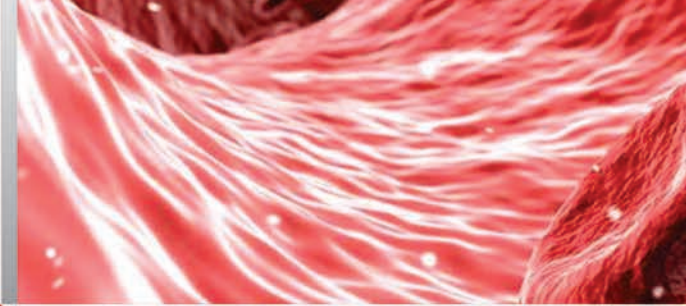




Section

I

Haematology

1. Blood Collection and Anticoagulants
2. Blood and Bone Marrow Smear Preparation and Staining Methods
3. Marrow Puncture Needle and Examination of Bone Marrow
4. Total Count of WBC, RBC and Platelets
5. Erythrocyte Sedimentation Rate (ESR) and Packed Cell Volume (PCV)
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Blood Collection and Anticoagulants

Blood is the most frequent body fluid used for analytical purpose. Blood is a mesenchymal tissue consisting of a liquid portion called plasma and particulate or formed elements (RBCs, WBCs, platelets) which are suspended in plasma.

Plasma

Blood plasma is a straw-coloured fluid component of blood which normally holds the blood cells in suspension. So, plasma can be called extracellular matrix of blood cells. It makes near about 55% of the total blood value. It is composed mostly of water (up to 95% by volume) and dissolved proteins (6–8%). These proteins are albumin (4.5 g%), globulins (2.5 g%) and fibrinogens (0.3 g%). Apart from proteins, glucose, clotting factors, electrolytes (Na^+ , Mg^{2+} , Ca^{2+} , Cl^- , HCO_3^- , etc.). Hormones, carbon dioxide and oxygen are present in the plasma.

Serum

When blood is collected in the test tube or vial without addition of anticoagulants, then blood is clotted. The clot is formed by using blood cells, clotting factor like fibrinogen. The fluid is separated from clot. So, this fluid contains proteins and other elements but lacks fibrinogen (used in the formation of clot).

So, serum is plasma minus fibrinogen.

BLOOD COLLECTION AND PROCESSING

Three general procedures for obtaining blood are (Fig. 1.1A and B):

1. Venipuncture for venous blood
2. Arterial puncture for arterial blood
3. Skin puncture for capillary (peripheral) blood

Venous blood is preferred for most haematological examinations.

Venipuncture or Venous Puncture

Venous blood is best withdrawn from an antecubital vein by means of a dry glass syringe or disposable plastic syringe. The steps are:

- i. Position the patient properly, depending on whether the patient is sitting or prone (ambulatory or non-ambulatory). This is to make sure for easy access to the antecubital fossa.
- ii. The patient is asked to make a fist, so that veins become more prominent and more palpable.
- iii. Select a suitable vein for venipuncture. For veins of the antecubital fossa, the median cubital and cephalic veins may be used alternatively. In case, the patient has an intravenous line, draw venous blood from other arm.
- iv. Cleanse the venipuncture site with 70% alcohol (isopropanol) or 1% iodine-

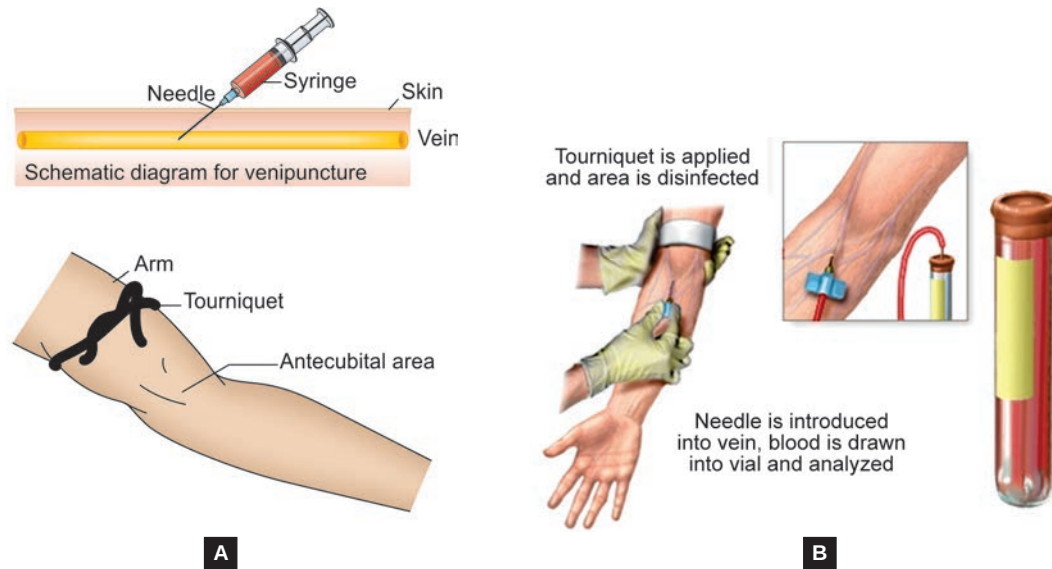


Fig. 1.1A and B: Blood collection procedure

saturated swab stick. Allow the area to dry.

- v. Apply a tourniquet few inches above the puncture site. But remember, do not keep the tourniquet for more than one minute.
- vi. Hold the vein firmly, both above and below the puncture site. For this, use either thumb and middle finger or thumb and index finger.
- vii. Perform the venipuncture. Enter the syringe needle (19 or 20 gauge) at approximately 15° angle to the arm. If using the evacuated system or vacutainer as soon as the needle is inserted in the vein, ease the collection tube forward in the holder as far as possible, firmly securing the needle holder in place.
- viii. Release the tourniquet when blood begins to flow.
- ix. After blood collection, place a clean cotton ball or gauze lightly over the site. Withdraw the needle, then apply pressure to site.
- x. Now apply an adhesive bandage strip over the cotton ball or gauze to stop bleeding or formation of haematoma.

Note

- i. The needles should not be too fine or too long, those of 19 or 20 SWG British standard, American standard 19 SWG = 18 (1.016 mm), 20 SWG = 19 (0.914 mm) are suitable.
- ii. If the veins are very small then 23 SWG (= 22 or 0.610) to be used to collect at least 2 ml venous blood.
- iii. If veins are selected from dorsum of the hand, it tends to bleed easily. So, care must be taken.
- iv. Beware of haemolysis of blood during collection. It can be avoided or minimized by using clean apparatus, withdrawing the blood slowly, not using too fine needle, delivering the blood slowly into the receiver and avoiding frothing during the withdrawal of the blood and subsequent mixing with the anticoagulant.

Arterial Puncture

Arterial blood is used rarely. It is used to measure oxygen and carbon dioxide tension, as well as pH (arterial blood gases or ABGs). These blood gas measurements are critical in the assessment of oxygenation problems encountered in patients with pneumonia, pneumonitis and pulmonary embolism. Also critically ill cardiovascular patients and

patients who are undergoing cardiac or pulmonary surgery are monitored for hypoxaemia.

Arterial puncture is technically more difficult than venous puncture. Increased pressure of the flowing blood, makes it more difficult to stop bleeding with the undesired development of a haematoma. The arteries selected for arterial punctures are radial, brachial and femoral arteries in order of choice. Unsuitable sites are oedematous, irritated, near a wound, or in an area of an arteriovenous (AV) shunt or fistula. Although venous blood yields adequate pH values if properly collected but venous blood yields incorrect values for arterial oxygen saturation and alveolar $p\text{CO}_2$.

Skin Puncture (Capillary Blood)

Skin puncture is the method of choice in paediatric patients especially infants. The large amount of blood collected from repeated venipuncture may cause anaemia (iatrogenic), especially in premature infants. Skin puncture is also preferred in geriatric patients because of thinness of skin and loss of skin elasticity which cause venipuncture difficult.

