: Faint turbidity

1+ : Distinct turbidity but no granulation.

2+: Turbidity with granulation but no flocculation

3+ Turbidity with granulation and flocculation

4+: Clumps of precipitate (1.0g/dL)



Sulphosalisylic acid test

Heat test for Bence Jones Protein:

Clinical significance

Presence of Bence Jones protein (a special protein formed in some lymphoproliferative disorders) suggests the diagnosis of multiple myeloma.

Principle

Bence Jones protein coagulates when heated to 45˚- 55˚C and re-dissolve partially or wholly on boiling. Albumin coagulates above 60˚C and does not re-dissolve on boiling.

Procedure

1. Centrifuge the urine specimen, filter the supernatant if it is turbid, and check the pH with litmus paper. Add few drops of dilute acetic acid, if necessary, to make the specimen slightly acidic.
2. Place 5 ml of the urine specimen in a test tube of 12ml capacity.

3. Place a test tube in a water bath with a thermometer. Temperature is kept at 55˚C.Turbidity will begin to appear above 40˚c and clearly seen at 55˚C to 60˚C. Now raise the temperature to 100˚c .The precipitate now disappears. Allow to cool & the precipitate will reappear.

6. b) Detection of glucose in urine:

Clinical significance:

The presence of chemically detectable amount of glucose in urine is called glycosuria. Various clinical conditions related to glycosuria are as follows:

1.. The main reason for glycosuria is hyperglycemia, that is elevated blood sugar level. Diabetes mellitus is the most common cause of hyperglycemia. Glucose is also found in urine of some hyperglycemic patients with endocrine hyperactivity,that is in hyperthyroidism, hyperpituitarism & hyperadrenalism.

2. There are various other non pathological cases of transitory hyperglycemia which will result into glycosuria. These conditions are as follows:

a) Pregnancy: Glycosuria is due to lowered renal threshold. Lactose may be present in urine.

b) Stress and anxiety: Hyperglycemia due to an increased output of epinephrine and glucocorticoids.

Benedict's qualitative test

Principle of the test: When Benedict's qualitative reagent (5ml) is heated with eight drops of urine (about 0.5ml), glucose present in urine reduces cupric ions present in the reagent to cuprous ions. Alkaline medium is provided to the reaction by sodium carbonate present in the reagent. The original colour of Benedict's reagent is blue. It changes to green, yellow, orange or red, according to concentration of glucose present in urine.

Note : The test is nonspecific for glucose since the reaction may be brought by other carbohydrates such as fructose, galactose , lactose & pentose and also by noncarbohydrates such as ascorbic acid,

salycylates, creatine and uric acid. Hence if Benedicts test is positive it is necessary to perform glucose oxidase (uristix) test to confirm whether it is due to only glucose.

Procedure: Take 5 ml of Benedict's qualitative reagent in a test tube & add 8 drops of urine. Heat carefully on spirit lamp or burner. Allow to cool & observe colour of reagent.

Benedicts Test

Observations

Color	Conclusion
1. Blue.	Sugar Absent
2. Green & slight yellow precipitate.	Sugar present, trace.
3. Green and thick yellow precipitate.	Sugar present, + to++.
4. Yellow and orange precipitate.	Sugar present, +++.
5. Orange and orange to red precipitate.	Sugar present, ++++

6. c)Detection of ketone Bodies:

Whenever there is inadequate carbohydrate in the diet or defect in carbohydrate metabolism the body tends to metabolize increasing amounts of fatty acids. Due to this the other intermediary products such as ketone bodies also increase in blood. These are 1. Acetone 2. Diacetic acid (acetoacetic acid) and 3. β– hydroxybutyric acid.

Ketonuria : Presence of ketone bodies in the urine.

Ketosis: Presence of increased amount of ketone bodies in bodyfluids.

Ketonaemia. : Increased ketone bodies in blood is called ketonaemia.

Clinical Significance

Diabetes mellitus is the most important disorder in which ketonuria occurs. Detection of ketonuria in a patient with diabetes mellitus is of great significance since a change in insulin dosage or other management is often indicated.

Ketonuria also accompanies the other conditions such as anorexia, fasting, starvation, fever and prolonged vomiting.

Rothera's test:

Principal: Nitroprusside used in this test reacts with both acetone & acetoacetic acid in the presence of alkali (ammonium hydroxide) to produce a purple colored compound.

Procedure: Take 5ml of urine in a test tube and saturate it with ammonium sulphate and add one crystal of sodium nitroprusside. Let liquor ammonia run carefully down side of the test tube so it forms a layer on the top of the urine. If ketone bodies are present, there is formation of pink – purple color ring at the junction of two layers.

This test is given positive by acetoacetic acid and acetone.

For this test Rothera's powder can be prepared as follows –

Sodium Nitroprusside – 0.75 gms

Ammonium sulphate – 20.0 gms

About 1gm of powder mixture is added in 5ml of urine.

Other tests for ketone bodies :

Gerhardt's test, Acetest (tablet test)

d)Detection Of Blood in urine :

Blood may be found in urine in form of red cells or when haemolysed, in the form of haemoglobin.

Haematuria: Presence of red blood cells in urine.

Haemoglobinuria : Presence of haemoglobin or blood pigments in urine.

Occult blood: Presence of blood in urine which is not visible but can be detected by chemical test or microscopic examination is called occult blood (hidden).

There are three types of tests to detect presence of blood in urine.

1. Microscopic examination: This detects the presence of intact RBCs.

2. Spectroscopic examination: Detection of specific absorption bands.

3. Chemical tests:

Principle of chemical test: Haemoglobin is a complex substance & one of it's constituents is 'Haem' which acts as a catalyst when hydrogen peroxide is mixed with substances like benzidine or guiac

a)Benzidine Test: Dissolve a small amount(knife- point full) of benzidine powder in 2ml of glacial acetic acid & add an equal volume of 3% hydrogen peroxide. Add 2ml of urine & mix. The appearance of green or blue colour indicates presence of haemoglobin.

Other tests which can be used are Guaiac test & Orthotolidine test**.**

Causes of Haematuria: Renal calculi, acute infections, nephrotic syndrome, infarction, trauma to kidney, tumours in kidney, ureter or bladder, Thrombocytopenia, coagulation factor deficiency etc.

Causes of haemoglobinuria : Mushroom poisoning, poisoning from snake venom, bacterial toxins, chemical poisoning, severe burns, complication of falciparum malaria, haemolytic anaemias.

d) Detection of Bile:

The constituents of bile may be excreted in urine as bile pigment (bilirubin & biliverdin), Bile salts, urobilin & urobilinogen.

Bilirubin appears in urine, when it is excess in blood, a condition is known as Jaundice. Urobilinogen is normally present in trace amount in urine.

a) Determination of bile pigments & urobillnogen-

i) Fouchet's test: Barium chloride solution is added in the urine. A precipitate of barium sulphate is produced onto which bile pigments are adsorbed after filtering this solution obtain precipitate on filter paper or alternatively centrifuge the mixture, pour off supernatant & obtain precipitate in the test tube. Add 2-3 drops of Fouchet's reagent on the precipitate on filter paper or sediment in the test tube. If colour changes to green, bile pigments are present.

Other tests which can be used are Foam test & Gmelin test.

b)Determination of bile salts:

Hay's sulphur test:

Principle: Bile salts when present lower the surface tension of the urine. When sulphur powder is added on the surface of urine, if bile salts are present they sink to the bottom. In case of normal urine, sulphur particles float on the surface of urine.

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f)Detection of urobilinogen & urobilin:

Ehrlich's test: To 10ml of urine add 1ml of Ehrlich's reagent, invert several times & let stand for 5 minutes. A pink colour is normal. Cherry red or dark red colour indicates abnormal amount of urobilinogen.

19.7 Chemical Examination of Urine By Using Multistix Reagent Strips:

Multistix reagent strips are clear plastic strips. Seven different reagent areas are affixed on the strip. These different cellulose areas are impregnated with the specific testing chemicals according to the test. The advantages of chemical examination of urine by multistix strips are as follows:

- It gives quick screening of urine chemistry.

- The method is very fast (requires only 2 to 3 minutes)
- The method is reliable, specific and sensitive.
- It avoids use of various corrosive reagents, different types of glassware and other laboratory material required for wet chemical testing of urine.
- It can be performed on uncentrifuged urine.

The various determinations possible by multistix reagent strip are (1) pH (2) Protein (3) Glucose (4) Ketones (5) Bilirubin (6) Occult blood and (7) Urobilinogen. The reagent strips are also available for only one or two tests.

Procedure:

1) Dip the test areas of the strip in the urine specimen (fresh, well mixed and uncentrifuged).
2) Remove excess of the urine by tapping the edge of the strip against the container.
3) Compare the test areas closely with corresponding color charts on the bottle at the times specified.

19.8 Microscopic Examination of Urine:

General Consideration:

The microscopic examination is a valuable diagnostic tool for detection and evaluation of renal and urinary tract disorders and other systemic diseases.

Principle:

The microscopic elements present in urine (in suspension) are collected in the form of deposit by centrifugation. A small drop of the sediment is examined by making a coverslip preparation under microscope.

Observations:

The various findings observed in the sediment may be as follows.

1) Leucocytes: The pus cells can enter in urine anywhere from the glomerulus to the urethra.

Normal urine can contain 2-3 pus cells/h.p.f.

These are mostly neutrophils.

Their presence indicates urinary tract infection.

Note: The addition of 2% acetic acid to the slide accentuate the nuclei of the cells.

2) Epithelial cells: These cells may originate from any site in the genitourinary tract from the proximal convoluted tubule to the urethra.

Normally few cells (3 to 5) per h.p.f. from these sites can be found in the urine due to sloughing off of old cells.

Three main types of epithelial cells may be recognized:
(a) tubular (b) transitional & (c) squamous.

3) Erythrocytes: - In fresh urine these cells have a normal, pale or yellow appearance. They appear smooth, biconcave discs about 7 µm diameter and 2 µm thick. They do not contain nuclei.
In dilute (or hypotonic) urine the red cells swell up and lyse.
Lysed cells appear as colorless circles (ghost cells).In hypertonic urine the red cells crenate.
Note: (1) It is important that contamination from menstrual flow be avoided in female patients.

(2) Yeast cells can be mistaken for RBCs. Yeast cells are ovoid and frequently having buds.

4) Casts: Urinary casts are formed in the lumen of the tubules of the kidney. The renal tubules secrete a mucoprotein called Tamm-Horsfall protein which is believed to form the basic matrix of all casts.
-Casts can form as the result of:

a) Precipitation of gelatin of Tamm-Horsfall mucoprotein.

b) Clumping of cells on other material within protein matrix.

c) The adherence of cells or cellular material to the matrix.

d) Coagulation of material within the lumen.

Cast formation takes place in the distal & collecting tubules (since the formation of casts require acidic conditions & high solute concentration).

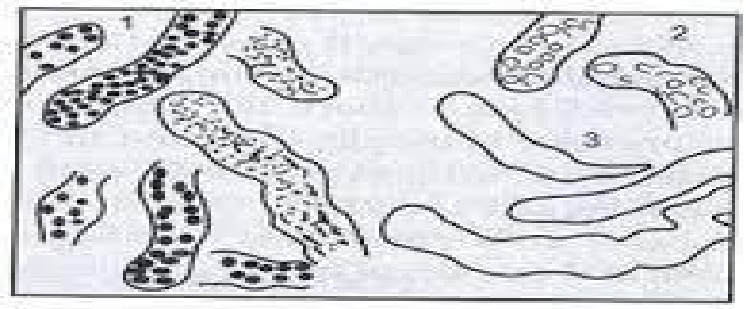
Casts dissolve in alkaline urine.

Casts have nearly parallel sides & rounded or blunted ends.

They may be convoluted, straight or curved.

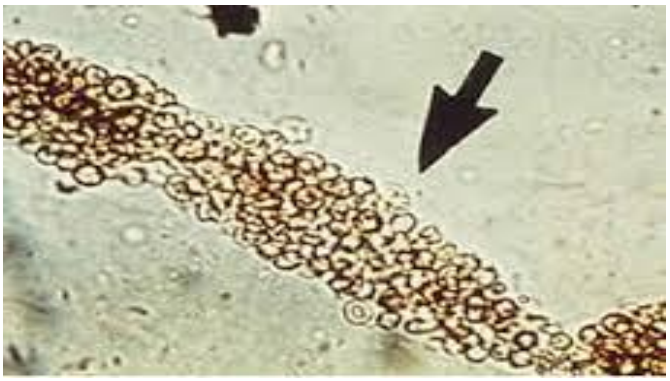
i) Granular casts: These always indicate significant renal disease. The casts are present due to degeneration of cellular casts or due to direct aggregation of serum proteins in a Tamm- Horsfall mucoprotein matrix.

ii) Hyaline casts: They are colorless, homogenous, and transparent & with rounded ends. These casts can be seen in increased number even in the mildest kind of renal disease. A few hyaline casts may be present in the normal urine.



1. Coarse and fine granular casts
2. Fat globules in casts
3. Hyaline casts

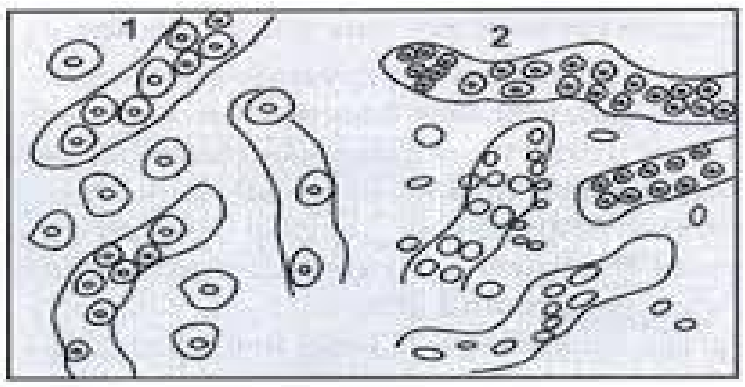
iii) Red cell casts: The casts may contain only a few RBCs in protein matrix or there may be many cells packed close together with no visible matrix. Their presence is always pathogenic. They are seen in acute glomerulonephritis, subacute endocarditis, severe pyelonephritis and in renal infarction.



RBC Casts

iv) White cell casts: Usually contains neutrophils. Seen in renal infections & noninfectious inflammations.

v) Epithelial cell casts: The epithelial cells may be arranged haphazardly and vary in size and shape. These casts are rarely seen in urine. Presence of these casts indicate tubular degeneration and necrosis. It may be due to nephrotoxic agents or viruses. These casts can also be present in severe chronic renal disease.



1. Epithelial casts and few free renal casts
2. Blood casts and few free RBCs

vi) Waxy casts: have very high refractive index. These are yellow grey or colourless and have a homogeneous appearance. Their presence indicates serious pathological condition.

vii) Fatty casts: fatty casts are formed by incorporated free fat droplets or oval fat bodies. These are seen when there is fatty degeneration of the tubular epithelium. These are frequently seen in

nephrotic syndrome, chronic glomerulonephritis and toxic renal poisoning.

5) Cylindroids: These resemble casts but have one end which tapers out like a strand of mucus. These are frequently hyaline. The exact site and mechanism of their formation are not known.

6) Mucus threads: These are long, thin waxy threads of ribbon like structures. These may be present in normal urine but found in high proportion in the presence of inflammation or irritation of the urinary tract.

7) Yeast cells : These are smooth, colorless and usually ovoid cells. These can vary in size and have doubly refractile walls. They often show budding. Unlike red cells, they are insoluble in acid and alkali. They also will not stain with eosin. Yeast cells may be found in urinary tract infections (mainly in diabetic patients). They may be present in urine as a result of skin or vaginal contamination.

Candida with pseudohyphae

8) Bacteria: Freshly voided normal urine is generally free of bacteria. Contamination may occur from bacteria present in the urethra, vagina or other external source. Presence of large number of bacteria with many pus cells indicates urinary tract infection.

9) Spermatozoa: These have oval bodies and long, thin and delicate tails. They may be present in urine of man after epileptic convulsions, nocturnal emission and in diseases of genital organs. After coitus they may be present in the urine of both the sexes.

1. Sperms
2. Microfilaria
3. Trichomonas
4. Eggs of *Schistosoma haematobium*

10) Oval fat bodies and fat droplets: Oval fat bodies are renal tubular cells containing highly refractile fat droplets. These may be macrophages or polymorphonuclear leukocytes which have ingested lipids or have undergone fatty degeneration. Free fat droplets may be present in urine in lipuria (excretion of lipids in urine). These may be present in nephrotic syndrome, diabetes mellitus, lipoid nephrosis, in chronic glomerulonephritis and in fat embolism.

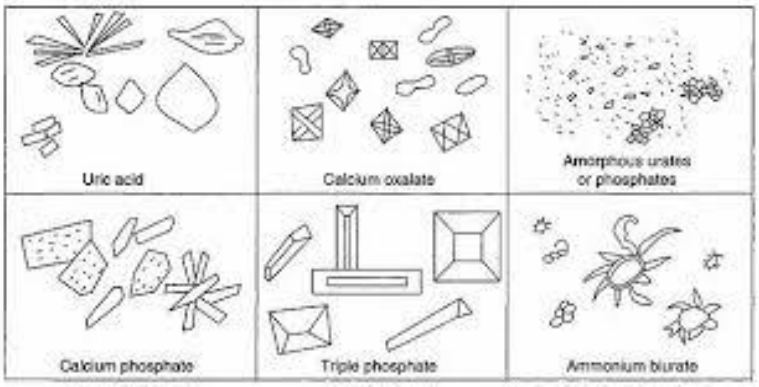
11) Crystals: Many of the crystals that are found in the urine have little clinical significance although they may be found in calculus formation, metabolic disorders and in the regulation of medication.