In the neonates and infants, the heel is often used for skin puncture. A deep heel prick is made at the distal edge of the calcaneal protuberance following 5–10 minutes prior exposure to prewarmed water. In the older paediatric population, and in geriatric patients, earlobes or fingers are preferred.

This capillary blood collected by skin puncture is good for making blood smear or for a single routine haematological test. A blood smear prepared from capillary blood without anticoagulant gives better information about blood cell morphology and differential count. But the total count is not very accurate. This is because of dilution by the tissue fluid and sometimes also due to lack of free flow of capillary blood. But if free flow capillary blood is received, then it is as satisfactory as venous blood.

Capillary blood obtained from heel puncture is not good for $p\text{CO}_2$ and $p\text{O}_2$ determination in the first day of life, probably owing to vasoconstriction and poor perfusion of the extremities. In infants with respiratory distress syndrome, heel blood deviates significantly from arterial blood in all parameters except standard bicarbonate and base excess.

The discrepancies between peripheral (capillary) and venous blood are more pronounced if earlobe than finger is chosen for skin puncture. However, if the ear is rubbed well with a piece of cotton or lint until ear is warm and pink, then a good spontaneous flow of blood can be obtained using sterile lancets as prickers. In this case, RBC count, leucocyte count and haemoglobin content almost close to venous blood.

Heel Puncture Method (Fig. 1.2)

A deep puncture in the heel is made after heel is really warm (5–10 minutes prior

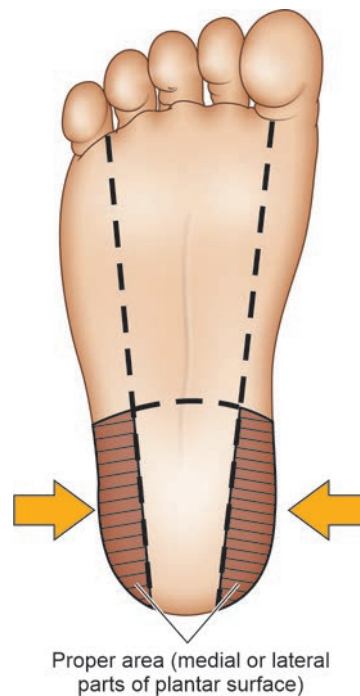


Fig. 1.2: Heel puncture method

exposure to prewarmed water) by using a steel lancet. Ideal sites are the medial or lateral parts of the plantar surface of the heel. Remember, the central plantar area and posterior curvature are not chosen in infants because of risk of injury to the underlying tarsal bones.

Earlobe Puncture Method

Rub the ear until it becomes warm and pink. Then with a sterile lancet prick the earlobe (as it has no bone or cartilage) to a depth of 2–3 mm by a single stab. Wipe and discard first few drops of blood. Collect the blood sample when it flows spontaneously (usually in about 30 seconds). Always use different lancet for different patients.

Finger Prick (Stick) Procedure (Fig. 1.3)

The best locations for finger sticks are the 3rd (middle) and 4th (ring) fingers of the non-dominated hand. Do not select tip of the finger or the centre of the finger. The second (index) finger tends to have thicker and calloused skin, so not preferred. The fifth finger (little finger) tends to have less soft tissue overlying the bone.

1. After selection of site of the finger, put on gloves and cleanse the puncture site with 70% alcohol (isopropanol).
2. Massage the finger toward the selected site prior to puncture.
3. Then with a sterile safety lancet make a skin puncture just off the finger pad.
4. Wipe away the first drop of blood which contains excess tissue fluid/plasma. Take subsequent blood drop into collection tube/device by gentle pressure on the finger; or put the blood drop onto a glass slide.
5. Cap, rotate and invert the collection device/tube.
6. Label it.

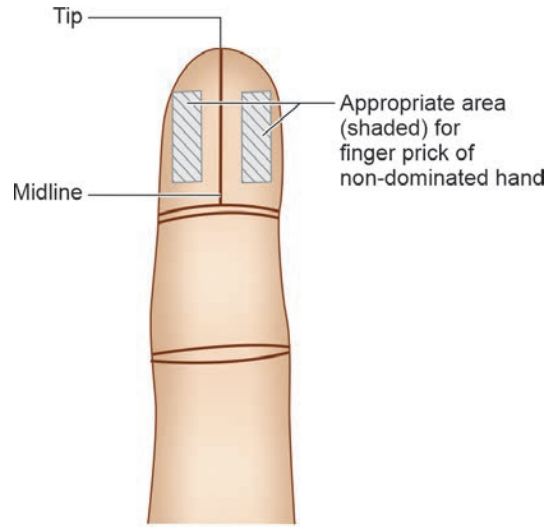


Fig. 1.3: Finger prick (stick) procedure

Differences between Venous and Peripheral (Capillary) Blood

- The platelet count is usually higher in venous than in peripheral blood (average 9% higher, but may go up to 32%). This is probably due to adhesion of platelets to the skin puncture site resulting in lower platelet count in peripheral blood.
- Venous blood and peripheral blood are not same, even though capillary blood is free-flowing which comes from arterioles after skin puncture.
- The RBC count, haemoglobin content and packed cell volume (PCV) are slightly higher in peripheral blood than venous blood. The total leucocyte count (TLC) and neutrophil counts are also higher about 8%, and monocyte count by 12% in peripheral blood. In children, it may be up to 100% higher, both for heel and earlobe punctured capillary blood.
- But the monocytes and neutrophils tend to accumulate in the earlobe if the blood is not free-flowing.

ANTICOAGULANTS

The anticoagulants prevent blood from clotting and as a result, plasma is formed (Table 1.1). Most anticoagulants bind with calcium ions (Ca^{2+}) and remove it by calcium chelating. Calcium is a factor in the coagulation cascade. As the calcium is chelated it cannot work. So, clot is not formed. Heparin, on the other hand, directly interferes in the coagulation process by destroying thrombin as well as thromboplastin.

Platelets + clotting factors + calcium → thrombin which converts fibrinogen → fibrin clot.

1. EDTA (Ethylenediaminetetra-acetic Acid)

It is also called sequestrene and probably the best anticoagulant for routine haematological investigations.

Mechanism of action: It is a powerful calcium chelating or binding agent and acts by binding the calcium in blood. So, active

Table 1.1: Some blood anticoagulants and their use

Name of anticoagulants (requirement per ml of blood)	Mechanism of action	Diagnostic use
1. No anticoagulant for serum in plain vial/tube	No anticoagulant; blood is clotted	Serum: Liver function test (protein, bilirubin, SGOT/AST, SGPT/ALT, alkaline phosphatase γ GT, etc.), lipid profile (cholesterol, triglyceride, HDL, LDL, VLDL), urea, creatinine, etc.
2. EDTA (1–1.5 mg/ml)	Binds Ca^{2+} and chelates it	Haemoglobin estimation, PCV, TLC, DLC, platelet count, parasite detection (microfilaria, malaria)
3. Trisodium citrate (3.2% aqueous solution; blood to anticoagulant ratio of 9:1 or 4:1 for coagulation studies and ESR respectively)	Binds Ca^{2+} and precipitates it as double salt (calcium sodium salt)	Coagulation studies, prothrombin time, ESR
4. Heparin (0.1–0.2 mg/ml)	Inhibits thrombin in the presence of antithrombin III. Also, it inhibits thromboplastin formation	• Osmotic fragility test • Plasma iron estimation • Demonstration of LE cells • Lymphocyte culture for karyotyping/genetic studies • Lymphoma/leukaemia panel for flow cytometry/immunophenotyping
5. Sodium or potassium oxalate (2 mg/ml)	Binds Ca^{2+} and chelates it	Blood urea and creatinine
6. Double oxalate (ammonium and potassium oxalate, 2 mg/ml)	Binds Ca^{2+} and chelates it	Hb, TLC, PCV, specific gravity
7. Sodium fluoride (6 mg powder/ml of blood)	• Blocks RBC enzymes for glycolytic inhibition of glucose • Also, chelates calcium	Blood glucose (sugar) estimation

calcium ions are not available for coagulation process.