Crystals found in acidic urine.

a) Uric acid crystals: These can occur in most characteristic diamond rhombic or rosette form. These are usually stained with urinary pigments as yellow or red brown .These are soluble in sodium hydroxide and insoluble in hydrochloric acid. Presence of uric acids can be a normal occurrence. Pathological conditions: Gout, chronic nephritis, acute febrile conditions.

b) Calcium oxalate crystals: These are colorless and octahedral or 'envelope' shaped. They also appear as oval spheres or biconcave disks which have a dumbbell shape (when viewed from the side). These

crystals are frequently found in acid and neutral urine. Occasionally they are also found in alkaline urine. They are soluble in hydrochloric acid and insoluble in acetic acid. These can be present in urine after the ingestion of tomatoes, spinach, garlic, oranges, asparagus and vitamin C.



Pathological conditions: Increased number of calcium oxalates in freshly voided urine suggest the possibility of oxalate calculi. They can be present in increased number in diabetes mellitus & liver diseases.

c) Amorphous urates: These are urate salts of sodium, potassium, magnesium and calcium. Usual form is noncrystalline and amorphous and appearance is yellow-red granular. These are soluble in alkali at 60˚C. They have no clinical significance.

d) Sodium urates: These may be present as amorphous or as crystals. These crystals are colorless or yellowish needles occurring in clusters. Sodium urates have no clinical significance.

Sodium urates

e) Calcium Sulphate crystals: These are long, thin and colorless needles or prisms. These are soluble in acetic acid. Calcium sulphate crystals are rarely seen in the urine and they have no clinical significance.

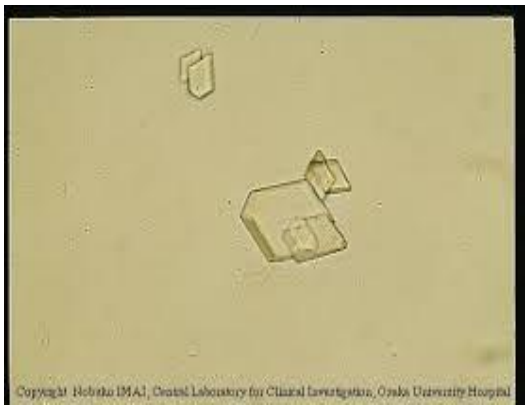
f) Hippuric acid crystals: These are in the form of elongated prisms or plates. The color may be yellow brown or colorless. These are soluble in water. They are rarely seen in urine and have no clinical significance.

g) Cystine crystals: These are colorless, refractile, hexagonal plates with equal or unequal sides. These are soluble in hydrochloric acid & ammonia. Pathological conditions: They occur in patients with either congenital cystinosis or congenital cystinuria. They can form calculi.

h) Tyrosine: These appear in the form of fine, refractile needles, occurring in clusters. These are soluble in ammonium hydroxide. Pathological conditions: Severe liver disease and tyrosinosis.

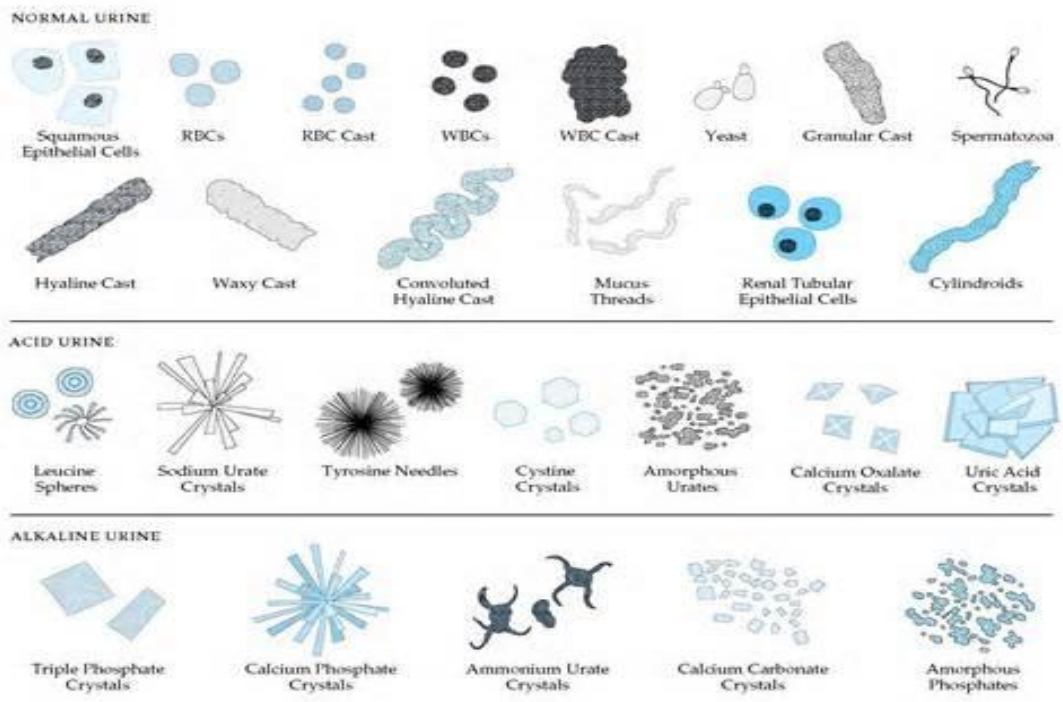
i) Leucine: These crystals are oily, highly refractile spheroids with redial and concentric striations. They have yellow or brown color. These crystals are soluble in hot alcohol and acetic acid. Pathological conditions: severe hepatitis, acute yellow atrophy and maple syrup urine disease.

j) Cholesterol: These are large, flat and in the form of transparent plates with notched corners. These are soluble in ether, chloroform and hot alcohol. Pathological conditions: Nephritis, nephrotic conditions, chyluria, excessive tissue break down.



Cholesterol crystals

k) Sulpha crystals: Most of the sulpha drugs precipitate out as needles. They may clear or brown in color and usually appear with eccentric binding. These drugs are soluble in acetone.



ll). Crystals found in alkaline urine:

a) Triple Phosphates: (Ammonium magnesium phosphates). The crystals are colorless prisms with three to six sides and frequently with

oblique end. These are soluble in acetic acid. These crystals are frequently found in normal urine. They can form calculi. Pathological conditions: chronic cystitis, chronic pyelitis , enlarged prostate.

b) Amorphous Phosphates: These are present in amorphous, granular form. These amorphous phosphates are soluble in acetic acid. They have no clinical significance.

c) Calcium carbonate: These appear as small, colorless and in the form of spherical, dumbbell shape or as granular type. These crystals are soluble in acetic acid. They have no clinical significance.

d) Calcium phosphate: These crystals are long, thin and colorless. The appearance is like prisms with one pointed end, arranged as rosettes or stars or appearing as needles. They may also appear as irregular, granular plates. Calcium phosphate crystals are soluble in dilute acetic acid. They may be present in normal urine. They may also form calculi.

e) Ammonium biurates: These are yellow brown spherical bodies with or without long, irregular spicules. These are soluble in acetic acid and dissolve by heating. Presence of ammonium biurates is abnormal if they are found in fresh urine.

12. Artifacts

a. Starch crystals

b. Fibers

c. Oil droplets

d. Hair

e. Air bubbles

f. Talcum powder particles.

13 Parasites

a. Trichomonas vaginalis trophozoites.

b. Enterobius vermicularis ova.

c. Schistosoma hematobium ovum.

Review Questions

1. Describe any two chemical tests of urine examination.
2. Name the important bile salts & bile pigment.

3. Describe the heat coagulation test of urine.
4. Why Benzedine test is done? Describe the procedure.
5. What are Ketone bodies? Describe Rothera's test.
6. Define the term Ketonemia, Ketonuria & Ketosis.
7. Describe Benedict's test.
8. Describe physical & microscopic examination of urine.
9. Give physical examination of urine.

Chapter **14** **STOOL EXAMINATION**

Objective- Students shall be aware of various tests done on stool sample and its significance.

14.1-Introduction

Stool is waste matter discharged from the bowel. Normally stool (Feces) consists of water, undigested and unabsorbed food residues (including cellulose), bacteria (forming 9% of the solids in dried feces), intestinal secretions, pigments like bile and urobilinogen, a little calcium and mucus and few desquamated cells from lower intestinal mucosa.

The frequency of defecation is a matter of habit and varies from 1-3 times a day to three times a week. The common pattern is once in a day. In infants it is 3-6 times a day. The stool tends to be small and dry on a diet high in meat, soft and bulky on a diet rich in vegetables and fibers. The normal brown color is from degradation of bile pigment and the odor results from no. of organic substances (indole and sketole) produced by bacteria, and degradation of proteins.

Indications for stool examination

- Detection of bacterial and parasitic infections of the intestine.
- Cultural examination will detect bacterial infections like enteric fever, bacillary dysentery, cholera, etc.
- Common intestinal parasites like hook worm, tape worm, thread worm, etc are detected.
- Chemical examination helps to detect defects in absorption, deranged liver and pancreatic functions, etc.

14.2-Collection of specimen

Precautions taken before collecting specimen-

- Collection of sufficient quantity- Morning specimen before the breakfast is most desirable specimen. Collected in a dry container.
- The container should not be completely filled.
- For bacteriological examination sterile container is used.
- The various types of containers (50ml capacity) are
  - Disposable wide mouth plastic or glass bottle.

- Waxed cardboard box.
- Glass jar with fitting lid

Figure 14.2

Disposable stool container with screw cap

Precautions taken after collection of specimen-

- The specimen container should be labeled, indicating identification number. Entry should be made in the register regarding name of patient, date and time of specimen collection, etc.
- Stool sample should not be left uncovered to prevent drying effect.
- Examination should be done within 1 hour of collection.
- Proper disposal should be done after examination.

For the preservation formal saline is used.

10% v/v formal saline-

- (Commercial 40%) Formaldehyde: 25 ml
- Normal saline (0.85 g/dl) sodium chloride: 75 ml

Mix and store in a clean and dry container

Mix 3 parts of 10% v/v formal saline with 1 part of stool.

This helps to preserve protozoan morphology and further development of certain helminth eggs.

- Apart from stool specimen other specimens used from intestinal tract are

1. For Pinworm- Enterobius vermicularis is universally found among children. The adult female worm migrates out of anus, usually at night and deposits

eggs at the perianal area. So they are not commonly found in feces. To detect them

Cellophane Tape preparation is used.

In this a piece of cellophane tape of about 4 inches in length is used. Its sticky end is applied against the skin across the anal opening with an even thorough pressure. Gently place the sticky side of the tape over a glass slide. Apply a small drop of xylene under the tape by lifting one side. Press the tape down again on the slide. Examine under microscope with low power and low illumination.

Anal swabs

A cotton wool swab is wiped round the anus. Dip the swab into a test tube containing 0.5ml of normal saline. Examine under microscope by preparing wet mount.

2. For amoebic trophozoites and cysts if not detected in feces of suspected case of amoebiasis then Sigmoidoscopy material is used. Material from the mucosal surface should be scraped or aspirated at least six different areas. Then it should be processed immediately and examined using direct wet mount, concentration method and permanent stained smear.

14.3-Laboratory investigations

- **Physical examination**

1. Consistency and form- Normally stools are soft and well formed. Abnormally stools are

Abnormal consistency	**Reason**
Watery	Diarrhoea- Bacterial infections, use of purgatives
Large amount of mushy, foul smelling bulky gray stools float on water	Steatorrhoea
Little firm spherical masses	Constipation
Narrow ribbon like	Spastic bowel or rectal narrowing
Rice water stools- Copious, thin with	Cholera

white flakes	
Semisolid	Mild diarrhea Digestive upset After taking laxative

2. Parasite- Normally absent. In parasitic infestation presence of adult worm like round worm, pin worm, whip worm, hook worm and Tape worm is seen.
3. Color- Normally feces are light to brown in color due to presence of bile pigments. Abnormal colors will be

Abnormal color	**Reason**
Clay	Obstructive jaundice, Presence of $BaSo_4$
Bright red	Blood from lower GIT, Beet consumption Contamination with menstrual blood
Black tarry	Bleeding from upper GIT Iron, Bismuth, charcoal consumption
Green	Ingestion of spinach, Presence of biliverdin Oral antibiotics

4. Odour- Normal feces may smell offensive but not foul smelling. In infections other odors like foul, putrid, sour, etc. can occur.

5. Pus- Not normally present. Found in chronic ulcerative colitis and chronic bacillary dysentery. Also in localized abscess or fistulas communicating with sigmoid colon or rectum. It is confirmed by microscopic examination of stool.

6. Blood - Normal stool does not contain any blood. There are different causes of presence of blood in the feces. It may be bright red in color in case of bleeding from lower GIT or has black tarry appearance in bleeding from upper GIT. Causes are as follows-

- Upper GIT-
  - Peptic ulcer
  - Erosive gastritis
  - Atrophic gastritis
  - Esophagitis
- Lower GIT-

Small and large bowel

- Polyps
- Infective diarrhoeas
- Inflammatory bowel syndrome (Crohn's Disease, Ulcerative colitis)
- Carcinoma

Rectum and anal canal

- Hemorrhoids
- Ano-rectal fissure

➢ Apart from this certain drugs like steroids can lead to appearance of blood in stool.

7. Blood and mucus- it is seen with amoebic and bacillary dysentery. Differences are as follows-

Amoebic dysentery	**Bacillary dysentery**
➢ Gross- o Appreciable amount of fecal matter. o Blood appears dark brown (altered) o Foul smelling o Acidic	➢ Gross o Very little fecal matter chiefly exudates o Blood appears bright red o No foul smelling o Alkaline
➢ Microscopic o Appreciable amount of fecal matter o RBCs tend to be clumped o Pus cells and macrophages absent. Entamoeba histolytica present. o Common intestinal bacteria in wet preparation seen. o Charcot Leyden crystals	➢ Microscopic o Chiefly exudates o RBCs discrete o Pus cells and macrophages are characteristic. o No bacteria in wet preparation. o No Charcot Leyden crystals found

❖ **Chemical examination**

1. pH- Normal pH range is 5.8-7.5

a. Strongly acidic stools (<5.5) -
    - Nonpathogenic- Excess carbohydrates in diet.
    - Pathogenic- Fermentation due to lactose intolerance.

b. Strongly basic stools (>7.5) -
    - Nonpathogenic-Excess proteins in diet.

2. Reducing substance- It measures unabsorbed sugar in the stool. It is used to evaluate body's ability to digest carbohydrates or to absorb nutrients from food or drinks. It is used to diagnose lactose intolerance.

Fecal Reducing Substances are reported as:

- Negative – this is the normal result and means that the body is digesting and absorbing sugars properly.
- Positive – this means there are substances in the stool that can act as 'reducing agents', i.e. there are forms of sugar in the stool that have not been absorbed by the body.

Lactose intolerance occurs by prolonged episode of viral gastro-enteritis.

3. **Occult blood-** It is a hidden blood. It is neither seen by naked eyes nor by microscopic examination. It is detected by chemical test. Most commonly used test is **Benzidine test.**

It is very sensitive test and can give positive result with 50-70% of normal adults whose diet contains abundant meat (False positive). False positive result can be overcome by boiling emulsion of feces for 1-2 minutes and then repeating the test.

Principle- A chemical reaction in which the reagent is oxidized by H_2O_2 at low pH and catalyzed by presence of haem- an intact iron containing porphyrine ring. All iron haem derivatives are active. But free iron or free porphyrine rings are not active.

Peroxidase type action of hemoglobin in the blood converts H_2O_2 to water and nascent oxygen. This Oxygen oxidizes benzidine (in acidic medium) to form green to blue color complex.

Result-

- No change in color-Negative i.e. Occult blood is absent.
- Green to blue color- Positive i.e. Occult blood is present.

It is reported as

Trace- Faint blue green after 1 minute

┼	Definite blue green color slowly
┼┼	Green blue color rapidly
┼┼┼	Blue almost immediately
┼┼┼┼	Dark blue immediately

Note-

- In case of a negative result check the reliability of reagent by treating with drop of blood sample.
- In case of positive result check the reliability of cleanliness of the slide by placing a drop of the reagent mixture on the plain part of the slide. It should not produce color.
- Now a day all occult blood tests are packaged into kit forms. Principle remains same E.g.
    - Okokit tablet test
    - Haemoccult method (Slide and Tape test).

Figure 14.3.1

Significance- Occult blood is tested to diagnose iron deficiency anemia, peptic ulceration or cancer of GIT.

4. **Fat estimation**- Most of dietary fat is absorbed in the small intestine with very little being excreted in the feces. Disorders of fat absorption lead to excess quantity of fat to be excreted in the feces. This condition is known as **steatorrhoea.** It is found in cases of post infective malabsorption, giardiasis, strongyloidosis and chronic pancreatitis.

❖ **Microscopic examination**

Examination of stool smear and wet mount is usually carried out for microscopic examination.

Stool smear-This is easy way for examination of formed stool. A small amount of feces is mixed with a drop of saline on microscope slide and examined under low power objective. This thick suspension looks like smear.

Wet mount- It is of two types-Saline and Iodine preparation

Saline preparation- A saline wet mount is used for preliminary microscopic examination of feces to detect protozoan trophozoites and cysts: and helminth larvae and eggs. Also it is useful to observe characteristic motility of parasite. Excessive cellular exudates in the form of pus (white cells) or blood (Red cells), macrophages or any other significant material can be detected.

Iodine Preparation- Iodine stains cysts particularly nuclear structures and glycogen. But motility cannot be detected as parasites are not motile in iodine.

Apart from above certain other wet mounts used for microscopic examination of stool are

a) Use of 1% eosin- Gives pink background against which cysts and amoeba stands out as clear unstained objects.

Figure 14.3.2 Use of Eosin wet mount

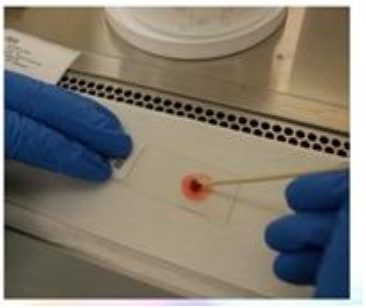


Figure 14.3.3

b) Use of Sergeant's stain- With this nuclear structures stain pale green and chromatid bars dark green. Used for cysts of E. histolytica mainly.
c) Use of Buffered Methylene blue (BMB)- Useful only when amoebic trophozoites are seen in saline wet mount of fresh, unpreserved fecal specimen.
d) Use of Sudan III stain- For fat staining.

Microscopic examination involves observation for

Figure 14.3.4

- Parasites- Free living amoebae, flagellates, eggs, larvae, cysts, etc.

Figure 14.3.5

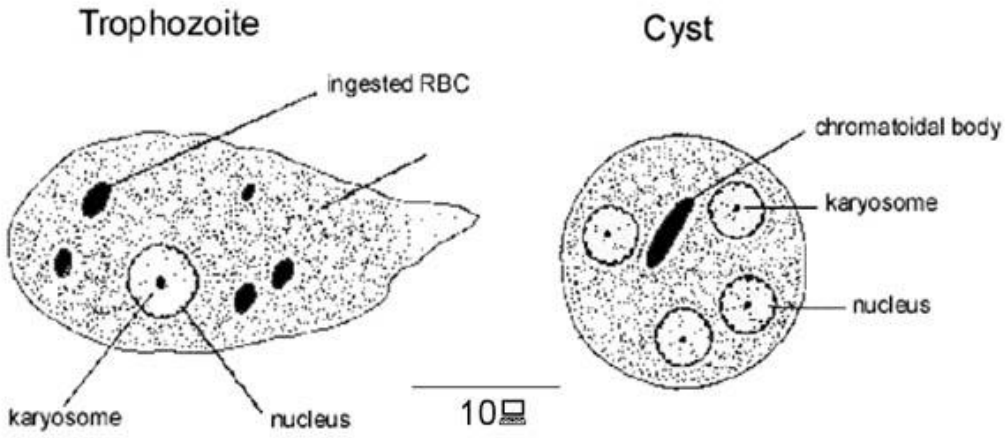


Figure 14.3.6 Trophozoite and cyst of E. histolytica

- In addition to parasites fecal sample may show various other cellular and noncellular structures, such as

  Blood cells

  - White blood cells (pus cells) - found in cases of bacillary or amoebic dysentery. It may be mistaken for amoebic cyst but cyst has rigid cell wall.
  - Red blood cell- Found in ulcerative and hemorrhagic conditions in the GIT.
  - Macrophages and epithelial cells- Macrophages found in bacterial or parasitic infections.

Fat globules- Found in malabsorption syndrome.

- Fungi- Candida species and other yeasts, and yeast-like fungi may be normally present in feces. Large number of budding yeast cells in a fresh stool indicates a source for a systemic infection, particularly in an immunocompromised patient.
- Vegetable cells and vegetable fibers- It is often found in feces of a patient on mixed diet.
- Muscle Fibers
- Mucus
- Bacteria

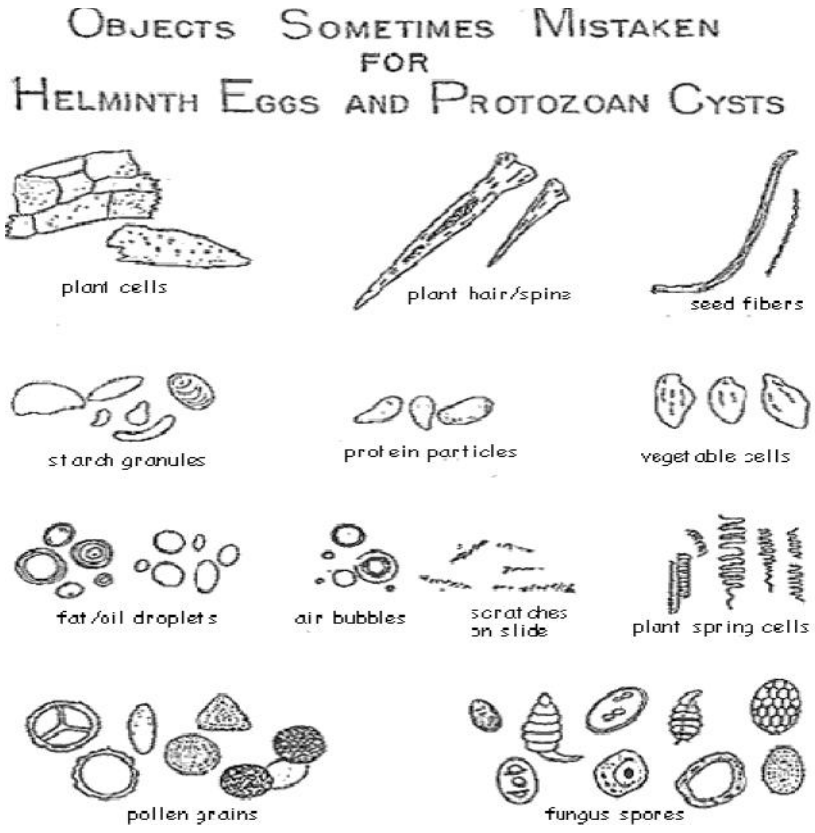


Figure 14.3.7

- Crystals- A variety of crystals may be found in normal stool.
  - Fatty acid crystals found after fatty meal. They take fat stain.
  - Crystals of inorganic substances (triple phosphate, calcium oxalate) may be seen but are of little significance.
- The most important **abnormal** crystals are

- **Charcot-Leyden crystals**- Sometimes found in amoebic dysentery, hookworm infection and other parasitic infections.


14.3.8 Charcot Leyden crystals

- **Haematoidin crystals**- found in intestinal hemorrhage. Rhombic in shape.

Reporting of Microscopic examination of wet mount

Count the number of each type of parasite in the entire wet mount and report as follows:

1-3 parasites per preparation	: Scanty
4-10 parasites per preparation	: Few
11-20 parasites per preparation	: Moderate number
21-40 parasites per preparation	: Many
More than 40 per preparation	: Very many

Also reporting of pus cells, red blood cells, crystals, etc. is done in the same format.

14.4- Supplementary techniques-

In addition to direct mount to detect and identify intestinal parasites other methods are used. They are

- **Concentration methods:** It is used
  - To see whether treatment of parasite has been successful or not.
  - To find ova of S. mansoni or Taenia (if few) and other ova and cysts if they have not seen on routine examination and suspected to be present.

There are two types of concentration techniques:

I. **Flotation technique-** using Zn sulphate solution

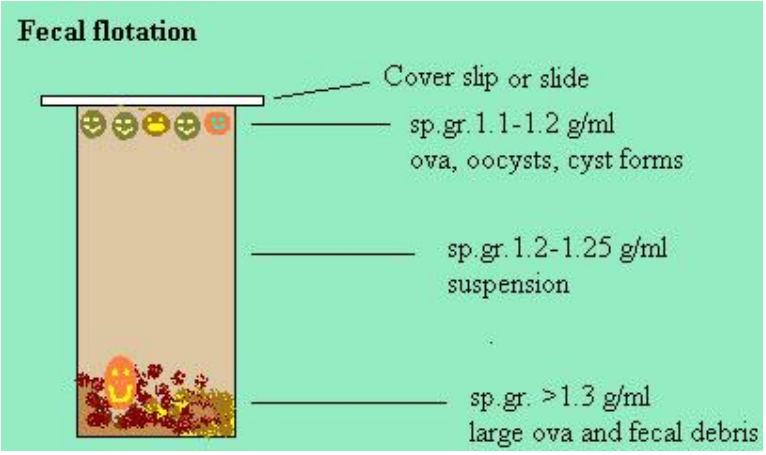


Fig. 14.4.1 Flotation technique

II. Sedimentation technique

- Simple sedimentation
- Formal saline ether sedimentation.

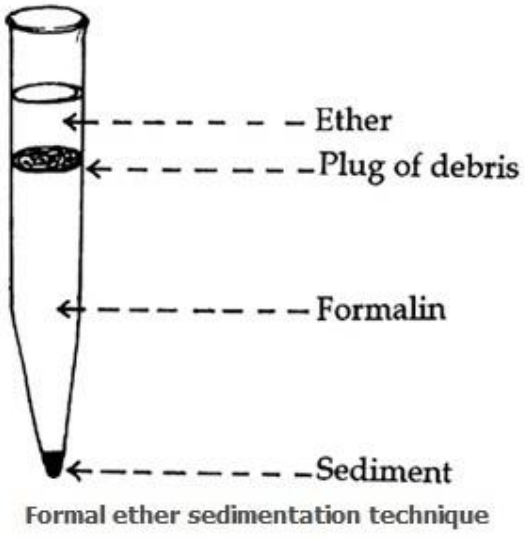


Figure 14.4.2

- **Permanent staining techniques** used for identifying trophozoites and cysts of protozoa.

Most of protozoa are identified on wet preparation. But sometimes for confirmation permanent staining methods are used. These stained smears provide permanent record which can be used later for reference purposes in doubtful cases.

Number of staining techniques is available but commonly **trichrome staining** and **iron-heamatoxylin** techniques are used.

❖ **Bacteriological examination**

Isolation and identification of bacteria from stool sample. This involves

- Direct smear- Gram staining
- Hanging drop preparation – Motility
- Culture- On suitable media to isolate Enteric and dysentery group of organisms.
- Biochemical- To identify organisms.

Review Questions:

- What are indications for stool examination?
- Note on collection of stool sample.
- Differentiate between amoebic and bacillary dysentery.
- What is occult blood? How will you detect it?
- How will you prepare wet mount for stool examination?
- Which different cells and crystals found in stool sample? Give its significance.
- What are concentration techniques? What are its indications?

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Lesson 15 SEMEN ANALYSIS

Objective - Students must know the different tests carried on semen along with its significance.

15.1 Introduction

The analysis of semen is one of the important clinical parameters of gonadal function. It is an important part of the overall evaluation of male infertility. In addition to infertility studies, as a part of forensic studies, semen examination may be asked to examine vaginal secretions or clothing stains for the presence of semen in alleged or suspected rape.

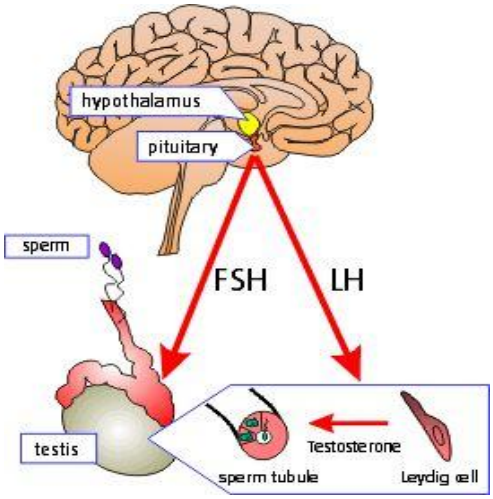


Figure 25.1

15.2 Composition of semen

Semen is a thick whitish secretion of the male reproductive organs discharged from urethra on ejaculation. It is composed of

1. Spermatozoa 5%
   (Derived from testis)
2. Seminal plasma 60%
   (Derived from seminal vesicles.
   It is viscid and yellowish in color
   Or opaque gray color)
3. Prostatic fluid 20%
   (Derived from prostate. It is
   Milky and slightly acidic pH due

to high content of citric acid. It
is also rich in proteolytic enzymes
and acid phosphtase.)

4. Other fluids 10-15%
(Derived from epididymis, vasa
Deferentia and urethral glands.)

15.3 Clinical significance

Semen analysis is carried out for following purposes-

- Evaluation of infertility
- Routine follow up of the patients who have undergone vasectomy.
- Artificial insemination
- Examination of stored semen sample. (This is for the case where husband is away from home for longer period of time and the wife is undergoing complicated infertility therapy.)
- In man whose future fertility is threatened may be due to radiotherapy or chemotherapy in the treatment of cancer.

15.4 Specimen collection and delivery

- Sample should be collected after 2-5 days of abstinence.
- It may be collected either at home or in hospital which is quiet, secluded and guarantee complete privacy.
- It should be collected either by masturbation or by coitus interruptus.
    - Sample collected by masturbation is generally complete sample.
    - Sample collected by coitus interruptus is not satisfactory but acceptable when subject cannot masturbate.
- Semen should be collected in a clean and dry wide mouth glass or plastic container. There should not be any traces of soap in container as it is toxic to sperms. Also same is about water so complete dry container is must. The lid should not have rubber lining as it destroys sperms.
- Proper labeling of container is must.
- Semen should not be collected in condoms as it consists of spermicidal agents.
- During transportation sample should not get exposed to extreme temperatures ($<20^0$c or $>40^0$c).

- The sample should be delivered within 2 hours of collection when collected outside the laboratory.
- For accurate evaluation of fertility status at least three samples should be assessed (collected on different days).
- If any portion of ejaculate is lost or if container leaks during transport then sample should not be used for analysis.

15.5 Laboratory investigations

Physical Examination

Freshly ejaculated normal semen is highly viscous, opaque, white or gray-white coagulum with distinct musty or acrid odour. On liquefaction the sample becomes slightly viscous, turbid and slightly alkaline (pH 7.7). On average volume is 2-5 ml.

1. Time of arrival-
    a. Time should be noted to find out interval between collection and commencement of tests.
2. Appearance-
    a. Freshly ejaculated normal semen is highly viscous, opaque, white or gray-white coagulum. Check is there any abnormal colour-yellow,brown or blood stained. It is due to presence of pus, blood, etc in the semen.
3. Viscosity-
    a. Viscosity can be tested by pouring the semen, if it falls drop by drop indicates normal. Increased viscosity may inhibit sperm motility.
4. Liquefaction time-
    a. Liquefaction is assessed visually. Unliquefied semen appears as a gel like coagulum. Partially liquefied semen may contain much small gel like clots. In completely liquefied semen no clots seen and it appears as completely fluid.
    b. Spontaneous liquefaction occurs within 10-20 minutes. It takes place with help of prostatic enzymes. It should be completed within 30 minutes.
    c. Unliquefied semen or delayed liquefaction time (> 1 hour) is considered as abnormal. It interferes with motility and sperm count.
5. Volume-
    a. A normal semen volume is 2-5 ml.

b. Decrease in volume i.e. <1.5 ml result in poor penetration of the cervical mucus by the sperms.
c. Excess volume i.e. >5 ml is associated with decreased motility.

Chemical Examination

1. Test for fructose-

   Fructose is the main sugar of semen. There is an inverse relationship between fructose level and sperm count.

   A low fructose concentration is due to low testosterone level or seminal vesicle insufficiency.

   Absence of fructose results in completely immotile sperms.

   It is detected by Resorcinol method.

   Principle- When fructose is heated with resorcinol in an acid medium, a red precipitate is formed. The red precipitate is due to conversion of fructose to hydroxymethyl furfural that condenses with resorcinol to give red precipitate indicating positive test.

Microscopic Examination

1. Sperm count-

   In liquefied semen sperms are counted as leukocytes on Neubauer chamber.

   Principle- First seminal fluid is diluted with semen diluting fluid which immobilizes sperms and then counting is done on Neubaur chamber similar to WBC count.

   No. of sperms are multiplied by 1000 to obtain count as count/ml.

   Normal count- 60-150 millions/ml

   Significance-

   - Count less than normal- Oligospermia
   - Absence of sperms- Azospermia
   - Both lead to infertility.

2. Sperm motility-

   Active motility is must for normal spermatozoa as they have to migrate from cervix to fallopian tube to fertilize ovum.

   Motility is observed by preparing coverslip preparation or wet mount technique.

   Note the proportion of motile to nonmotile sperms.

Observe the slide at 3,6,12, and 24 hours. Upto 3 hours there is no change in motility but as the time increases motility decreases.

Grading of sperm movement-

Definition	Description
0-None	An absence of forward progression
1-Poor	Weak or sluggish forward progression
2-Moderate	Defining forward progression
3-Good	Good forward movement with progression
4-Excellent	Vigorous rapid forward progression

Normal Value-

- 80% are actively motile
- 20% sluggishly motile or nonmotile.

Significance-

- Asthenozoospermia- Decreased motility or nonmotility leads to infertility.
- Oligoasthenozoospermia- Abnormalities of sperm movement associated with reduction in sperm number.

3. Sperm morphology-

The morphological characteristics are important for the complete assessment of the seminal fluid.It is done by performingdifferential count of morphologically normal and abnormal sperms on stained preparations.

Stains are Giemsa and carbol fuchsin stain.

a. Morphology of normal sperm

A normal sperm measures 50-70µ in length. It has large oval head measuring about 3-6×2-3µ , a short middle piece and a long tail.