Concentration of EDTA: A concentration of 1.2 mg of anhydrous salt per ml of blood is required. For dipotassium salt, a concentration of 1.5 ± 0.25 mg/ml of blood is recommended as per International Council for Standardization in Haematology (ICSH). The dipotassium salt is very soluble and is preferred over disodium salt which is less soluble.

Advantages of EDTA

- i. Very good anticoagulant for routine haematological investigations. EDTA has the advantage over oxalate anticoagulant because it prevents clumping of platelets *in vitro*. So, platelet count can also be performed on venous blood.
- ii. The dilithium salt of EDTA has the advantage that same blood sample can be used for chemical investigations apart from haematological investigations. But dilithium salt is less soluble compared to dipotassium salt and is less preferred.

Disadvantages of EDTA: Excess of EDTA (>2 mg/ml), irrespective of its salts, cause shrinkage and degenerative changes in RBCs and WBCs. Also, excess salt causes significant decrease in PCV and increase in MCHC (mean corpuscular haemoglobin concentration). Excess EDTA causes platelets to swell and then disintegrate, resulting in spuriously high platelet count as the swollen platelet fragments are large enough to be counted as normal platelets.

2. Trisodium Citrate

A 3.2% aqueous solution of trisodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) is the anticoagulant of choice for coagulation studies. It is also most widely used anticoagulant for ESR (erythrocyte sedimentation rate) also. But for coagulation studies (prothrombin time) 9:1 blood to anticoagulant is used, whereas in ESR determination 4:1 venous blood to anticoagulant is used.

Mechanism of action: Coagulation is prevented by precipitation of blood calcium in the form of a double salt (calcium sodium salt) which is very weakly dissociated.

3. Heparin

Heparin powder or liquid is used in a concentration of 10–20 IU (0.1–0.2 mg) per ml of blood.

Mechanism of action: It acts by inhibiting thromboplastin formation. Also it has antithrombin activity, i.e. inhibiting the action of thrombin on fibrinogen in the presence of plasma of co-factor antithrombin III.

Uses

- i. Osmotic fragility test
- ii. Chemical investigation like plasma iron estimation
- iii. Demonstration of LE cell in SLE patients
- iv. Lymphocyte culture for karyotyping/genetic study
- v. Lymphoma/leukaemia panel for flow cytometry or immunophenotyping
- vi. Nitro blue tetrazolium (NBT) test to assess phagocytic activity of phagocytes.

Advantages: It is an effective anticoagulant and does not alter the size of RBCs. It minimizes chances of haemolysis. Some consider heparin in the form of lithium salt is the ideal universal anticoagulant for blood.

Disadvantages

- i. It is expensive
- ii. It is inferior to EDTA as anticoagulant
- iii. Heparinized blood should not be used for making blood films as it gives a faint blue colouration to the background when blood smears are stained with Romanowsky stains. Also, it causes leucocytes to clump, so TLC and DLC will be erroneous.

4. Oxalate

Potassium, sodium and ammonium oxalates act as calcium chelating agent like EDTA and trisodium citrate. They interact with blood

calcium and form calcium oxalate after chelation.

- i. Potassium and sodium oxalate: Used mainly for chemical analysis. Concentration of anticoagulant 2 mg/ml of blood.
- ii. Double oxalate (Wintrobe's mixture): It is a mixture of two types of oxalates (ammonium oxalate and potassium oxalate in a ratio of 3:2). It is used in a concentration of 2 mg/ml of blood. Preparation of double oxalate mixture is as follows:
 - Ammonium oxalate: 1.2 g
 - Potassium oxalate: 0.8 g
 - Distilled water: 100 ml

This solution contains 20 mg of oxalates (both ammonium and potassium oxalate)/ml solution. So, 0.2 ml of this solution containing 4 mg of oxalates is sufficient for 2 ml of blood (up to 5 ml blood) as anticoagulants.

Uses

- i. Determination of haematocrit value or PCV.
- ii. Determination of haemoglobin and total leucocyte count (TLC)
- iii. Determination of specific gravity of whole blood or plasma.
- iv. Single powder in the form of sodium or potassium oxalate can be used for blood urea and creatinine estimation.

5. Sodium Fluoride

It is used in the concentration of 30 mg powder/5 ml of blood or 6 mg powder/ml of blood. **Mechanism of action:** (i) It chelates calcium and forms calcium fluoride, (ii) it prevents glycolysis by blocking acid phosphorylase enzymes in RBCs but increases amylase activity.

Uses: It is anticoagulant of choice for blood sugar estimation.

For glucose estimation, fluoride may be added to heparin also.

Note

Fluoride inhibits glycolysis of blood cells (RBCs) which may otherwise destroy glucose at the rate of about 5%/hour.

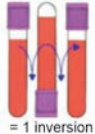
BLOOD COLLECTION TUBES/VIALS AND COLOUR CODE (Figs 1.4 and 1.5)

1. **Red or gold top clot tube:** Contains no anticoagulant. Blood will clot and serum will be formed.
2. **Purple top tube:** Contains EDTA anticoagulant.
3. **Blue top tube:** Contains 3.2% buffered sodium citrate anticoagulant for coagulation studies.
4. **Black top tube:** Contains 3.2% sodium citrate for ESR only.
5. **Light green top tube:** Contains lithium heparin anticoagulant.
6. **Dark green top tube:** Contains sodium heparin anticoagulant used for amino acid and cytogenetic studies.
7. **Gray top tube:** Contains glycolytic inhibitor or sodium fluoride for glucose estimation.
8. **Yellow tube:** Contains acid citrate dextrose (used in blood banking).

SOURCES OF BLOOD COLLECTION ERROR

1. Wrong patient identification or labelling error.
2. Haemoconcentration: Prolonged tourniquet time (>1 minute) restricts blood flow causing false high results, e.g. cell counts.
3. Haemodilution: If blood is collected from an arm with an IV (intravenous), the blood can be diluted and/or contaminated causing false low cell counts.
4. Haemolysis: Caused by traumatic blood drawing technique, vigorous shaking of the blood tube or forcing the blood

Draw blood culture bottles first, then proceed with blood tube order of draw (1–12) below:			
1.	Light blue top plastic tube PT, PTT, fibrinogen, fibrin D-dimer, other coagulation testing Note: Invert gently 3–4 times	7.	Bright green top (sodium heparin) plastic non-gel tube Mycobacteriology (AFB) blood culture, HLA-B27, chromosome studies
2.	Gold gel plastic tube Most chemistry tests and immunology tests, hepatitis tests, serologies (do not use for troponin, BNP)	8.	Lavender top plastic tube Haematology: CBC, platelet, sed, rate Chemistry: CD4, CD8, G6PD, haemoglobin A1C and haemoglobin variants
3.	Red top plastic tube For tests requiring serum Note: Contains clot activator	9.	White top plastic tube (PPT) Hepatitis and HIV viral loads, BNP
4.	Royal blue top plastic tube Copper, zinc, trace elements	10.	Pink top plastic tube for blood bank only
5.	Light green top (lithium heparin) gel plastic tube Troponin, metabolic panels, lipid, liver panels, ammonia (ice), HIV rapid antibody	11.	TAN top plastic tube Lead
6.	Dark green top (lithium heparin) plastic non-gel tube Ionized calcium (not part of blood gas), ammonia (ice)	12.	Gray top plastic tube Glucose, lactate (lactic acid) on ice
Important: Please follow the correct order of draw as numbered above and thoroughly mix all specimens (except light blue top) by inversion 8–10 times.			



✓ Properly align patient labels reduce lab errors!

Using the coloured sidebar as your guide, align the label lengthwise on the tube with patient's name at the top of tube learning the coloured sidebar exposed.

Colour coded sidebar

Patient's name at top end of tube

✗ Misaligned patient labels increase lab errors!



Fig. 1.4: Colour code of top of blood tube/vial and their significance



Fig. 1.5: Different blood collection tubes/vials with colour code

through syringe needle into the tube. Rupture of blood cells causes release of cell constituents like potassium, tissue factors and responsible for low RBC counts.

5. Use of wrong anticoagulant/tube: As for example, heparin causes platelet clumping. Hence, unsuitable for platelet counts.