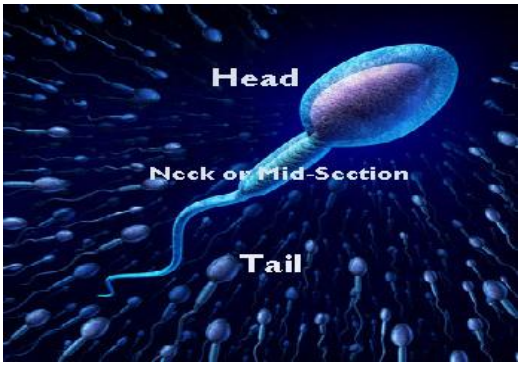


Figure 15.5.1

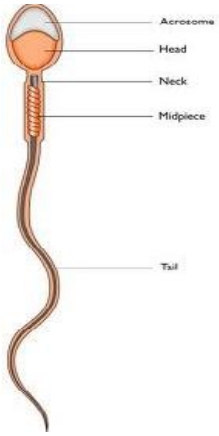


Figure 15.5.2

a. Normal value-

80% are normal sperms and 20% show abnormal morphology.

b. Significance

Abnormal sperms usually show

Head abnormalities

- Marked reduction in head size
- Marked increase in head size
- Double head
- Tapering head
- Presence of vacuoles within head
- Amorphous head - Nuclear chromatin unevenly distributed.

Tail abnormalities

- Double tail or triplicate tail
- Coiled tail
- Hairpin deformity
- Short stubby tail
- Midpiece defect

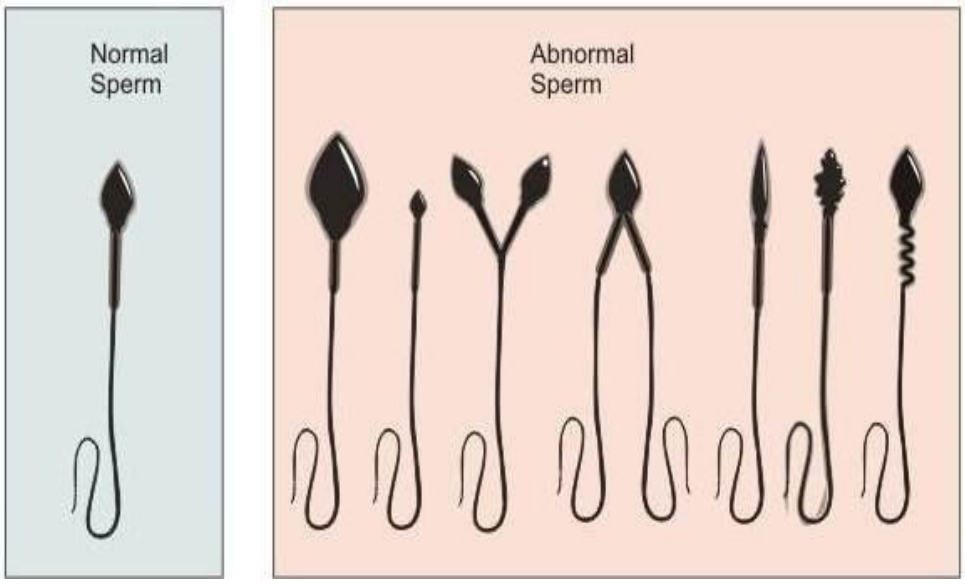


Figure 15.5.4

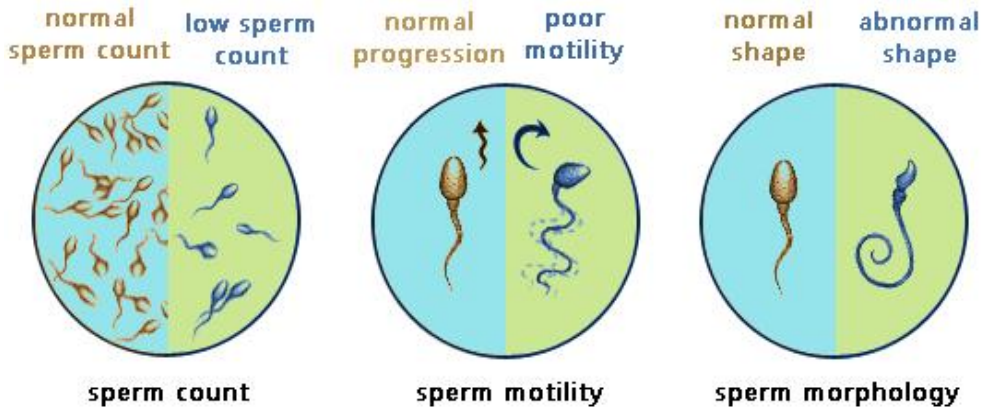


Figure 15.5.5

4. Apart from normal and abnormal sperms smears are examined for
   - White blood cells-

     Presence of WBCs in large indicate inflammation.
   - Epithelial cells-

     Arise from lining epithelium of genital tract and always present in semen.

     Not associated with any pathology.
   - Red blood cell-

     Presence of RBCs indicates possibility of infection or malignant disease within genital tract.
   - Protozoa and bacteria-

     Presence of protozoa like trichomonas and bacteria indicate infection.

Bacteriological Examination

Semen culture-

This is required to exclude morphological causes of infertility. This involves isolation of N. gonorrhea, Chlamydia trachomatis, etc. If pus cells seen in smear then culture is done.

Review Questions:

- Give composition of semen.
- What are different methods of collection of semen?
- What precautions are to be taken while collecting and delivering semen sample?
- Give significance of
    - Liquefaction time
    - Sperm motility
    - Sperm count
    - Semen volume
- Describe morphology of sperm.
- What are different abnormal forms of sperm?

Lesson16

EXAMINATION OF CEREBROSPINAL FLUID (CSF)

Objective-Students shall carry out various tests on CSF and understand the significance of CSF examination.

16.1 Introduction

Cerebrospinal fluid examination helps in diagnosing clinical disorders of central nervous system- brain and spinal cord.

16.2 Physiology

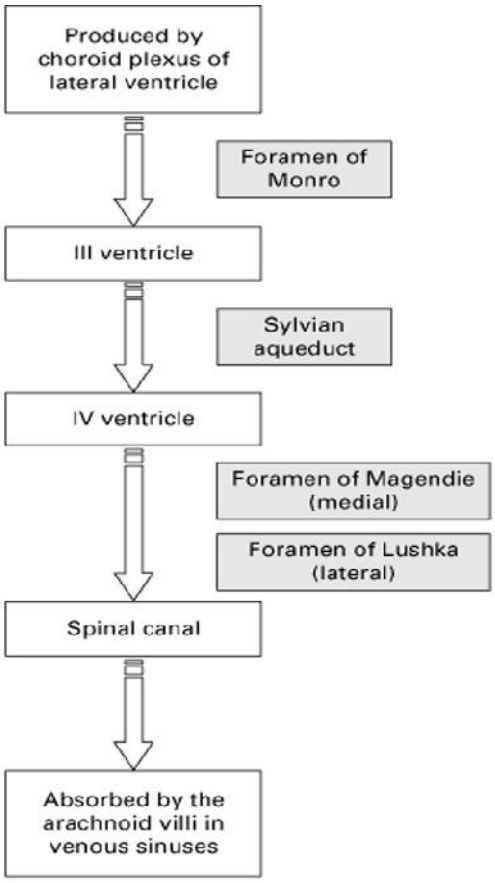
The brain is present within cranial cavity and weighs about $1/5^{th}$ of total body weight. Structurally it is divided into cerebrum, the brain stem (midbrain, pons varolli and medulla oblongata) and the cerebellum. The brain is protected by cranium and spinal cord by vertebral column. The brain and spinal cord are covered by three membranes known as meninges. The meninges consists of

- Dura mater- A outer protective membrane
- Arachnoid mater- a middle membrane
- Pia mater- an inner membrane

The space between dura and arachnoid mater is subdural space and that between arachnoid and pia mater is subarachnoidal space which contains cerebrospinal fluid.

The cerebrospinal fluid is formed by selective dialysis of plasma by the choroid plexuses of the ventricles of the brain and cerebral vessels.

The lateral ventricle is a large irregularly shaped chamber lying deep within each cerebral hemisphere. Within these lateral ventricles choroid plexuses are present which form CSF.The fluid passes into third ventricle through interventricular foramen of Monro, from their in to fourth ventricle through cerebral aqueduct. The fourth ventricle lies between pons and cerebellum of the brain. The CSF then passes from the fourth ventricle down the small central canal of the spinal cord and through openings into subarachnoid space to completely surround the brain and the spinal cord. Finally it is absorbed into cerebral veins and dural sinuses through arachnoidal villi.


Produced by choroid plexus of lateral ventricle
Foramen of Monro
III ventricle
Sylvian aqueduct
IV ventricle
Foramen of Magendie (medial)
Foramen of Lushka (lateral)
Spinal canal
Absorbed by the arachnoid villi in venous sinuses

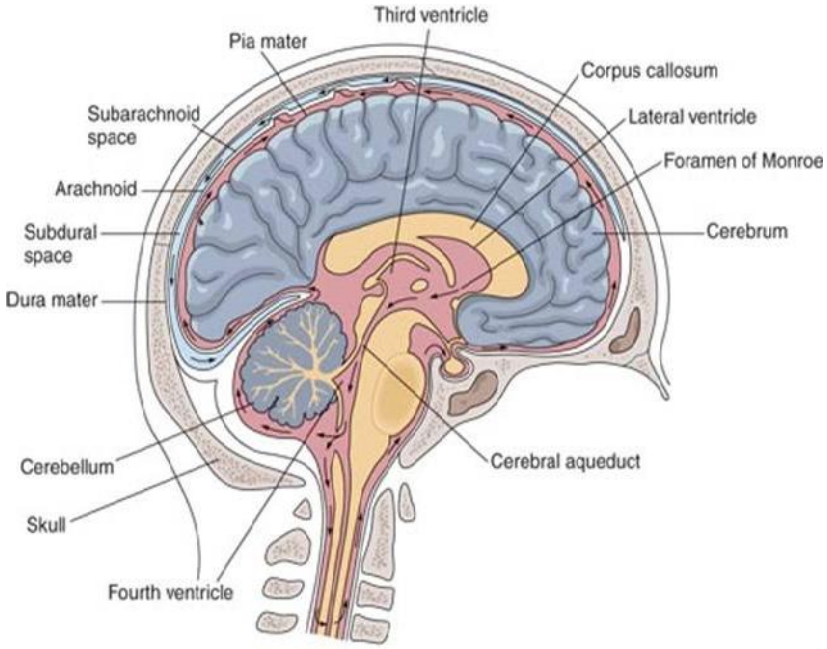

Third ventricle
Pia mater
Corpus callosum
Subarachnoid space
Lateral ventricle
Foramen of Monroe
Arachnoid
Subdural space
Cerebrum
Dura mater
Cerebellum
Cerebral aqueduct
Skull
Fourth ventricle

Figure 36.2.1

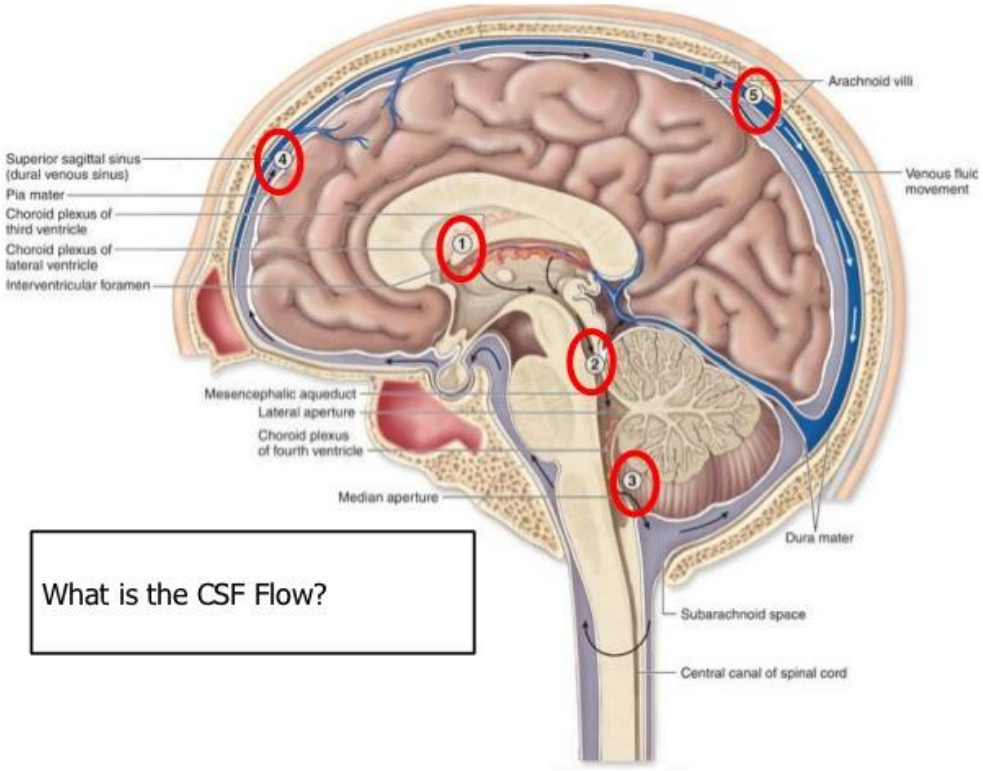


Figure 16.2.2

Functions of CSF

- To serve as a ‘‘fluid cushion’’ to support and protect the brain and spinal cord.
- It acts as a shock absorber for the brain and spinal cord.
- It serves as a medium for the exchange of substances i.e. carries nutrients to brain and spinal cord and removes waste products.
- It helps to maintain a constant pressure inside the head and around the spinal cord.
- It keeps the brain and spinal cord moist.

16.3 Collection of CSF

CSF is collected by a method known as **Lumbar Puncture (LP)**.It should by physician or specially trained nurse or technician.

For this **lumbar puncture needle** is used. It is long needle about 10cm long having stylette fitting into the bore of the needle. For children short needle is used.

LP is done by puncturing the subarachnoidal space between 3rd and 4th lumbar vertebra to a depth of 4-5 cm. This space is nearly at the level of iliac crest.

Figure 16.3.1 Lumbar puncture needle

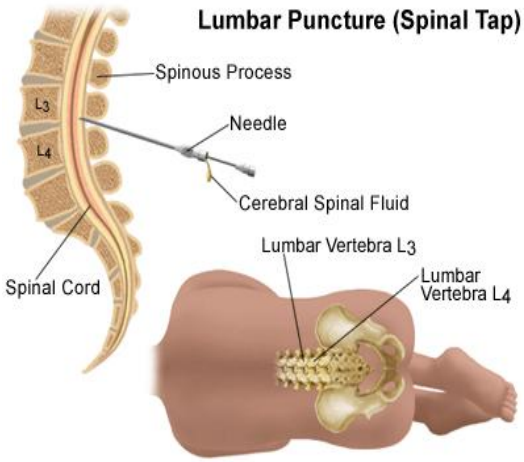


Figure 16.3.2 CSF collection

Technique

- LP should be done under all aseptic precautions.
- Proper position to the patient is given.
- Prepare the site of puncture. Clean the area with spirit swab, iodine swab and spirit swab.
- Local anaesthesia is given.
- The needle with stylet is inserted inside in the midline between 3rd and 4th lumbar vertebra. When needle enters in to subarachnoidal space there is feeling of loss resistance. Remove the stylet. The CSF comes freely.
- CSF is collected in three sterile tubes. For routine examination 5-6 ml is sufficient. First few drops (about 0.5 ml) in 1st tube used for culture, 2nd tube for biochemical examination and 3rd for cytological examination.
- After collection insert stylet and remove the needle along with stylet.
- Apply benzoin seal or small dressing can be done.

Precautions

- CSF should be collected by doctor or specially trained Nurse or technician.
- The collected CSF must be examined immediately (at least within one hour of collection).
- The sample collected for culture should not be refrigerated as N.meningitidis is killed by cold. The sample for biochemical test can be stored at 2-8^{0}c for 2-3 hours.
- Cells and trypanosomes are rapidly lysed after the collection so urgent analysis is must.
- The specimen is difficult to collect, so once it is collected it should be analyzed carefully and economically.
- The specimen may contain virulent organisms so it should be handled very carefully.

Complications of LP

1. In case of spinal cord tumors paresis may progress to paralysis as a result of LP.
2. Production of cerebellar pressure cone in patients with increased intracranial pressure.
3. Introduction of infection can occur by
    - Use of unsterile equipment
    - By passing the needle through superficial or deep sepsis in the lumbar region.
    - Poor technique.
4. Post puncture headache as a result of leakage of CSF. This is reduced by using small bore needle.

Indications for CSF examination and LP

1. Diagnosis of meningitis, subarachnoidalhemorrhage, encephalitis CNS syphilis spinal cord tumors or multiple sclerosis.
2. Differential diagnosis of cerebral infarction from intracerebralhemorrhage.

Indications for LP

1. Confirming increased intracranial pressure.
2. Instillation of antibiotics in meningitis.
3. Repeated LP in subarachnoidalhaemorrage and head injury.
4. For spinal anaesthesia

16.4 Normal composition of CSF

The CSF is clear and colorless fluid mainly composed of water and dissolved oxygen and solids. Average volume is 100-150ml which is produced at the rate of 500ml/day. It is similar to plasma in composition but it has less sugar, calcium and NPN. It contains very little cholesterol and no bilirubin at all. As it is poor in colloids it doesn't clot on standing. Its specific gravity is 1.003.

Criterion	**Normal value**
Volume	100-150ml
Color	Colorless
pH	7.3-7.4
Appearance	Clear
Clot formation	No
Specific gravity	1.003-1.008
Total proteins	15-45mg% (Albumin-50-70%) (Globulin-30-50%)
Glucose	40-80mg%
Chlorides	700-750mg%
Sodium	144-154mEq/l
Potassium	2.0-3.5mEq/l
Creatinine	0.5-1.2mg%
Cholesterol	0.2-0.6mg%
Urea	6-16g/dl
Uric acid	0.5-4.5mg%
Iron	0.2-0.4mmol/l
Cells	0-5 lymphocytes/mm^3

16.5 Routine examination of CSF

I. Physical examination

1. Appearance and color

Normally CSF is clear and colorless. It doesn't clot on standing. Presence of any color is abnormal.

- Most commonly color is due to presence of **blood**. Blood may be due to trauma during LP or it may be because of subarachnoidal hemorrhage. In case of trauma first few drops are heavily contaminated with blood and later CSF becomes clear but in subarachnoidalhemorrhage blood is present throughout.
- **Yellow**color This is known as 'Xanthochromia'. It is associated with breakdown of hemoglobin as a result of earlier subarachnoidalhemorrhage, cerebral tumor or jaundice. Yellow color is also due to increased protein levels (over 100mg) or contamination of CSF with iodine used to disinfect the skin.

2. Turbidity

   Normal CSF is clear. Turbidity is found in pyogenic meningitis when large no. of bacteria are present. Also when there are large number of pus cells present in CSF.

3. Coagula (Formation of clot)

   Clot formation on standing occurs when there is enough fibrinogen. It indicates increased protein concentration. It is also found in TB meningitis where CSF when kept for few hours on standing forms web like clot (cob-web). Cob-web contains TB bacilli.

II. **Chemical examination**

1 Proteins

Protein content of normal CSF is very low. It is 15-45mg%.

Proteins are detected by

- Turbidometric methods-using Sulphosalicylic acid or Trichloroacetic acid method.

Principle- proteins in CSF are precipitated by either dilute trichloroacetic acid or sulphosalicylic acid in sodium sulphate solution. The turbidity of resultant uniform suspension is measured in a colorimeter against known standard.

- Globulins are detected by
  - Pandy's test-

  Principle- In a saturated solution of phenol globulin molecule absorbs water and phenol is displaced from the solution causing fine persistent turbidity. This turbidity is read against dark background.
  - Nonne-Apelt's method-

Globulin is precipitated by solution of saturated ammonium sulphate. A thin white ring of precipitate appears at the junction of two liquids. This may disappear on mixing indicating a 1+ reaction. Heavy cloudiness indicates 4+ reactions.

a. Radial immunodiffusion test- This is useful test to measure concentration of albumin and globulin in the CSF.

Principle- In this there is diffusion of an antigen (IgG or albumin) in the CSF through a semisolid medium containing an antibody (anti-IgG or anti albumin) thereby forming visible zone of precipitation. This zone of precipitation corresponds to the concentration of antigen.

- **Clinical significance**- Increase in CSF proteins is found in
  - Mild increase in viral meningitis, neurosyphilis, subdural hematoma, cerebral thrombosis, brain tumor.
  - CSF globulins get increased in multiple sclerosis, neurosyphilis.
  - Marked increase in CSF proteins is found in acute bacterial meningitis, TB meningitis, spinal cord tumors, cerebral haemorrage,etc.

<u>Glucose-</u>

Concentration of glucose in CSF depends on

- Blood sugar concentration
- The permeability of lining of choroid plexuses and meningeal vessels.
- Rate of glycolysis.

In normal person the CSF glucose is 60-70% of blood glucose. It is 40-70mg%. It is detected by same method as blood sugar detection i.e. GOD-POD method.

- **Clinical significance-**

Increase in CSF glucose is found in

- Diabetes mellitus
- Nonmeningeal syphilis
- Epidemic encephalitis

Decrease in CSF glucose

- Different types of meningitis
- Hyperinsulinism

<u>Chlorides</u>- CSF chlorides are higher than serum chlorides**.** It is 650-750mg%.

- **Clinical significance-**

  Decrease in CSF chlorides is found in

  - TB meningitis (450mg %)
  - Suppurative meningitis (600-650mg %)

III. Cytological examination-

It consists of total and differential count. Normally there are no red cells. It consists of very few white cells 0-5 cells/mm^3 and they are mainly lymphocytes.

As number of cells are very less,**total count** is done on chamber either undiluted or in very small dilution (9 parts of CSF and 1 part or CSF diluting fluid). CSF diluting fluid lyses all red blood cells and white cells are made prominent.

For **differential count** centrifuge the sample. Prepare the smear from deposit. Stain with Leishmanor Giemsastain and then count.

Cells found are

- Polymorphs
- Lymphocytes
- Endothelial cells

Neutrophils and lymphocytes are commonly seen.

Clinical significance- Various cells found in different disease conditions are

1) Increase in polymorph nuclear leukocytes-
   - Pyogenic meningitis
   - Viral meningitis (sometimes)
2) Increase in all cells
   - Sub-acute bacterial meningitis
   - TB meningitis
   - Mycotic meningitis
   - Viral meningoencephalitis
3) Increase in lymphocytes and monocytes
   - Viral meningoencephalitis
   - Multiple sclerosis
   - TB meningitis
   - Fungal meningitis
   - Syphilitic meningitis

4) Blasts cells in leukemic infiltrate.

IV. Bacteriological examination

It is carried out to isolate and identify pathogenic organism.

It includes

- Gram staining
- Z-N staining
- Culture on suitable media
- Serology

V. Serological examination

This is carried out in case of Neurosyphylis.

16.6 Comparison of CSF values in various diseases

Diseases → Charcters ↓	Normal	Purulent meningitis	TB meningitis	Viral meningitis	Syphilitic meningitis	Brain tumour	Cerebral haemorrage
Appearance	Clear and colorless	Turbid and big clot	Opalescent and cobweb	Clear and opalescent	Clear and opalescent	Clear	Clear or haemorragic
Proteins mg/100 ml	20-40	500-1500	45-50	20-200	45-500	20-300	20-2000
Sugar mg/100 ml	40-70	Decreased or absent	Decreased	Normal	Decreased	Normal	Normal
Chlorides mg/100 ml	700-750	650-700	500-600	650-700	700-750	700-750	700-750
Total cells/mm^3 Predominant cell	0-5 cells Lymphocytes	5-20000 Neutrophil	25-500 Lymphocyte	10-1000 Lymphocyte	20-500 Lymphocyte	Normal or 10-200 Lymphocyte	Full of red cells
Other finding	---	Gram staining	Cobweb for M. tuberculosis	---	wasserman test	--	--

Review questions:

1. How circulation of CSF takes place?
2. Describe lumbar puncture technique.
3. What are complications of lumbar puncture?
4. What are indications for lumbar puncture?
5. What is cobweb?
6. What are the causes of blood in CSF?
7. Explain cytological examination of CSF.
8. Give normal composition CSF.
9. Give normal value and significance of
    a. CSF-proteins
    b. CSF-sugar
    c. CSF-chlorides
    d. Total count
10. What precautions are to be taken in collecting and processing CSF?

****

LESSON 17

ABO BLOOD GROUP SYSTEM

Objective – *At the end of the topic the students will be able to identify the normal blood groups and know the clinical significance of the various blood group systems.*

Introduction

Blood transfusion is undertaken as a routine procedure and administration of blood to a needy patient is a potentially lifesaving procedure, if sufficient care is taken with the grouping and cross matching of donor and recipient blood the result may be fatal.

It should be remembered that blood is a connective tissue in a fluid state and transfusion of blood is in effect a tissue transplantation and may be subject of problem if rejection.

In history little progress was made until 1628, when William Harvey advanced his theory of the circulation of blood.

James Blundell of Guy's hospital in London became the first recorded doctor to perform successful transfusion between humans.

Karl Landsteiner discovered ABO blood group system in 1900 which resulted in understanding why some donor blood was incompatible with some recipient.

Landsteiner and Levine discovered two further blood group systems in 1927 the MNS system and P system but major advance in blood group system was made after discovery of Rh system in 1939, it led to the recognition of the cause of hemolytic disease of the new born.

The advent of new techniques in the field of blood grouping hastened the discovery of a number of new blood group system including Lutheran, Kell, Lewis, Dulty and Kidd system.

However out of the above all blood group system only ABO and Rh are important with relation of blood banking.

17.1 ABO Blood group System

Karl Landsteiner in 1900 in series of experiments mixed the red cells of a number of individual with sera of the others in the experimental group. After experimenting he found that he could classify the agglutination pattern into

three group A, B and O, after two years one of this student found out fourth group AB Landsteiner's group became known as ABO blood group system

Antigen (Red cells)	Antibody (serum)	Blood group
A	B	A
B	A	B
A & B	-	AB
-	A & B	O

Inheritance, Genetics and Antigen of ABO Blood group system Genetics

Blood group of parents is inherited to their children. According to the Mendelian half the chromosomes of father and half of mother inherited to their children.
Nucleus of each human cell has 46 chromosome (except gametes which has only 23) 23 of which are inherited from each parents and these genes are called alleles and composition of each alleles from parents on off springs chromosome are called genotype and because of this composition genotype the character which is expressed in that offspring (child) is called phenotype.

If parents share similar alleles of genes it is called homozygous combination and different alleles then it is called heterozygous combination.

If one allele gene from any parent is more powerful in expressing its character it is called dominant gene and if less powerful in expressing then it is called recessive gene.

The ABO blood group system is controlled by the allelic genes A, B & O on chromosome no. 9 and H & h on chromosome no 19

The AB & H genes are dominant genes and they express their character and O & h are recessive gene and do not express their character.

ABO Inheritance

Each individual inherits a single A B gene from each parents which means that there are six possible ABO genotype namely AA, AB, BB, AO, BO & OO, These genotypes translates into four possible phenotypes (character) namely A, B, AB AND O, Because of the O recessive genes AO and BO genotypes are expressed as A and B blood group respectively.

ABO antigen

An antigen is defined as a substance which stimulates the production of antibodies when introduced in an individual & there produced antibodies bind specifically with their antigen when mixed it with them.
These antigens are present on the red cell substance in the protein form.

These antigenic character of red cells are inherited and expressed as blood groups in an individuals are detected by the principals of haemagglutination. It is a serological reaction of red cells with the corresponding antibody as determined in the laboratory.

Incidence of various blood groups in the population

.

No	Blood group	British	Indian
1	A	41.7 %	24.7 %
2	B	8.6 %	37.5 %
3	AB	3 %	5.3 %
4	O	46.7 %	32.5 %

Bombay blood group

If h recessive gene is shared from both the parents then h gene (h h alleles) will make individual blood group without A, B & H antigen.

In routine blood grouping test they do not react with anti A, anti B and anti H antibodies. The lack of reaction with anti A & anti B suggests that the individuals' blood group is O and lack of reaction with anti H suggests it is a rare blood group without antigen H.

This rare abnormality which has an incidence of less than 1 per million worldwide is known as Bombay phenotype or Bombay blood group & indicated as Oh. It has been shown to have a higher incidence in the Marathi speaking people in the Mumbai (previous Bombay) and was first repeated in 1952 by Dr. Bhende and his associates.

Bombay blood group Oh has no antigens on the red cells & contains anti H antibodies in the serum in addition to anti A & anti B. This anti H antibody make these Bombay blood group individual difficult to receive blood from normal O blood group individual, Bombay blood group individual will receive blood only from Bombay blood group individual.

Blood group	Antigen	Antibody
O (normal)	H	anti A & anti B
O^h (Bombay)	No H	anti A, anti B & anti H

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Antibodies

An antibody is a protein formed in the bone marrow, spleen as lymphnode in response to the presence of an antigen which react specifically with antigen.

Antibodies are a two types, Natural and immune antibodies.

Natural antibodies: ABO antibodies are natural antibodies which are also known as agglutinin. These are naturally present in the circularity blood from birth without any antigenic stimulation. Natural antibodies are complete antibodies and predominantly of IGM class with large molecular weight & more antigenic sites which binds more red blood cells. Example of antibodies are anti A and anti B.

ABO antibodies are having following properties.

1) React maximally of 4^0C but the thermal range of activity includes 37^0C
2) Agglutinate cells suspended in saline.
3) The agglutinated cells adhere very strongly & agglutination are very difficult to break up Anti A and anti b antibody levels are highest between the ages 5 and 10 years after which titres decreases to normal.

Immune antibodies

These antibodies are not present in the blood since birth. These are produced when stimulated by antigen example - anti D antibodies are immune antibodies these are produced in the Rh negative individual when Rh positive blood (Rh antigen) is injected

These immune antibodies are usually IgG type and react best at 37^0C body temperature.

Antigen antibody reaction

The red cells antigen is referred to as agglutinogen and its antibody as agglutinin

ABO antibodies anti A and anti B react with corresponding antigen A and B in the saline medium at room temperature whereas immune antibodies anti D are incomplete react with its corresponding antigen D only in albumin solution with incubation of 37^0c.

The haemagglutination reaction first result in coating of the antibody on the red cell followed by binding of the red cells. This results into agglulination but may remain invisible which is called sensitization.

This sensitized antibodies on the red cell can be detected by their reaction with Coomb's reagent (Antihuman globulin reagent)

17.2 ABO blood grouping

The performance of any blood grouping technique demands a high degree of concentration and technical competence.

All blood for grouping and cross matching must be correctly labeled . If there is any doubt about the identification of the sample it must be discarded & a fresh properly identifiable sample obtained. The information given on the sample tube must also agree with that given on the signed request form.

Clerical errors can only be avoided by uninterrupted concentration repeated and careful checking & attention to detail

Serum for blood grouping should be stored at 4^0c

Blood grouping

Principle – ABO blood grouping (determination) is based on the principal of agglutination that is normal human red cells which posses antigen react with corresponding similar antibodies. Example – antigen A will react with antibody A and antigen B will react with antibody
ABO grouping can be done by direct as cell grouping or indirect (reverse) or serum grouping.

The most reliable result is achieved by both these methods
Blood grouping by these two methods can be done on a slide or in a tube at room temperature.

Direct method (cell grouping)

In direct method identification or detection of antigen on red cells surface of the patient's blood is done with the use of known antisera.

Slide method

Requirement : Glass slide, pasteur pipettes applicator sticks, centrifuge etc

Reagents : Anti A sera (blue color)
Anti B sera (yellow color)
Normal saline

Blood sample

Patient's blood is collected preferably in plain bulb, after clotting, blood cells and serum are separated and labeled, blood cells are used for direct grouping and serum for indirect grouping and serum for indirect grouping or for confirmation.

Procedure –

1. First prepare 10 % suspension of red blood cells of the patient's blood. M normal saline as follows.
    a. Mix 5 drops of sedimented red cells with 2 ml of normal saline

b. Centrifuge at 1500 RPM for 1 to 2 minutes discard supernatant fluid.
c. Add 2ml of normal saline to the sedimented washed cells & mix well. This gives 10% red cells suspension for direct grouping.

1. One half of a glass slide place 1 drop of anti A blood grouping serum
2. On the other half of the slide place on drop of anti B blood grouping sera
3. Using a pastern pipette add 1 drop of the patients cell suspension to each drop on the slide.
4. With separate applicator sticks, mix each cell serum mixture well
5. Tilt the slide back and forth and observe the agglutination.

Note –

a. Blood obtained by finger prick may be tested directly by the slide method..
b. Do not interpret peripheral drying or fibrin strands as agglutination.

By tube method

Additional requirements

1) Test tube (10 X 75 mm or 12 X 75 mm)
2) Microscope
3) Prepare 5 % red cell suspension – procedure is same as above but final mixture take 4 ml normal saline instead of 2ml

Procedure

1) To a small test tube add one drop of anti A blood grouping sera
2) To a second tube add one drop of anti B blood grouping sera.
3) Using a pasteur pipette, add one drop of 5 % cell suspension into each of the two test tubes.
4) Mix well and centrifuge both the tubes at 1500 rpm for one or two minutes
5) Resuspend the cells by gently agitation to find out whether cells are truly agglutinated or not
6) Observe for agglutination macroscopically and microscopically.

NB – To avoid false positive results over centrifugation must be avoided.

Reaction with anti A	Reaction with anti B	Blood Group
+	-	**A**
-	+	**B**
+	+	**AB**
-	-	**O**

Note-
(-) indicates no agglutination
(+) indicates agglutination

Reverse or serum grouping

One of the most important controls on direct grouping is called reverse grouping or serum grouping. Patient's unknown serum is tested with suspension of known group A and B cells,. These control cells can be prepared in the blood bank as a standard pool cells. Example – A pooled cells are prepared by collecting only A cells from 3-4 different blood samples, washing and then preparing 2 – 5 ml red cells suspension.

Serum grouping by tube method

1) Take 2 test tubes – place 2 drops of patients unknown serum into each test tube
2) Add 2 drops of 2 – 5 % red cell suspension of standard A cell into one tube and 2 drops of red cells suspension of standard B cell into another tube
3) The tube is shaken and centrifuged at 1000 rpm for one minute
4) Read macroscopally as well as microscopically for agglutination.

Result

Reaction with A cell	Reaction with B cell	Blood group
-	+	A
+	-	B
-	-	AB
+	+	O

Note-
(-) indicates no agglutination
(+) indicates agglutination

Review Questions:

1. Name the different types of blood group systems.
2. Write in short on ABO blood group system.
3. Describe the procedure of ABO grouping.
4. Describe the clinical importance of blood grouping.