6. Partially clotted blood draws improper mixing of anticoagulant containing tubes or blood obtained using poor blood drawing technique (e.g. too slow) may clot. Cells are trapped in the fibrin clot causing falsely low cell counts.
7. Insufficient fill: All tubes should have minimum draw amounts to maintain the proper anticoagulant concentration to blood volume. As for example, blue top tubes for coagulation test must be full.
8. Proper instruction for the said test not followed, e.g. certain tests have time limits for testing.

EFFECT OF STORAGE OF BLOOD

- When blood is kept in room temperature (18–25°C) for prolonged time, certain changes take place regardless of the anticoagulant use.

- This is obvious in EDTA blood (tripotassium salt > dipotassium salt).
- RBCs begin to swell; as a result, MCV increases, osmotic fragility and prothrombin time increase slowly and ESR decreases.
- The TLC and platelet count gradually fall. It is best to perform TLC and platelet count within 2 hours.
- The fall in leucocyte count is more if there is excessive amount of EDTA (>4.5 mg/ml).
- Reticulocyte count is unchanged for 24 hours at 4°C but at room temperature, it begins to fall within 6 hours.
- Nucleated RBCs (normoblasts) disappear from stored blood within 1–2 days at room temperature.
- Haemoglobin content is relatively stable for days unless it is infected.

For different investigations, blood is collected in tubes with or without anticoagulants. If there is anticoagulant, after mixing the blood cells of whole blood can be analyzed. Centrifugation of whole blood separates the cells from fluid plasma. In the bottom there will be RBCs. Above, there will be plasma containing fibrinogen. In between these two layers, there will be a **buffy coat** containing WBCs and platelets.

If there is no anticoagulant, fibrinogen will be used up to form fibrin strands which entrap blood cells. Centrifugation of this blood will separate the clot from the fluid serum. Serum lacks fibrinogen.

Serum and plasma can be obtained on standing also and they can be taken out by micropipette without disturbing fibrin clot or blood cells respectively. But, centrifugation will give better quality serum or plasma (Fig. 1.6).

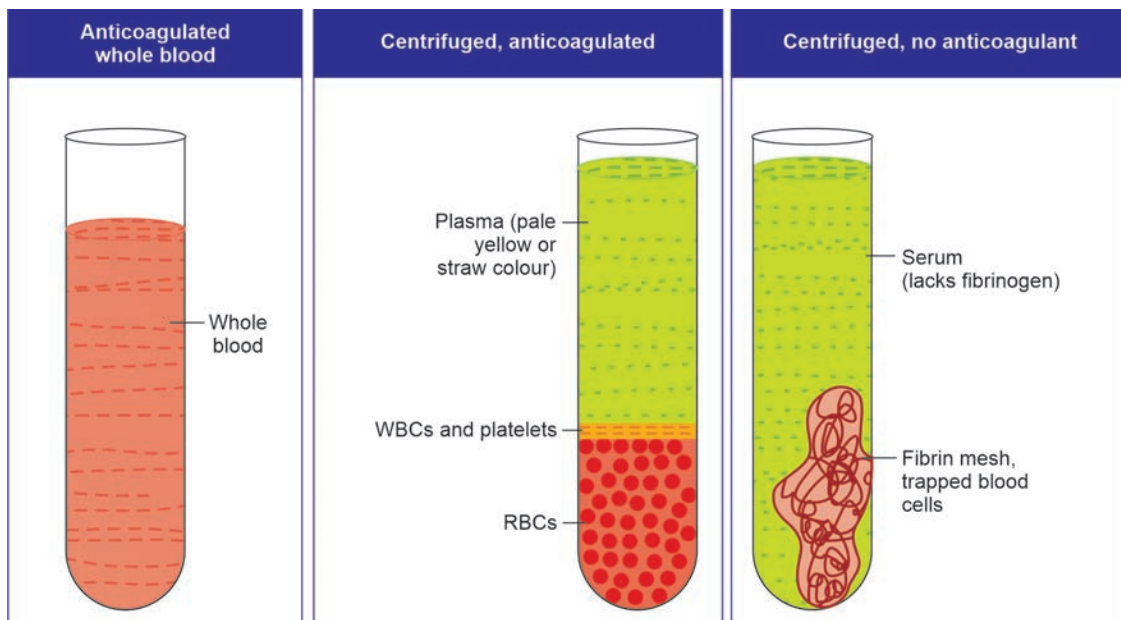


Fig. 1.6: Left tube contains anticoagulated whole blood, middle tube showing plasma and last tube showing serum



Q1. What are differences between serum and plasma?

Ans:

Serum	Plasma
1. Serum is formed as supernatant when blood undergoes clotting 2. Serum does not contain fibrinogen, prothrombin and other clotting factors like V, VII, VIII, IX, X, XI and XII which have been used in clotting. 3. It is used to estimate different biochemical parameters and serum enzymes like uric acid, electrolytes, urea, creatinine, SGOT, SGPT, alkaline phosphatase, etc. 4. Serum is clear fluid.	1. Plasma is obtained by centrifugation of anti-coagulant mixed blood. Anticoagulant mixed blood after standing may also give plasma but of poor quality compared to plasma after centrifugation. 2. It contains all the clotting factors including fibrinogen. But calcium ion is absent. 3. It is used mainly for coagulation studies like PT, APTT, TT, etc. Also used for detection of plasma glucose, plasma calcium, plasma ammonia, etc. 4. Plasma is yellowish or straw coloured.

Q2. What is the ideal gauge needle (bore size) for collection of venous blood?

Ans: If the needle is too large for the vein for which it is intended, it will tear the vein and cause bleeding (haematoma). If the needle, is too small, it will damage the blood cells especially RBCs. So, laboratory tests which require whole blood cells or haemoglobin or plasma will give inaccurate results.

The gauge refers to the inner measurement or opening of the needle. Usually needle gauge of 21G to 23G is preferred for venous blood collection. Small bore needles of 25G or less cannot be recommended and reserved only for problematic venous accesses and newborns. Usually 25G or lesser size may cause haemolysis and inaccurate results of electrolytes especially potassium. Nonetheless, 21G needles are most commonly used for routine tests.

Q3. Why is middle or ring finger preferred for capillary blood collection?

Ans: The best locations for finger sticks are the 3rd (middle) and 4th (ring) fingers of the

non-dominated hand. Do not select tip of the finger or the centre of the finger. The second (index) finger tends to have thicker and calloused skin, so not preferred. The fifth finger (little finger) tends to have less soft tissue overlying the bone. Ulnar side of the tip of ring finger is comparatively less innervated. So, needle prick is less painful to the patients.

Q4. Why too much pressure is not given during blood collection from finger prick?

Ans: Needle prick should be deep enough so that free flow blood comes out. Gentle pressure may be applied to start the blood. But too much pressure to finger tip should not be given as tissue fluid will come out which will dilute the blood. So, haematological values will be lowered.

Q5. Why double oxalate is preferred over single oxalate as an anticoagulant?

Ans: Ammonium salt (ammonium oxalate) causes swelling of RBCs while potassium salt (potassium oxalate) causes shrinkage of RBCs. Hence, mixture of these two salts or

double oxalate will cause neither RBC swelling nor RBC shrinkage. Normal shape and size of RBCs are maintained. Potassium oxalate: Ammonium oxalate = 2:3.

But oxalates are not preferred as anti-coagulant for Hb/TLC/platelet count as they induce morphologic alterations in WBCs and RBCs. So, smear morphology cannot be studied.

Q6. Why excess EDTA is bad as anti-coagulant?

Ans: Excess of EDTA (>2 mg/ml), irrespective of its salts, cause shrinkage and degenerative changes in RBCs and WBCs. Also, excess salts cause significant decrease in PCV and increase in MCHC (mean corpuscular haemoglobin concentration). Excess EDTA causes platelets to swell and then disintegrate, resulting in spuriously high

platelet count as the swollen platelet fragments are large enough to be counted as normal platelets.

Q7. What is a vacutainer?