LESSON 18

Rhesus Blood Group System

Objective – *At the end of this topic the students will be able to identify Rh blood groups and will know their importance.*

Introduction

In 1940 Landsteiner and Wiener discovered Rh blood group system. They performed experiments on Rhesus monkey & rabbit. They injected RBCs of Rhesus monkey into rabbits. Rabbits developed antibodies against monkey's RBC. These antibodies agglutinated red cells of monkey. as well as 85% human RBCs.

As these people apparently possessed an antigen similar to the Rhesus monkey, they were designated as Rhesus positive and the remainder whose red cells did not agglutinated were called Rhesus negative.

The Rhesus antigen was called D antigen & corresponding antibody anti-D. Later on Rhesus was substituted by Rh.

Subsequent investigation showed the existence of further Rh antigens and antibodies and the basis of the Rh blood group system was explained.

The Rh blood group system is clinically the second most important blood group system in human being after ABO system.

The D antigen is the most antigenic and therefore the most clinically significant red cell antigen after A and B antigen of ABO system.

Introduction of the D antigen into an individual lacking that antigen (Rh-negative individual) is more likely to stimulate antibody production than other Rh antigens.

Due to this reason, blood for transfusion is routinely typed for ABO and Rh D status only. Although testing for all other Rh antigens (namely C , D , E , c , e) anti seras are available, routinely only presence or absence of D-antigen is tested.

18.1- Clinical Significance

Rh blood group system has great clinical significance due to its immune antibodies.

Rh-positive blood cells are antigenic for Rh-negative individual. If an Rh-negative individual is transfused with Rh-positive blood Rh immune antibodies(Anti D) will form in them within few days. At first transfusion, there is formation of antibodies but no haemagglutination reaction takes place .But in second transfusion of Rh-positive blood in same Rh-negative individual ,there will be haemagglutination reaction. which can be fatal.Therefore, Rh-negative individual should receive only Rh-negative blood.

Rh blood groups and Pregnancy – HDNB

Rh factor has considerable significance in certain pregnancies. If a woman possesses Rh-negative blood and her husband is Rh-positive, there is strong possibility that the foetus will inherit the father's dominant Rh-positive factor & this may be the case of haemolytic disease of new born (HDNB)

The first child is seldom affected by Haemorrhagic disease of New born (HDNB). Since the development or stimulation of antibody in the mother is frequently due to a transplacental haemorrhagic from the foetus to the mother during delivery in first pregnancy. If a pregnancy with a second Rh-positive foetus occurs then small bleeds from foetus to mother may further stimulate antibody formation.

If the antibody titre is high, the foetus may die and be expelled before the end of the normal gestational period.

If the titre is low, the child may born alive and will develop haemolytic jaundice and severe anaemia condition is known as Erythroblastosis foetalis. New born baby with this haemolytic condition may survive by exchange transfusion.

18.2-Determination of Rh blood group

Principle

The procedure is based on the principle of agglutination – Normal human red blood cells possessing D antigen will clump in the presence of blood typing serum containing anti-D antibody.

Tube method

Requirements – Pasteur pipettes, Test tubes, Centrifuge, Microscope etc.

Reagents – 1) Blood typing serum anti-D 2) Normal saline

Procedure –

1) Prepare a 5% suspension of red blood cells in normal saline
2) To a test tube, add one drop of anti-D serum.
3) Add one drop of cell suspension (by Pasteur Pipette)

4) Mix well and centrifuge at 1500 RPM for one minute.

5) Resuspend the cells by gentle agitation & examine macroscopically and microscopically.

6) Observation

Reaction with Antisera D	Blood group
+	Rh positive
-	Rh negative

Note

(+) agglutination

(-) No agglutination

Determination of Rhblood group by slide method

Principle

The procedure is based on the principle of agglutination – Normal human red blood cells possessing D antigen will clump in the presence of blood typing serum containing anti-D antibody

Requirements – Glass slides, Pasteur pipettes, applicator sticks

Reagent – Anti-D serum, Normal saline

Procedure – 1) On a prewar med (40°c to 50°c surface temperature) glass slide place one drop of Anti-d serum

2) By using a Pasteur pipette, add one drop of whole blood

3) With a applicator stick mix cell serum mixture well

4) Tilt the slide back and forth and observe for agglutination

Reaction with Antisera D	Blood group
+	Rh positive
-	Rh negative

Note –

(+) Agglutination

(-) No agglutination

Questions :

1. Which are Rh Antigens? Give their clinical significance.
2. Differentiate between anti A and anti Rh antibody.
3. Write a note on HDN.

LESSON 19

THE COMPATIBILITY TEST OR CROSS MATCHING

Objective – *At the end of this topic the student will be able to perform the cross matching testing of blood in the blood bank before transfusion.*

Introduction

Also known as compatibility test means to see whether the patient's serum (antibodies) is compatible with donor's cells & donor's serum is compatible with recipient's (patient's) cells, so that blood can be transfused in the patient safely.

19.1-Clinical significance

One of the most critical test performed in the blood bank is the cross matching of blood for transfusion. If this test is improperly performed and interpreted, there is a risk of mismatched transfusion. .This will cause transfusion reactions which can prove fatal.

Medical lab technician or technologist are actually responsible for this test because they select the unit of blood from the cold storage of blood bank which is going to be transfused in the patient.

So cross matching test should be performed very carefully.

Cross matching test consists mainly of two parts, the major cross match and minor cross match.

Major cross match

In major cross matching donor's cells are allowed to react with recipient's or patient 's serum and observed for cmpatibility. This will detect incompatible antibodies in the recipient or patient's serum.

The major cross matching is considered more important because of the possible destruction of the donor's cells. If massive destruction of red blood cells of donor's cells occurs, then more quantity of bile pigments will form & in high concentration, these pigments will cause toxicity in the patient as lower nephron nephritis which may result in kidney failure. In severe cases it may cause death of the patient by this haemolytic transfusion reaction. So major cross matching is considered more important.

Minor cross match

This test is designed to detect incompatible antibodies in the donor's serum and it is done by mixing recipients cells with donor's serum.

This test is less important because if any reaction occurs it is minor & very negligible.

But if the donor's incompatible antibodies are in high titre, or antibodies are increased due to repeated transfusion then this high titre of incompatible antibodies of donor's may cause severe reaction which may be dangerous to the patient as mention in the major incompatibility reaction.

Therefore cross matching test before blood transfusion should be done with more precautions as follows.

1. First find out the blood group of patient's blood sample and separate it into serum and cells in separate tube with proper label on it.
2. The technician in the blood bank or hospital where the blood will be used selects the blood group identical to that of the patient's blood group in both the ABO and Rh system.
3. Selected unit of blood from the cold storage should be thawed or its cold temperature is brought back to room temperature before doing cross matching test.
4. A cross match test is the performed on the selected unit of blood .After testing the donors & recipient's blood ,if they are found to be compatible then technician should release the checked blood to the patient for transfusion.

Factors which affect cross matching or agglutination reaction

1. **Clotted blood** - Clotted blood sample is preferred to carry out cross matching test.
2. **Centrifugation -** Centrifugation brings the cells into close proximity and hastens the agglutination reaction.
3. **Incubation -** (not heating) Incubation at 37^0c may detect some warm antibodies missed at room temperature.
4. **The bovine albumin -** (22 %) This bovine albumin if added to the mixture of antigen & antibody some incompatible antibodies (incomplete) may be detected. Example Rh incomplete antibodies.
5. **Coombs Reagent -** Some Rh negative blood may not show positive cross match but may cause incompatibility reaction due to incomplete adsorbed antibodies and these adsorbed antibodies are detected by coomb's reagent.

Complete antibodies are detected in saline medium at room temperature but incomplete antibodies requires addition of albumin, incubation or/and coomb's reagent for its detection. Therefore cross match test should be performed in two ways.1 saline cross matching or saline phase and bovine albumin test 2 Coomb 's cross matching or anti humanglobulin phase or test.

19.2 Saline cross matching

Requirements

small tubes (10 X 75 mm)
pasture pipettes
normal saline
centrifuge

22 % bovine albumin

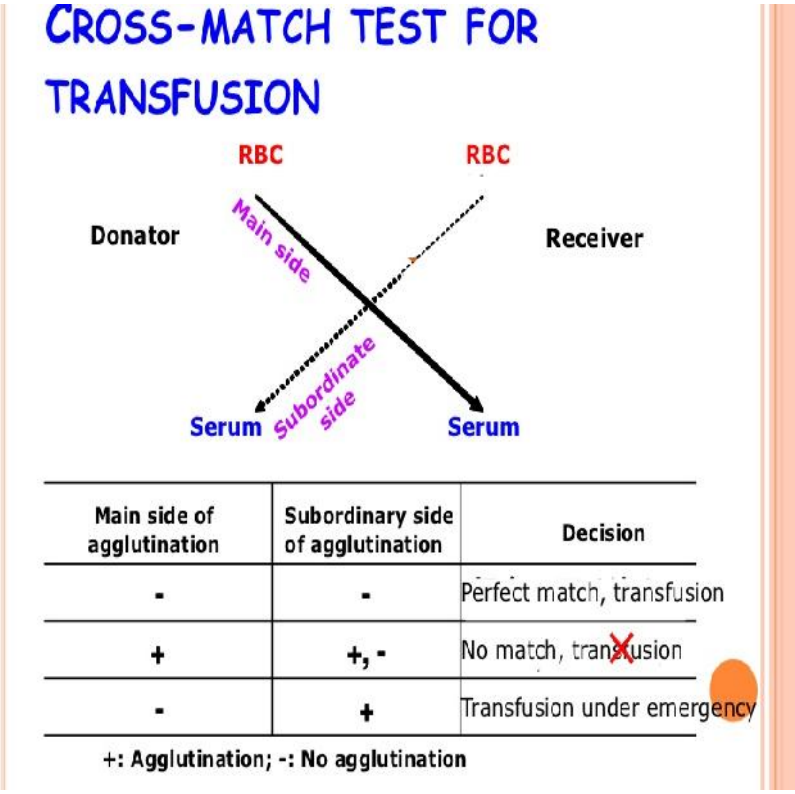
Specimen - 1) Patient’s blood specimen (clotted or EDTA blood sample)

2) Donor’s blood specimen (selected from blood bank)

Procedure -

1. Separate patient’s cells & serum. Prepare 5% red blood cell suspension. Label adequately.
2. similarly separate red blood cells and serum from donor’s blood sample. Also prepare red cell suspension from donor 's blood sample and label accordingly.
3. Take two tubes and label them as major & minor cross match.
4. In tube labeled as **major** add 0.5 ml of cell supension of donor 's blood sample & two drops of patient's (recipient)serum.
5. In tube labeled as **minor** mix donor's serum & 0.5 ml of patient 's cell suspension.
6. Mix and centifuge the tubes at 1500 rpm for 10 to 15 minutes.
7. Check for agglutination microscopically as well as macroscopically. Interpretation-If there is no agglutination in both tubes donor's and recipient's blood are compatible with each other. Agglutination indicates mismatch between two blood samples.

In addition major tube 22% bovine albumin is added to enhance the reactivity of antibodies. No agglutination even after adding albumin can confirm compatibility. Both the tubes are incubated at 37 for 15 to 30 minutes and again centrifuged and observed for agglutination.No agglutination indicates compatibility between donor 's blood & patient's blood.



2. Coomb's cross matching (Antihuman globulin cross matching)

This test should detect almost any blood group antibody that failed to react in previous two tests i.e.saline and albumin cross matching

Procedutre- 1 Take one tube and mix donor's cells & patient's serum.
2 Incubate the tube at 37 for 30 to 45 minutes and wash the cells three times .
3 Add few drops of coomb's serum to the tube and again centrifuge the tube .
4 observe for agglutination.

Note - Washing the cells is extremely important to remove all unreacted antibodies in tube because if any antibodies remain in the tube they may inactive coomb's reagent & result in false negative result.
Result - If there is still no reactivity observed, one final control must be made coomb's control cells or coomb's check cells should be added to the tube, mixed and centrifuged and examined macroscopically.

A truly negative compatibility test will show agglutination in final stage with coombs control cells. This coombs test detects incompatible antibodies which were actually present in the patient's serum during cross matching reaction but because of no visible agglutination in test I & II they are not detected.

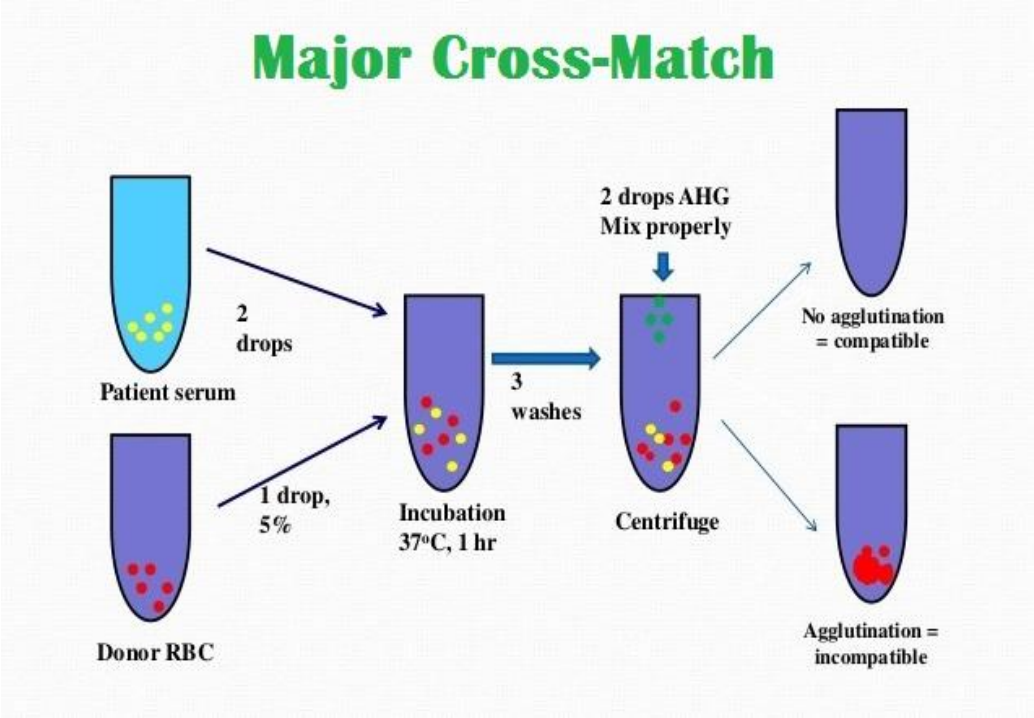
This is because these incompatible antibodies are incomplete in nature & when they react with cells they bind only from one side (other binding site is absent)

That is why they get adsorbed on red cells and remain as single cells with reacted adsorbed antibody on its surface therefore there is no visible agglutination.

Coomb's reagent is antihuman globulin reagent which when added to these cells with adsorbed antibody get agglutinated immediately.

To make coomb's phase perfect test washing of red cells is necessary because incomplete antibodies in the serum may inactivate coomb's reagent & test will show false negative result.

And when there is truly no incompatible antibodies are present then in final check coombs control cell will agglutinate coombs reagent and will show agglutination reaction.
Thus when selected unit of blood passes all these tests then only technician should release tested unit of blood to transfuse into the patient



Review Questions:

1. How Cross Matching of blood is carried out in Blood bank?
2. Why cross Matching is required? What are the types of cross matching?

LESSON 20
COOMB'S TEST

Objective-*At the end of this topic students will understand importance & application of Coomb's test in blood bank*

Introduction

The anti human globulin (AHG) test was introduced in 1945 by Dr. R. Coombs. It is considered to be one of the most sensitive techniques for the detection of compliment or IgG antibody (anti D) sensitization of red cells or other antibodies.

Poly specific antihuman globulin (AHG) reagent used is made by injection of human serum (anti D) into animals which result into development of antibodies in the animals to human serum
(Antihuman globulin)which is then purified and used as coombs reagent in the coombs test, compatibility test etc.

These days AHG or coombs reagent consists of a carefully controlled mixture of monoclonal antibodies directed against IgG, IgA, Igm and the compliment C3d, C3b, C4d, C4b

Addition of this coombs reagent to carefully washed sensitized red cells triggers agglutination by forming immunoglobulin bridges between adjacent red cells as follows.

The coombs test is of two types.

1) Indirect coombs or antihuman globulin test
2) Direct coombs or antihuman globulin test

20.1 Indirect coombs or antihuman globulin test

Importance

This test is performed to detect presence of Rh antibodies or sensitizing antibody in the patient's serum.

In case of Rh negative mothers married to Rh Positive husband may develop anti D in her serum due to Rh positive foetus. This test detects Rh antibodies in mothers blood during pregnancy.

Rh antibodies may be produced in the blood of any Rh negative person if he or she exposed to Rh positive blood, Indirect coombs test detects there antibodies in the blood of Rh negative person.