Ans: Vacutainer: This is a blood collection tube which is sterile glass or plastic tube with a coloured rubber stopper creating a vacuum seal inside of the tube facilitating the drawing of a predetermined volume of blood/liquid. Vacutainer tubes may contain anticoagulant/additives to stabilize and preserve the blood/liquid specimen prior to analytical testing. Tubes containing gel can be easily handled and transported after centrifugation without the blood cells and serum mixing.

Vacutainer tubes were invented by Joseph Kleiner and Becton Dickinson in 1949.

Q8. What are different vacutainers used in haematology?

Anticoagulant/additives	Colour	Blood volume	Uses
1. Plain	Red	6 ml	Most biochemistry including drug levels, serological tests, which uses serum, cross-matching.
2. EDTA-K2	Purple/ Lavender	3 ml	Most haematological tests, HbA1C, molecular genetic tests using blood DNA, blood grouping, crossmatch.
3. Lithium heparin	Green	4 ml	Cytogenetic tests using blood DNA, osmotic fragility test, STAT biochemistry like electrolytes, renal screen, ammonia, etc. After blood is drawn inside by vacuum, it should be inverted gently at least 6 times to prevent clotting.
4. Sodium citrate	Blue	2.7 ml	Coagulation studies. This tube should be inverted at least 3–4 times.
5. Sodium fluoride/ potassium oxalate	Grey	6 ml	Glucose test. It should be inverted gently at least 6 times.
6. SST II, clot activator and serum gel separator, plain	Yellow	5 ml	All tests requiring serum except those few that need red cells as well

Blood and Bone Marrow Smear Preparation and Staining Methods

Examination of blood and bone marrow smear/film are important haematologic evaluation and to diagnose a haematologic disease. Blood or bone marrow smears should be prepared immediately as delay can cause spurious results.

Here, three methods of making blood smears/films are described

1. Wedge method
2. Spinner or spin method
3. Cover glass method

WEDGE METHOD

This is perhaps the most common method to prepare a blood film (Fig. 2.1A to D). Place a small drop of blood (2–3 mm in diameter) about 1–2 cm from the end of a clean, dust-free slide which is on flat surface. Then without any delay, a spreader (second slide) is held between thumb and forefinger of the right hand against the surface of the first slide at an angle of 30–45° and move it back to make contact with the blood drop to spread it. Then push the spreader slide at a moderate speed forward until all the blood has been spreaded over the first slide forming a moderately thin film. Ideally, the spreader slide should be clean, dry and slightly narrower than the first slide so that

blood does not cross the edge of the first slide and edges of blood film can be examined under the microscope.

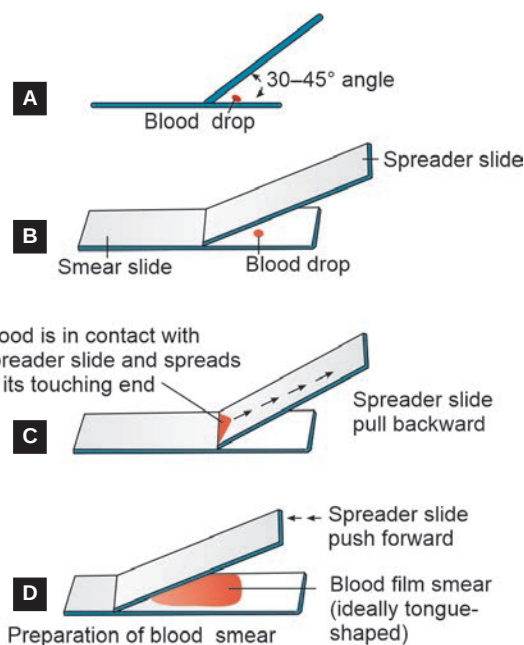


Fig. 2.1A to D: Preparation of blood smears. (A) Small blood drop from 1 to 2 cm from one end of glass slide; (B) Place the spreader slide at an angle of 30° to 45° over the smear slide; (C) Pull back the spreader slide so that it touches the blood drop and spreads throughout the edge of the spreader slide; (D) Push forward to make blood/smear

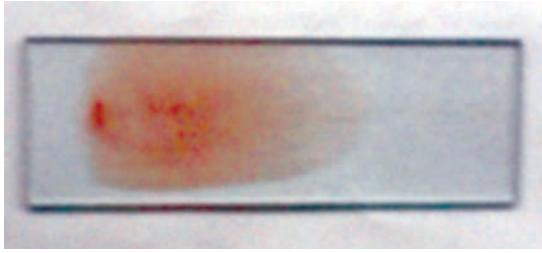


Fig. 2.2: Ideal thin blood smear

Note

- The blood drop should be such that it can produce the blood film 3–4 cm in length.
- The ideal thickness of blood film should be such that there will be some overlap of RBC (red blood cells) throughout much of the blood film's length. But the RBCs are separated at the tail end of the film.
- The film should not cover the entire surface of glass slide.
- Ideally, there will be a thick portion and a thin portion in a good film and there will be gradual transition from one to the other (Fig. 2.2).
- The blood film should have an even, smooth appearance and should be free ridges, waves or holes.
- The edge of spreader slide must be very smooth. Roughed edges will produce ragged tails containing many leucocytes (WBCs).
- The thickness of blood film can be adjusted by changing the speed of spreading or by changing the angle of the spreader slide or by using a larger or smaller blood drop (Figs 2.3 and 2.4).
- At a given angle, increasing the speed of spreader slide will cause increase in the thickness of the film.
- At a given speed, increasing the angle of spreader slide will also cause increase in the thickness of the film.
- The faster the blood film is air dried, the better the spreading of the individual cells on glass slide. Slow drying of film (as in humid weather) may cause contraction artifacts of the cells.
- There may be disproportionate monocytes at the tip of the feather (tail) edge or neutrophils just in from the feather edge and both at the lateral edges of the film.

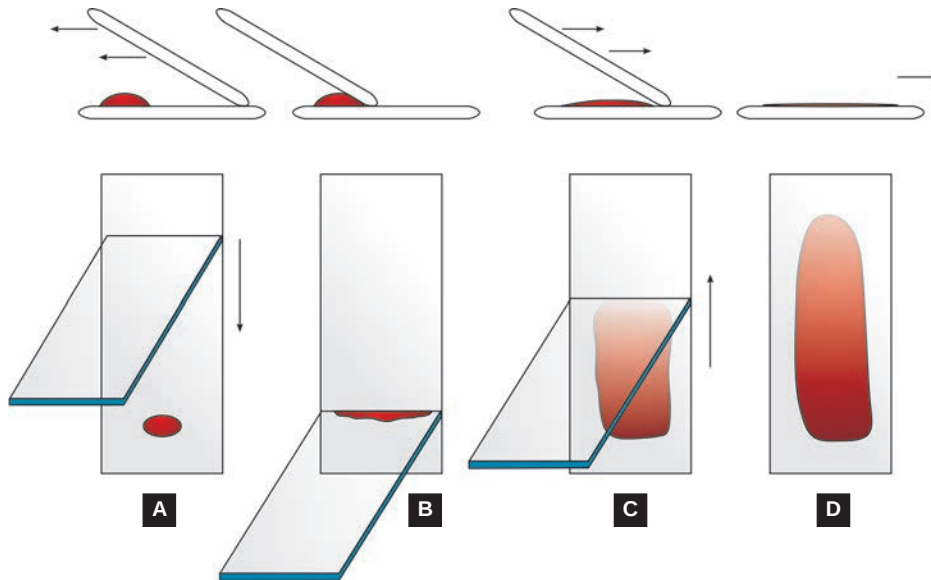


Fig. 2.3A to D: Upper row showing side view of different steps of making a thin blood film. Lower row showing front view of different steps of making a thin blood film

Thin film

- Good preparation—feathered end of the film should be centrally located on the slide with free margins on both sides, when properly prepared, it will be only one cell layer thick at this end.

- Badly prepared smears can cause presence of streaks—as a result of chipped spreader.

- Holes in the film indicate faulty preparation and dirty or greasy slides, respectively.

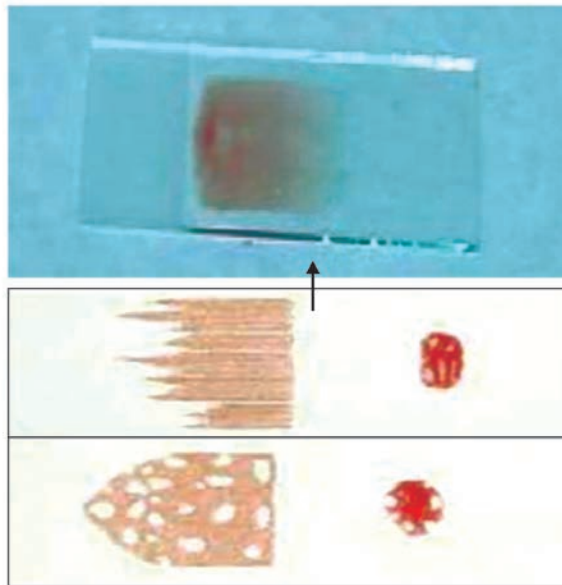


Fig. 2.4: Good and badly prepared thin blood film

SPINNER OR SPIN METHOD

Spinner or spin method is an automated method in which 1–2 drops of blood drops are placed in the centre of a glass slide. Then it is spun at a high speed in a spherical centrifuge (e.g. cytospin) for a short period. The blood drops are spreaded on the glass slide in a monolayer. With this method leucocytes and platelets are distributed uniformly without any distortion.

Note

1. The RBCs may be distorted. To overcome this problem, mix one volume of 9 g/L NaCl (sodium chloride) to 2 volumes of blood for diluting the blood and then put the diluted blood on the glass slide.
2. White blood cells (WBCs) can be easily examined on any spot in the film made by spinner method.
3. Unlike wedge method, it does not produce disproportionate monocytes and/or neutrophils at the tail or lateral edges.

COVER GLASS METHOD

For this, 22 mm square (number one or one and a half cover glasses) are recommended. Touch a cover glass to the top of a small blood drop without touching the skin. Now place it, blood side down crosswise onto another cover glass, so that the corners appear as an eight point star. The small blood drop will spread out quickly and evenly in a thin layer between the two surfaces. Prepare blood film by pulling the cover glasses quickly and firmly apart on a plane parallel to their surfaces. After that, cover glasses are placed on clean paper (film side up) and are air dried.

Blood film from venous blood may be prepared likewise by placing a blood drop on a coverslip and follow the above mentioned steps.

THICK SMEAR AND ITS PREPARATION

While the blood film mentioned above, is suitable for studies of cellular morphology, sometimes thick smears are prepared to detect microfilariae and malarial parasites (Figs 2.5 to 2.7). Thick smears are very useful

when parasites (malaria and microfilaria) are scanty but identification of the parasites is less than thin films. Mixed infection (both *Plasmodium vivax* and *falciparum*) may also be missed. Thick smear is also useful when there is severe leucopenia. It helps to perform differential count or at least the proportion of polymorphonuclear to mononuclear cells.

Preparation of Thick Film

A drop of blood is placed in the centre of a glass slide and is spread out with a corner of another slide to cover an area about four times its original area. The film may be air-dried or dried at 37°C for 30 minutes in an incubator. If the film is satisfactory, then printed matter (small print of newspaper) is just visible.

Alternatively, four small blood drops may be taken in the mid-portion of a glass slide. They are joined together to form a blood film (square shaped) in the mid-portion of the slide.

Sinton proposed to make thin and thick smears onto same glass slide. For this, a large blood drop is taken near one end to make thick smear and one small blood drop is taken in the centre of the slide. Thick smear is

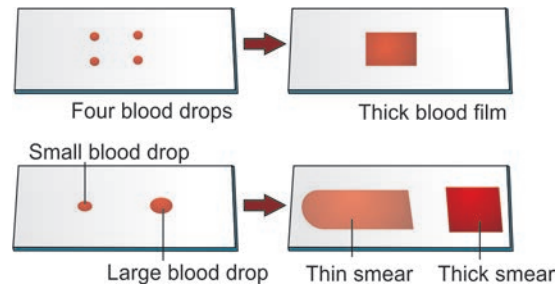


Fig. 2.5: Preparation of thick and thin blood films in the upper panel. Preparation of both thin and thick blood films on the same slide.

prepared from the large blood drop (square shaped) while thin smear is prepared from the small blood drop (tongue shaped) in the same manner as previously described.

Fixation of Blood Films

Blood films need to be fixed before staining to prevent haemolysis when they come in contact with water during water-based (aqueous) stains or water is poured during staining. For this, blood films are coated with acetone-free methyl alcohol for 1–2 minutes. This alcohol (methyl alcohol) denatures the proteins present in the blood and hardens

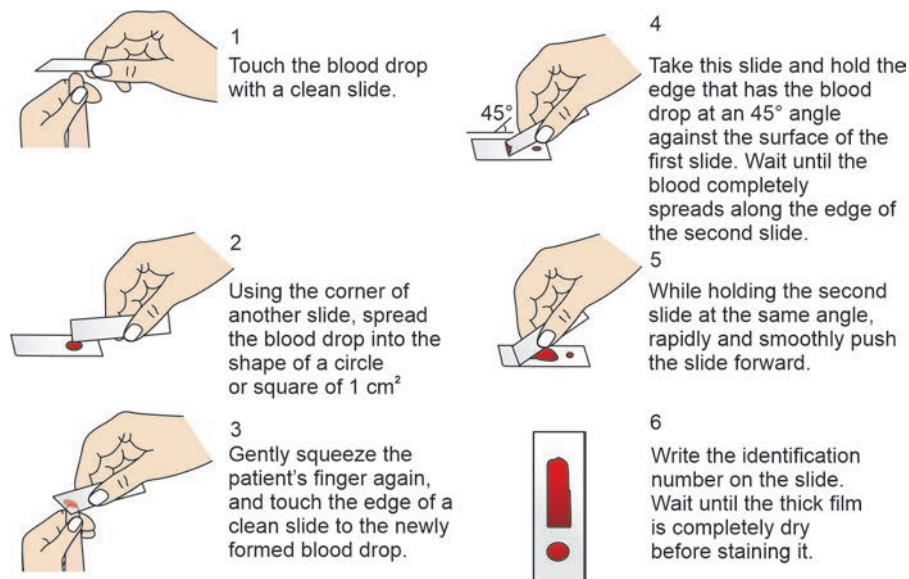


Fig. 2.6: Preparation of a thin and a thick blood film on the same slide

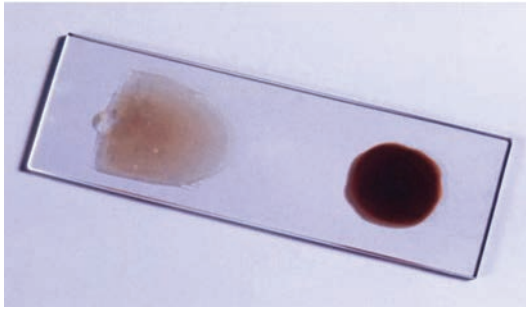


Fig. 2.7: Thin and thick blood films on the same slide. Thin blood film is typically tongue-shaped while thick blood film is circular or square-shaped

the blood cells. As Leishman's stain and Wright's stain contain acetone-free methyl alcohol in the staining solution, the blood films do not require prefixation with alcohol. But Giemsa staining needs prefixation with alcohol as the ready to use staining solution contains only 5% alcohol (suboptimal for fixation).

BONE MARROW ASPIRATE FOR EXAMINATION

1. **Bone marrow films:** Put one drop of aspirate onto slides about 1 cm from one end. Then quickly suck off most of blood present in the aspirate with the help of a fine Pasteur pipette applied to the edge of each drop. Alternatively, keep the glass slides on a slope (to tilt them) for draining away of the blood.

While the blood is removed, the irregularly shaped marrow fragments adhere to the slide. After that make bone marrow films which will be 3–5 cm in length by using a smooth edged glass spreader of less than 2 cm width. The marrow fragments are dragged behind the spreader and place the marrow cells trailing behind the spreader (trail of cells). The differential count should be made in these cellular trails, starting from the marrow fragment and working back towards the head of the film.