Even abortion of Rh positive foetus in Rh negative mother may develops Rh antibodies in mothers blood. These antibodies are also detected by indirect test

Requirement

1) Test tube (10 X 75 mm)
2) Pasteur pipettes
3) Incubators
4) Centrifinge

Specimen - Patients serum (need not be fasting)
Reagent - anti human serum (coombs reagent)
Anti - D serum

Additional requirement- Coombs control cells

Preparation

a) Make pooled O Rh positive cells from at least three different O positive blood persons.
b) Wash these cells three times in normal saline to remove free antibodies in the serum
c) Make 5% saline suspension of these cells

Procedure

1) Label three test tube
   a) Test serum as T
   b) Positive control tube as PC
   c) Negative control tube as NC
2) In the tube T (test serum) add two drops of test serum
3) In the tube PC (positive control) add one drops of Anti D serum
4) In the tube NC (negative control) add one drops of normal saline
5) Add one drop of 5% saline suspension of the O positive cells in each tube
6) Incubate all the tubes for one hour at 37^0c
7) Wash the cells three times in normal saline to remove excess serum (free antibodies in case washing is inadequate false negative result may be obtain)
8) Add two drops of coombs serum to each tube, keep it for 5 minutes & then centrifinge at 1500 / rpm for one minute
9) Resuspend the cells and examine macroscopically and microscopically. Observe result as follows.

	Observation	Conclusion
Positive control	a) Agglutination	Procedure is correctly performed
	b) No agglutination	Not correctly performed or coombs'reagent is faulty
Negative control	No agglutination	No antibodies
Test serum (T)	Agglutination	Pt. serum contains anti D

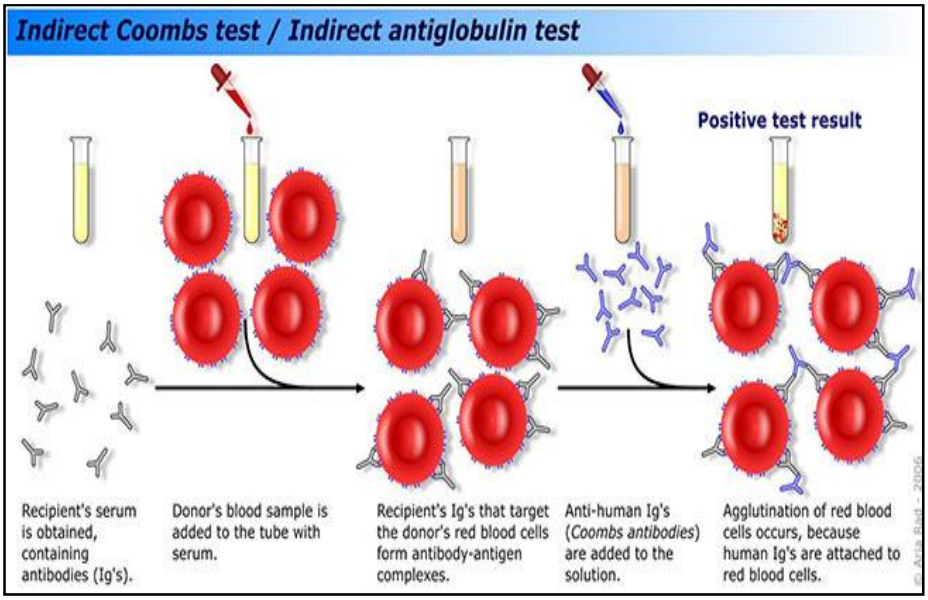


Fig 20.1 -Indirect Coomb's test

20.2 Direct coombs' test

Importance

This test is designed to detect red cells with adsorbed antibodies means antibodies (Rh) attached to red cells which have not agglutinated

This condition occurs in following cases. Rh negative mother who have developed Rh antibodies due to Rh positive foetus cross placental barrier & enter foetus blood and get adsorbed on fetus red cells. This reaction may cause agglutination and haemolysis which results into haemolytic disease of new born or get adsorbed on it.

Such baby with adsorbed antibodies when born blood is collected from umbilical cord and tested by direct coombs test to detect adsorbed antibodies.

Direct coombs test is also performed to detect adsorbed cells in compatibility test and also in auto immune haemolytic anaemia

Requirement - Same as Indirect coombs test

Specimen - Blood drawn into EDTA is preferred but oxalated, citrated or clotted whole blood may be used (no need to be fast)

Procedure

1) Prepare a 5 % suspension in normal saline of the red blood cells to be tested.
2) With a clean Pasteur pipette add one drop of the prepared cells suspension to a small tube
3) Wash three times with normal saline to remove all the traces of serum
4) Decant completely after the last washing
5) Add two drops of coombs reagent or antihuman globulin serum
6) Mix well and centrifuge for one minute at 1500 rpm
7) Resuspend the cells by gentle agitation & examine macroscopically or microscopically for agglutintation

Note -

1) The sensitivity of the test can be increased by incubation at room temperature for 5 to 10 minutes & recentrifugation.

Result -

If adsorbed antibodies are presents on the blood cells, coombs reagents will agglutination them .Agglutination indicates positive test & no agglutination means the test is negative.

Review Questions:-

1. What is the importance of Indirect Coomb's test ?
2. Describe direct Coomb's test.
3. What is the use of Coomb's test in blood transfusion?

LESSON 21

BLOOD BANK

Objective-*At the end of this topic students will know about operation ,organisation and functions of blood bank*

Introduction

Blood Transfusion Service (BTS) is a vital part of healthcare system. Regular supply of safe blood and blood products is priority of every nation and this can be achieved by well-equipped blood Centre with adequate infrastructure, well trained manpower and advanced techniques. The blood bank also called as 'blood Centre' is required for collecting, processing, testing, storing and distribution of blood in the blood transfusion system.

21.1 Definition of Blood bank:

A place where blood is collected from donors, typed, separated into components, stored, and prepared for transfusion to recipients. A blood bank may be a separate free-standing facility or part of a larger laboratory in a hospital.

Layout of Blood bank:

1. Location: The blood Bank should be situated in the hospital where there is maximum hospital traffic. It should be on the ground floor.
2. Area: the minimum area should be 100 square meters for operation of the blood bank and 50 square meter additional are for preparation of blood components.
3. Designing: the blood bank should have following rooms with necessary furniture. All the rooms should preferably Air conditioned.
   (a) Waiting Room or Reception Room: it should be spacious to accommodate god number of donors. It should be well decorated and comfortable for donors. It should have a registration counter, a doctor's cabin where proposed donor is examined for fitness and a small side laboratory for checking Hb content and other parameters of donor screening.
   (b) Blood donation room: it should be pleasant and safe. It should be well lighted and attractive. It should be well ventilated, preferably air-conditioned. It should have comfortable beds for donors. It should be equipped with all the materials and instruments required for bleeding donors.
   (c) Donor Refreshment Room: A small air-conditioned room where donor is served with light refreshments and allowed to rest for a while. The room should have proper seating and one or two comfortable beds.

(d) Serology Laboratory: it is for blood group serology. it should be spacious and should be air-conditioned. Its main functions are grouping of the donor's blood, grouping of the recipient's blood and cross-matching.
(e) TTD Laboratory: Laboratory for testing transfusion transmissible diseases like Hepatitis B, Malaria, Syphilis, HIV and cytomegalo virus (CMV).
(f) Sterilization and washroom: For sterilization of the equipment used for blood collection and processing.
(g) Store and record room: Record keeping is very important part of blood bank. The record includes blood donor's record, master record of blood and its components, issue register, records of components supplied, record of blood donation bags, transfusion adverse reaction register, etc. Store is required for storing blood collection kits and other equipment.
(h) Room for preparation of blood components: this is a separate room, air-conditioned and used for separation of blood in to different components. It should be kept sterile.
(i) Cloak roam or toilet for the staff and donors

4. Staff: Every blood bank shall have following staff :-
(a) Medical Officer, possessing the qualifications specified.
(b) Blood Bank Technician(s), possessing – (i) Degree in Medical Laboratory Technology (M.L.T.) with six months' experience in the testing of blood and/or its components; or (ii) Diploma in Medical Laboratory Technology (MLT) with one year's experience in the testing blood and/or its components.
(c) Registered Nurse(s).
(d) Technical Supervisor(where blood components are manufactured), possessing - (i) Degree in Medical Laboratory Technology (M.L.T.) with six months' experience in the testing of blood and/or its components; or (ii) Diploma in Medical Laboratory Technology (MLT) with one year's experience in the testing blood and/or its components.

21.2 Equipment:

Equipment used in the collection, processing, testing, storage and sale/distribution of blood and its components shall be maintained in a clean and proper manner and so placed as to facilitate cleaning and maintenance. The equipment shall be observed, standardized and calibrated on a regular basis. The important ones are :-

a) Refrigerator
b) Centriguge
c) Auto clave
d) Incubator
e) Microscope with lamp
f) Set of Haemocytometer
g) All necessary glassware & chemical including the blood collection bottle or bags
h) Blood collection tubes etc.
i) All types of group serum

j) Diagnostic kits for serological test which includes VDRL, HbsAg, HIV.

21.3 Functions of Blood Bank

1. The blood bank selects the donor & collects the blood, which can be used for the needy patients.
2. The blood which is collected from the donor is processed. The grouping of the blood is done. Various serological test are carried out on the collected blood, specially the VDRL, HbsAg, & HIV to see that the blood is totally safe for transfusion.
3. The blood banks stores the blood till it is used by the needy patients.
4. The grouping & cross-matching of the donors & the patient's blood is done in the blood bank.
5. The blood bank arranges blood donation camps in the society to promote voluntary blood donation.
6. The blood bank processes blood & produces some of the important blood products such as packed red cells, fresh frozen plasma, platelet concentrate etc.

21.4 Blood Transfusion

Introduction

Blood transfusion is of great help in management of patient. It is nothing but intravenous administration of blood to a needy patient .For providing blood transfusion services, somebody should donate blood .Blood bank is always demanding for voluntary blood donors .At the same time in blood transfusion ,safety of donor as well as patient is considered most important.

22.4.a.BLOOD DONORS

When you require blood for transfusion you must have a blood donor. In order to keep the blood transfusion safe & harmless you must screen the donors in order to find out whether he or she is suitable for donation. The criteria for selection of a blood donors are as follows:

selection of donor

1. Age: Any person between 18 yrs. to 60 yrs.can become blood donor.
2. Weight: The weight of the donor should be minimum 45 Kg.
3. Interval between donations: There should be minimum 3 months gap between two successive blood donations. In other words one preson can donate blood maximum four times in a year.

4. Haemoglobin level: The donor should not be anaemic. The male must have Hb level 13.5 gm% and the female must have Hb level minimum 12.5 gm%. The Hb level is checked before the blood donation. Haemoglobin is checked by specific gravity method using copper sulphate solution or it is also checked by Drabkin 's technique in some blood banks.In blood donation camps specific gravity method is preferred when number of donors are more and fast screening is required.

Physical Examination

1. Temperature: It should be normal between 98 to 98.6^{o}F or 37^{0}c
2. Pulse: It should be within normal limits i.e. 70 to 80 per minutes. It must be regular.
3. Blood pressure: It should be normal. Systolic blood pressure should be between 110 to 140 mm of mercury & the Diastolic blood pressure is to be between 70 to 90 mm of mercury.
4. Examination of donor: Donor is examined by doctor (blood transfusion officer) in blood bank with respect to general examination as well as examination of respiratory system & cardiovascular system using the stethoscope. The person with a heart disease or a lung disease is not allowed to donate blood.

History of the Donor:-

1. The donor is asked about history of any recent illness or history of any surgery or history of blood transfusion in the past.
2. He is also asked history of any recent vaccination. Donors who have received killed vaccine such as cholera, typhoid, diphtheria etc. can donate one week after vaccination; but the donors who have received killed vaccines such as BCG, Polio, rabies should not donate for at least 3 weeks after vaccination.
3. The donors are also asked about history of any major illness in the past such as jaundice, tuberculosis, syphilis, diabetes, malaria ,etc.
4. The donor is also asked about history of medicines for regular consumption. Donors receiving anticonvulsant, anticoagulant, anti-hypertensive, anti-histaminic or anti-pyretic drugs, particularly aspirin and donors receiving corticosteroid drugs cannot donate blood.
5. A person who had undergone surgery can donate blood six weeks after a minor surgery and six months after a major surgery.
6. The female donors are asked about history of abortion, pregnancy ,recent delivery and lactation.

21.4.b Collection of blood from the Donor

1. Always ask the donor to lie down on a bleeding table and get sure he is comfortable.
2. Tie the blood pressure cuff around the arm and inflate up to 60 mm of Hg.
3. Select the site for venipuncture. Clean the skin first with tinc. Iodine and then with spirit.
4. Allow the skin to dry.
5. Select the most prominent vein and proceed for venipuncture.
6. Nowadays plastic bags containing CPD or ACD anticoagulants are used for blood collection. These bags are provided with a bleeding set which has a needle usually 18 gauge which is kept sterile under a cover.
7. The bag is kept on a weighing machine about 60 cm below the level of the arm.
8. Make a loose loop about 10cm away from the distal end of the needle.
9. Remove the cover of the needle and pierce it through the vein with the bevel of the needle facing upwards.(Nowadays local anesthesia is not preferred as it was used before.)
10. Observe the flow of the blood in the bleeding tube.
11. When the blood starts entering the bag, the bag is agitated gently to allow mixing of the blood with the anticoagulant 1 ml of blood approximately weights about 1.05 gms. Hence, 350 ml of blood will weight 367 grams.
12. When the required amount of blood is collected as indicated by the scale on the weighting machine, make the loop tight, release the pressure in the BP instrument and withdraw the needle gently by applying a light pressure.
13. Put a cotton swab over the venipuncture site and ask the donor to press it lightly for 10 minutes.
14. Apply a small sticking plaster over the punctured site and take the donor to a refreshment room where he is given coffee and biscuits.
15. The bag is removed from the weighing machine and about 6 to 8ml of blood is divided equally in 3 numbered pilot tubes. The number on pilot tubes is same as that on the bag. These samples are used for grouping, cross-matching as well as for HIV, HbsAg, and VDRl testing.

16. The needle is removed from the set and the blood bag is kept immediately in a refrigerator of donor room at 4 to 6 C.

17. According to Indian standards one unit of blood contains about 350ml of blood, which requires 49ml of CPD anticoagulant.

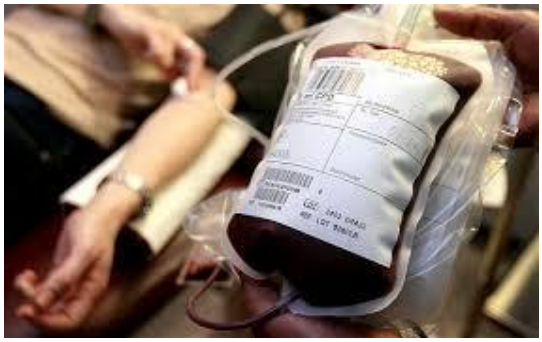


Fig 22.2 blood collection bags

21.4.c.Preservation of blood (anticoagulants used)

The blood which is collected should be added to a preservative which will not only prevent it from clotting, but it will also provide nutrition to the cells so as to maintain the cell metabolism and release of energy. Trisodium citrate is the most suitable anticoagulant which acts by chelating the calcium ions. The nourishment for the cells is provided in the form of dextrose. Trisodium citrate is alkaline and hence to maintain an optimum pH, a weak acid is added which is citric acid. An ideal anticoagulant preservative was introduced in 1943 called ACD Solution (Acid citrate dextrose Solution) with a shelf life of 21 days. After this a new anticoagulant preservative CPD (Citrate Phosphate Dextrose) was introduced with a shelf life varying between 28 to 35 days. When Adenine hydrochloride was added to above solution it was found to increase the shelf life up to 42 days. This solution is called as CPDA.

Anticoagulants used in blood bank

Composition	ACD A	ACD B	CPD	CPDA
Trisodium citrate	22gm	13.2 gm	26.3 gm	26.3 gm
Citric acid	7.3 gm	4.4 gm	3.2 gm	3.2 gm
Na hydrogen phosphate	-----	-----	2.2 gm	2.22 gm
Dextrose	24.5 gm	14.7 gm	25.5 gm	31.9 gm
Adenine hydrochloride	----	----	----	0.35 gm
Water	1000 ml	1000 ml	1000 ml	1000 ml
Volume/100ml	15ml/100ml	25ml/100ml	14 ml/100ml	14ml/100ml
Shelf life	21 days	21 days	28 days	28-35 days

21.4.d.Pretransfusion tests

In order to have safe transfusion ,donor's blood is screened for some diseases like malaria, syphylis etc .These tests are carried out in the blood bank laboratory .In case some of the test result is positive ,then that blood bag is discarded & not used for transfusion. This assures safety of recipient. Various screening tests commonly done are as follows.

HbsAg-(Australia antigen)- .HBsAg also known as Australia antigen is the surface antigen of the hepatitis B virus(HBV).It indicates current hepatitis B infection This test will detect about the disease Hepatitis -B. If HbSAg test is positive, it indicates that donor's blood is carrier for Hepatitis-B and there is risk that patient can suffer from Hepatitis -B

V.D.R.L.Test-(Veneral disease Research Laboratory)- The Venereal Disease Research Laboratory test(**VDRL**) is a blood test for syphilis. The VDRL test is used to screen for syphilis (it has high sensitivity), whereas other, more specific tests are used to diagnose the disease. . The venereal disease research laboratory

(**VDRL**) **test** is designed to assess whether or not the donor have syphilis, a sexually transmitted infection (STI). Syphilis is caused by the bacteria Treponema pallidum.

ELISA Test (Enzyme linked immunosorbant assay)-ELISA test is an antibody test (also called immuniassay) which checks for antibodies to HIV virus.If this result is negative or non-reactive, then donated blood is HIV-negative.If the result is positive though, this does not mean that donor is HIV-positive. A small percentage of people can test positive with ELISA who are not HIV-positive (called a 'false-positive' result).

All positive results need to be confirmed by a second, more sensitive antibody test called Western Blot. The Western Blot test takes longer (usually a week or so) and is the most accurate at identifying genuine positive results. When it is used to confirm a positive result the Western Blot test is 100% accurate.