Fix the bone marrow films and stain them with Romanowsky dyes as for peripheral blood films. But for high quality, a longer fixation time is needed (>20 minutes in methanol).

Some advocate to add the aspirated marrow material to an anticoagulant like EDTA in a tube and to prepare a marrow films on returning to the laboratory. But there may be a possibility of using excess anticoagulant (only 0.2–0.3 ml of marrow aspirate compared to 2–5 ml of blood). The stained marrow film may show pink-staining amorphous material and some of the erythroblasts and reticulocytes may clump together due to excess anticoagulants.

2. **Bone marrow imprints:** Bone marrow fragments/particles of imprints may also be used for preparation of imprints. One or more visible particles are picked up with a capillary pipette, a toothpick or the broken end of a wooden applicator. The bone marrow particle(s) are transferred immediately to a slide and made to adhere to it by a gentle smearing motion. The slide is air dried rapidly by waving it and then is stained.
3. **Crush preparations:** A small drop aspirate containing a slide near one end. Another slide is placed over the first slide. Slight pressure is given to crush the bone marrow and the slides are separated by pulling them apart in a direction parallel to their surfaces.

All bone marrow films should be dried quickly by moving them in the air (air dried) or by exposing them to a fan.

As the bone marrow aspirate is being spreaded, the fat appears as irregular holes and make it sure that the marrow, material not only the blood has been aspirated.

POOR BLOOD SMEARS AND ITS COMMON CAUSES

1. The glass slides should be very clean as dirty slides do not give an even smear.

2. Put an appropriate size of blood drop onto glass slide and make the smear immediately. Delay will cause uneven distribution of WBCs.
3. The spreader slide should be moved steadily and confidently. Jerky movement or loss of contact between spreader slide and smear slide will give poor smears.
4. Angle between the spreader slide and smear slide should be 30° to 45°. Increasing the angle may result in a thick smear, whereas decreasing the result in a thin smear.

FIXING AND STAINING OF BLOOD SMEAR

The smears should be stained immediately after the preparation. Methanol (acetone-free) present in the common Romanowsky stains fix the smear slides in the staining procedure. If staining is delayed then smears must be fixed with methanol for 2–3 minutes. Fixation of smears will prevent distortion of blood cells and smears can be stored for future staining.

In the blood cells, some structural components are acidic while others are basic. Acidic substances stain with basic stain like methylene blue, azure B, etc. and are called basophilic. Examples of basophilic substances are nuclei and nucleic acids. Some basic structures like haemoglobin are stained with acid stains like eosin and are called acidophilic or eosinophilic. Other structures stained by combination of the two are called neutrophilic.

Stains which are composed of both acid and basic dyes are known as “Romanowsky” stains. These stains have the ability to make subtle distinctions during staining of cell and can stain the granules differentially. Neutrophilic granules are weakly stained by azure complexes, whereas eosinophilic granules get stained by acidic component of the dye and basophilic granules which contain acid heparin are stained by basic component of the dye.

The thiazine’s basic component consists of methylene blue (tetramethyl thionine) and in

varying proportions, its analogues produced by oxidative demethylation: Azure B (trimethyl thionine); azure A (asymmetric dimethylthionine), azure C (monomethyl thionine) and symmetric dimethyl thionine.

As already said most Romanowsky stains are dissolved in methyl alcohol and combine fixation with staining. Various modifications of the original Romanowsky combination of methylene blue (basic stain) and eosin (acid stain) are now used. Usually combination of azure B and eosin Y is used as Romanowsky stain. Common Romanowsky stains are:

1. Leishman’s stain
2. Wright’s stain
3. Giemsa stain
4. May-Grünwald-Giemsa (MGG) stain
5. Field’s stain
6. Jenner’s stain
7. MacNeal stain.

Leishman’s stain is mostly used in the routine staining of blood film though Wright’s stain and Giemsa stain are also very popular (Table 2.1). Giemsa stain is ideal for staining and detecting malarial parasites and other protozoa. Field’s stain is used for staining thick film to detect malarial parasites and it offers rapid staining and screening of blood smears. MGG stains are used not only for blood/bone marrow films but also for cytology/FNAC smears.

Leishman’s Stain

Reagents

1. Leishman powder (eosin-methylene blue powder): 0.15 g
2. Methyl alcohol (acetone-free): 100 ml

The Leishman powder is placed in a conical flask to which methyl alcohol is added. Then the mixture is warmed to 50°C for 10–15 minutes. It is then filtered. The dye is ripened by keeping the filtrate in sunlight for 3–4 days or in an incubator at 37°C for 7 days.

Method: Dry the film in the air and flood the slide with the stain. After 2 minutes, add double the volume of water and stain the

Table 2.1: Common causes of faulty staining and their corrections

Faulty staining pattern	Causes	Corrections
1. Excessive blue stain	Thick films, prolonged staining time, inadequate washing or too high alkaline pH of stain or diluent.	Staining for less time or using less stain and more diluent. The pH of the buffer should be lowered.
2. Excessive pink stain	Insufficient staining, prolonged washing time, mounting the coverslips before they are dry, too high acidity (very low pH) of the diluents buffer or stain.	Staining time or washing time as advocated pH of buffer and stain should be adjusted.
3. Precipitates on the film	Drying during period of staining, inadequate washing of slide after staining, inadequate filtration of the stain, dust particles on smear or slide and use of unclean slides.	Act as per the cause

film for 7–10 minutes. Then wash the smear in a stream of buffered water until it has acquired a pinkish tinge (up to 2 minutes). After the back of the slide has been wiped clean, set it up right to dry.

Wright's Stain

Reagents

1. Wright's stain powder: 0.2 g
 2. Methyl alcohol (acetone-free): 100 ml
- The solution is kept at 37°C for a few days before use.

Method: Almost same as in Leishman's staining. When the stain is ripe, a scum of film is formed over the surface of the stain.

Giemsa Stain

Reagents

- Giemsa powder: 0.6 g
- Glycerol: 50 ml
- Acetone-free methyl alcohol: 50 ml

Giemsa powder (0.6 g) is placed in a conical flask. Then 50 ml of glycerol is added. This mixture is warmed at 50°C for 15 minutes with occasional shaking and then 50 ml methanol is added to the mixture. It is now filtered and filtrate is ready for use. But

before use the stain should be diluted 1:10 (1 part stain + 9 parts distilled water).

Method: Unlike Leishman or Wright stain, here the blood films should be fixed with methyl alcohol (acetone-free) separately for 3–5 minutes and then dried. Because after 1:10 dilution acetone-free methyl alcohol becomes 5% only (from 50% in original), which is suboptimal for fixation of blood cells. Diluted Giemsa stain (1:10) is poured on the fixed smear and kept for 20–30 minutes. Wash the smear with neutral/distilled water and dry.

May-Grünwald-Giemsa Stain

Reagents

1. May-Grünwald powder: 0.3 g
2. Acetone-free methyl alcohol: 100 ml

Dissolve the 0.3 g powder (dye) in 100 ml methyl alcohol and warm it at 50°C for 10 minutes. During warming shake it from time to time, filter after 24 hours.

Method: Fixed the smear in methyl alcohol for 3–5 minutes. Then stain the film with diluted (1:10) May-Grünwald stain for 5 minutes. Then stain the film with diluted (1:10) Giemsa stain for 15–20 minutes. Wash with buffered water and dry in the air.

Field's Stain

Reagents

1. Stain A (polychromed methylene blue)

- Methylene blue: 0.26 g
- Azure B (optional): 0.1 g
- Disodium hydrogen phosphate: 2.5 g
- Potassium dihydrogen phosphate: 1.25 g
- Water: 100 ml

Dissolve the phosphates in warm freshly boiled water. Then mix the azure B with phosphate solution and dissolve it well. Lastly the dyes (methylene blue) are added and mix well. Filter it.