Malarial Parasite-Blood is checked for presence of malarial parasite by usual method. If the blood is positive for malarial parasite then chances are there that the

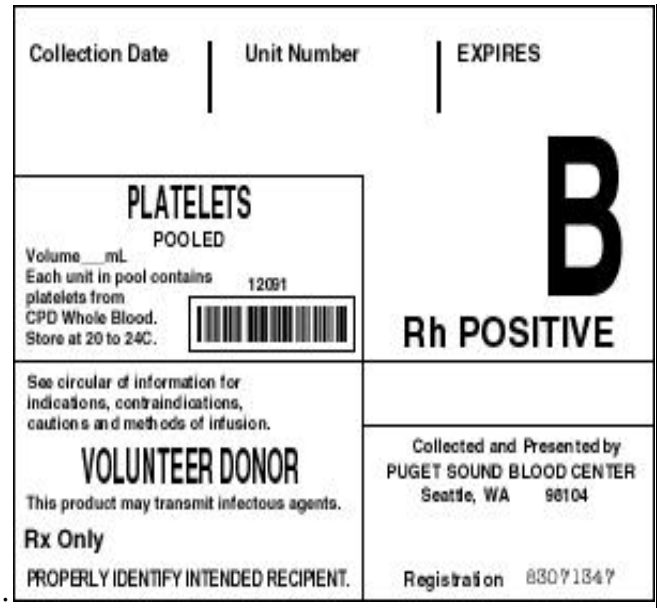
21.4.e.Storage of blood

Blood must be stored at the temperature 4^0c with range from 2^0 c to 4^0c.It is stored in a specially constructed refrigerator which has a high insulation properties and a very sensitive thermostat. The temperature of the refrigerator must be maintained at 4^oC with a range from 2^o C to 6^o C. The temperature should not exceed 6^oC or fall below 2^o C otherwise damage to the RBC's may occur. The door of the refrigerator should not be opened or closed constantly. There should be an alarm system if the door remains open and also an adequate battery backup in case of electricity failure.In some of the blood banks special walk in cold rooms are available for storage of blood. After all the pretransfusion tests are over final label will be applied to the blood bag. The label contains information regarding blood group, date of collection ,date of expiry and the number of blood bag according to numbering system

STANDARD COLOR LABELS

Group	Color of label
O	
A	
B	
AB	

Fig 22.5 Standard colour coding



.Fig 22.5 blood bag label

Review Questions:

1. Describe the various functions of a blood bank.
2. How will you set up a Blood bank?
3. How Donor's screening is done?
4. Which are the different anticoagulants used in Blood bank?
5. How the blood is stored in a blood bank?
6. Write an essay on the blood bank.

LESSON 22

Complications of Blood Transfusion

Objective- Students shall understand various transfusion reactions that can occur during or after transfusion of blood or blood components.

Transfusion Reaction is any unfavorable transfusion-related event occurring in a patient during or after transfusion of blood components.

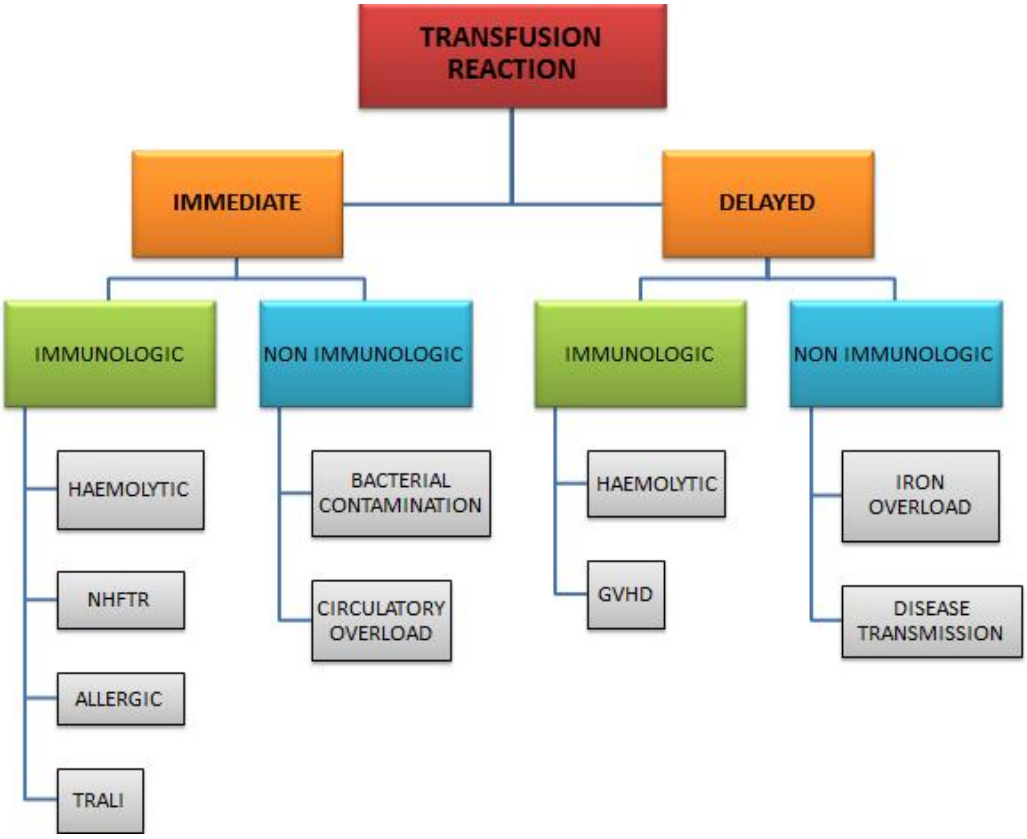
Classification:

1. Acute (Immediate) reactions:

   Symptoms appear within minutes or up to 24 hrs post transfusion.

2. Delayed reactions

   Reactions occurring more than 24 hrs following transfusion (up to months)



1. Acute Hemolytic Transfusion Reaction

The most severe reactions occur when transfused red blood cells (RBCs) combine with recipient antibodies and lead to increased RBC destruction. Most of these cases result from transfusion of ABO-incompatible red cells, and can be life-threatening.

Symptoms of acute hemolytic transfusion reaction may begin mildly, after infusion of as little as 10-15 mL of incompatible blood.

The following reactions may occur in an acute hemolytic immune-mediated transfusion reaction:

- When incompatible RBCs bind with patient antibody, the immune complex on the cell surface can activate complement which leads to cell lysis and the release of anaphylatoxins C3a and C3b.

- Hemolysis that occurs intravascularly releases hemoglobin, RBC stroma, and intracellular enzymes into the plasma which result in the manifestation of symptoms.

2. Febrile Non-Hemolytic Transfusion Reactions

Febrile non-hemolytic transfusion reactions occur in about 1% of transfusions. Along with allergic reactions, febrile reactions are the most commonly encountered type of transfusion reaction. The commonly accepted definition of a febrile transfusion reaction is an increase in temperature of 1°C or 2°F or more associated with transfusion and without any other medical explanation. These reactions may be caused by antileukocyte antibodies present in the recipient's plasma directed against antigens present on transfused monocytes, granulocytes, and lymphocytes.

3. Delayed Hemolytic Transfusion Reaction

Hemolytic transfusion reactions are usually due to non-ABO incompatibilities. The mechanism of RBC destruction for most delayed hemolytic transfusion reactions occurs when the sensitized RBCs are removed from the circulation by the reticuloendothelial (RE) system. The patient experiences less severe symptoms, including mild fever, chills, and moderate jaundice, than in the acute hemolytic reaction.
Recipients may be sensitized to RBC antigens during pregnancy or previous transfusion (alloimmunization). The corresponding antibodies may be undetectable in pre-transfusion laboratory testing, but when transfused, an anamnestic response leads to antibody production and clinical symptoms in about three to seven days post-transfusion. Unexpected or unexplained decreases in hemoglobin or hematocrit values following a transfusion should be investigated as a possible delayed hemolytic transfusion reaction.

Non-Immune Mediated Transfusion Reactions

Many of the clinical symptoms of these transfusion reactions are nonspecific, but include facial numbness, chills, numbness, muscle twitching, cardiac arrhythmias, nausea, vomiting, altered respirations, and anxiety. Laboratory tests for investigation and diagnosis of these conditions include electrolyte levels, calcium, pH, glu-cose, urinalysis, hemoglobin, hematocrit, platelet count, prothrombin time, and activated partial thromboplastin time.

Embolic

Air embolism is a concern if blood is infused in an open system, or if air enters the system when containers or tubing are changed. Proper use of pumps, apheresis equipment, and tubing connectors is essential. Patient symptoms may include cough, dyspnea, chest pain, and shock.

Metabolic

During massive transfusions, depletion of platelets and coagulation factors may occur if the patient is not sup-ported with transfusion of these components. Hypothermia may occur when large volumes of cold fluids are infused.

Citrate toxicity may result from apheresis procedures and when large volumes of fresh frozen plasma (FFP), whole blood, or platelets are transfused. The citrate will bind the recipient’s free calcium ions and produce hypocalcaemia. This is also a concern when exchange transfusion is performed on infants who are already ill. RBCs lose intracellular potassium during storage and may be the cause of transfusion-induced hypokalemia when washed RBCs are administered.

Circulatory Overload

Whereas TRALI is associated with transfusions of blood volumes that do not produce hypervolemia, circulatory overload may be caused when blood volume is rapidly increased.

Transfusion that proceeds at too fast a rate may lead to congestive heart failure and pulmonary edema. The administration of 25% albumin may also be implicated as it causes a shift of large amounts of interstitial fluid into vascular spaces. The transfusion should be stopped, or at least slowed when completion of the transfusion is critical.

Patient support generally includes administration of oxy-gen, diuretics, and in severe cases, therapeutic phle-botomy to reduce blood volume. For patients at risk of developing circulatory overload, blood units may be split into aliquots to allow for trans-fusion over longer time periods, and the use of washed RBCs may help diminish the plasma load administered to the recipient.

Metabolic Iron Overload

A complication of long-term RBC transfusion is an accumulation of iron that may affect heart, liver, and endocrine gland function. Also called hemosiderosis, symptoms of iron overload include muscle weakness, fatigue, weight loss, jaundice, anemia, and cardiac arryhythmias. Laboratory investigation will include iron and ferritin levels and possibly tissue stains for iron.

Bacterial Contamination Reactions

No matter how carefully blood is collected and processed, bacteria can never be completely eliminated. Bacterial contamination is responsible for one-eighth of reported transfusion-associated fatalities.

It is believed that bacteria in blood products originate with the donor, either from the venipuncture or from inapparent donor bacteremia. Bacterial multiplication in stored blood may occur with the production of endotoxins that give rise to symptoms including fever and chills, hypotension, hemoglobinuria, muscle pain, shock, renal failure, and disseminated intravascular coagulopathy (DIC).

Gram negative organisms capable of growing at cold temperatures include Pseudomonas species, Citrobacter freundii, Escherichia coli, and Yersinia enterocolitica. Gram positive organisms are more likely to be found in products stored at room temperature. Before release to the transfusionist, examine each unit of blood for visual evidence of contamination. Color change to dark purple or black, clots in the bag, and hemolysis may suggest contamination.

Review Questions:

1. What are transfusion reactions? How they are classified?
2. Describe the Immediate transfusion reactions.
3. Describe the delayed transfusion reactions.
4. How bacterial contamination of transfused blood can occur? Which are the common bacteria?

LESSON 23

Blood products

These are classified as blood components prepared in the blood transfusion center (red cells, platelets, fresh frozen plasma and cryoprecipitate) or plasma derivatives manufactured from pooled plasma donations in plasma fractionation centers (such as albumin, coagulation factors and immunoglobulins). Plasma derivatives are covered by the Medicines Act and, like any other drug, must be prescribed by a licensed practitioner.

Blood components

Whole blood is now rarely used for transfusion. Blood component therapy makes clinical sense as most patients require a specific element of blood, such as red cells or platelets, and the dose can then be optimised. Each component is stored under ideal conditions (e.g. red cells must be refrigerated, platelets must not) and the use of precious blood donations becomes more efficient. Precious blood donation can be used economically for more number of patients. The use of blood components in clinical practice is covered in Chapters 7 to 10.

The process of producing blood components and plasma derivatives is summarized in Figure below.

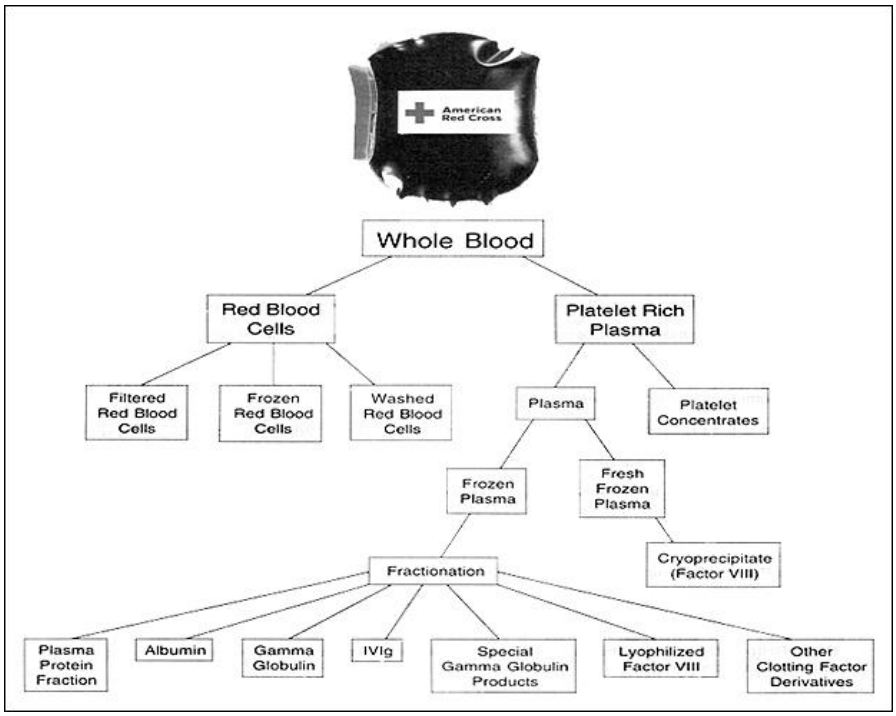


Figure 23.1 Different Blood Component

Specifications of blood components:

Whole blood donations of 405–495 mL (mean 470 mL) are collected into 63 mL of citrate phosphate dextrose (CPD) anticoagulant.

All blood donations are filtered to remove white blood cells (pre-storage leucodepletion) to leave <1×106 leucocytes in the pack. This was introduced in 1998 as a vCJD risk-reduction measure but also reduces the incidence of febrile transfusion reactions and alloimmunisation to white cell (including HLA) antigens.

Figure 23.2 Aphaeresis machine

BLOOD AND BLOOD PRODUCTS

1. Whole blood

In most instances the doctor requests and needs whole blood including the cells as well as plasma. Whole blood is obtained by adding anticoagulant (ACD solution) to the blood collected from donor. Fresh blood is required when platelets, WBCs are required or in haemophilia since all these blood components are lost on storage.

2. Packed Red Cells

Red cells are transfused in anaemic patients with low haematocrit. These red cells are separated from the plasma and are called packed red cells. The blood bottles kept in the refrigerator shows sediment of settled RBCs at the bottom and plasma above it. The plasma is aspirated in to a separate sterile bottle leaving the cells in the original

bottle. The plasma bottle is labeled with the date, bottle number and group and type of blood from which it is obtained. The packed cells are transfused to the patients after, diluting with sterile saline. The packed cells should be transfused within 4 hours.

3. Plasma

The ACD plasma aspirated from the sedimented cells may be stored for months at 4 – 6 C and is safer for transfusion. However, it should be checked frequently of development of small floating granules or cloudiness due to growth of bacteria Minor cross match should be done before giving plasma transfusion. Plasma can be used as-

(i) Liquid Plasma : It should contain 5% dextrose to prevent precipitation of fibrin at room temperature. It may be kept at room temperature for 3 years.

(ii) Fresh Frozen Plasma : It is separated from the whole blood within 2 hours after collection of blood from the donor. It can be stored indefinitely at-20 C. Before administering it is reliquified at 37 C in a water bath and used promptly. FFP is rich in all coagulation factors and hence is given to patients with general bleeding disorders.

4. Cryoprecipitate

This is prepared from fresh plasma, which is frozen solid in a mixture of solid CO_2 (dry ice) and ethanol and is allowed to thaw slowly at 4C for 24 hours. Thawing leaves a cold insoluble precipitate, which is rich in AHF. The precipitate is stored at -20^{o} C and for use thawed at 37^{o} C and transfused. Cryoprecipitate contains about 50% of the original factor VIII together with small amount of fibrinogen, but no other coagulation factors. IT is used predominantly in the treatment of haemophilia and patient's with Von Willebrand's diseases. Administration of cryoprecipitate does not require any compatibility testing.

5. Platelet Concentrate

It is prepared from whole blood by differential centrifugation. In this process, the red cells are first separated at low speed followed by high-

speed centrifugation for sedimentation of platelets, Platelet concentrate is given to patients with thrombocytopenia with history of bleeding. ABO, compatibility is not required for platelet transfusion.

Review questions:

1. Name the different Blood products.

2. Name the different clinical conditions in which following products are used.

(a) Packed Cell Volume

(b) Whole Blood

(c) FFP

(d) Cryoprecipitate

(e) Platelet Concentrate

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