2. Stain B (eosin)

- Eosin Y (yellow eosin, water soluble): 0.26 g
- Disodium hydrogen phosphate: 2.5 g
- Potassium dihydrogen phosphate: 1.25 g
- Water: 100 ml

Dissolve the phosphates in warm freshly boiled water. Then mix the dye (eosin Y) with phosphate solution and dissolve it well, filter it.

Staining Method

- Fix the film for 10–15 seconds in methanol.
- Pour off the methanol and put 12 drops of diluted stain B (1:4 dilution in water)
- Immediately add 12 drops of stain A.
- Agitate the slides to mix the stains.
- After 1 minute, rinse the slide in water.

Note

- A pH to the alkaline side of neutrality accentuates the azure component of Romanowsky stain at the expenses of the eosin and vice versa.
- A pH of 6.8 is usually recommended for general or routine use.
- To look, malarial parasites a pH of 7.2 is recommended in order to detect Schuffner's dots of *Plasmodium vivax*, *ovale* and *malariae*. (Remember Maurer's dots in *P. falciparum* and Ziemann's dots in *P. malariae*).

- Differentiate the slide in phosphate buffer for 5–10 seconds at pH 6.6.
- Wash the slide in water.
- Place it on end to drain and then dry.

Chemical Theory of Romanowsky Staining

The mechanism by which certain components or structure of a cell stain with particular dye, depends on complex differences between the different dyes. As for example, azure B in dimer form is bound to anionic molecules, e.g. phosphate groups of DNA, whereas eosin Y is bound as a monomer to cationic sites of proteins.

As early as the dyes are bound to particular structure of the cell, either electron interaction occurs with dye-dye aggregation or the eosin Y molecule is inserted between the azure B molecules and the complex is held together by charge effect.

So, the acidic groupings of the nucleic acids and proteins of the cell nuclei and primitive cytoplasm determine their uptake of the basic dyes (like azure B). On the other hand, the presence of basic groupings on the haemoglobin molecules determines its affinity for acidic dyes and its staining with eosin (acid dye).

Examination of Romanowsky Stained Blood Smear

At first, examine the stained blood smear under low power for screening. Note the background colour and distribution of WBCs. In an ideal stained smear, three zones can be identified visually. The starting area or the "head" of the smear (where blood drop was originally placed), following which is the "body" and the thin end of the smear known as "tail" (Fig. 2.8).

At the tail end, RBCs lie singly and the neutrophils and monocytes predominate. In the body, RBCs overlap each other to a certain extent and lymphocytes predominate. The ideal area is in-between these "body" and "tail" of the smear where blood

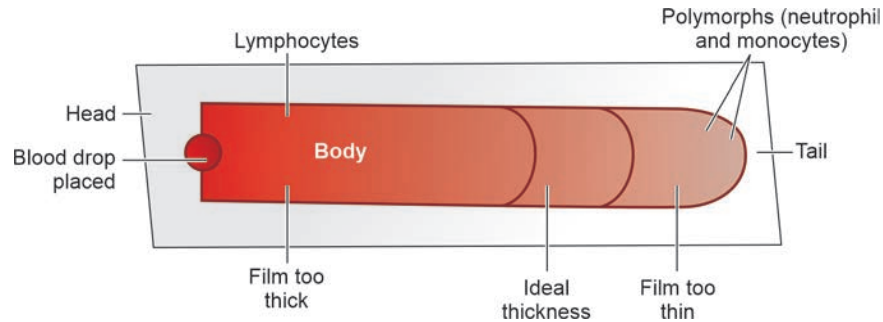


Fig. 2.8: Three zones in stained smears. The head, body and tail

cells are uniformly distributed. Here, the RBCs do not overlap and touch each other slightly.

For differential count of WBCs (Figs 2.9 and 2.10), two methods can be adopted:

1. The original drop of blood spreaded out between spreader and slide (C-C₁). The film is made in such a way that representative strips of film like A-A₁ and B-B₁ are formed from point of application A and B respectively. In order to make an accurate differential count, all leucocytes in one or more strips (like A-A₁, B-B₁, etc.) should be inspected and classified.

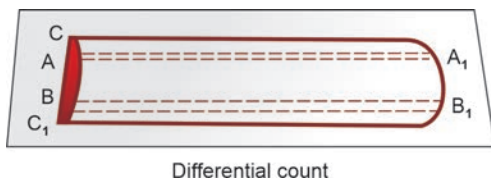


Fig. 2.9: Linear or straight line method of differential count of WBC

2. Choose the ideal thickness of the stained smear. Then inspect and classify all the leucocytes in a serpentine counting pattern (shown diagrammatically) (Fig. 2.10).

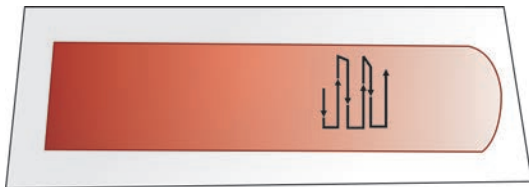


Fig. 2.10: Serpentine counting pattern (area is in between body and tail end of smear)

EXAMINATION OF STAINED BLOOD FILMS

Erythrocytes

The erythrocytes when not crowded together, appear as circular, homogenous discs of nearly uniform size, ranging from 6–8.5 mm in diameter. As for haemoglobinization, normally a small area of central pallor is seen (central 1/3rd) in RBCs.

Colour

1. **Normochromia:** Normal RBC appears pinkish brown due to presence of haemoglobin. Peripheral part looks deep brown while the central part (1/3rd) is pale because of biconcave shape of RBC.
2. **Hypochromia:** When RBC contains less haemoglobin, the central pale area becomes larger and paler. The MCHCs are also decreased.

Example: Iron deficiency anaemia.

3. **Hyperchromia:** The RBCs become thicker and larger and they stain deeply and less central pallor because of increased haemoglobin content (MCH), but the haemoglobin concentration (MCHC) is normal.

Example: Megaloblastic anaemia.

4. **Polychromasia:** Theoretically means many colours but practically RBCs appear bluish grey. This is due to presence of residual RNA in RBC (normally absent in mature RBC). So, young red cell shows polychromasia and larger than mature red cell and may lack central pallor. These

young red cells are called reticulocytes. It is most marked in **haemolysis and blood loss**.

4. **Anisochromia:** It means unequal haemoglobin content due to different populations of RBCs. Hence, different staining patterns of individual RBC. Example: **Iron deficiency anaemia treated with blood transfusion**.

Size

1. Normocytes: **Normal RBC** (6–8 μm in diameter; average 7–7.5 μm)
2. Microcytes: Decrease in size of RBC which may result from fragmentation of normally sized red cells (normocytes) or larger red cells (macrocytes). It occurs in many types of abnormal erythropoiesis, e.g. **iron deficiency anaemia and thalassaemia**.
3. Macrocytes: They are large RBCs having a diameter more than 8 micro mm, a MCV (mean corpuscular volume) more than 95 fl and higher than normal Hb concentration (MCHC). Example: **Megaloblastic anaemia, chronic liver diseases**.
4. Anisocytosis: This is a general term which describes any variation in size of RBC. Example: **Anaemias, thalassaemias**.

Shape

- i. **Poikilocytosis:** This is a general term which describes any variation in the shape of RBC.
- ii. **Spherocytes:** They are nearly spherical RBC in contrast to normal biconcave disc. Their diameter is smaller than normal and thickness is greater than normal. Tiny bits of membrane (in excess of Hb) are removed from adult RBC resulting the cell with a decreased surface/volume ratio. Example: Hereditary spherocytosis and in some cases of autoimmune haemolytic anaemia.
- iii. **Target cells (leptocytes):** These refers to leptocytes (unusually thin red cells), and when stained show a peripheral ring of

Hb with a dark, central, Hb containing area. Example: Haemoglobinopathies like thalassaemia, chronic liver disease, following splenectomy, HbC disease.

- iv. **Schistocytes (cell fragment):** It indicates the presence of haemolysis as seen in severe burn, megaloblastic anaemia or in microangiopathic haemolytic anaemia.
- v. **Acanthocytes:** These are irregularly spiculated RBCs in which ends of spicules are bulbous and rounded. Examples: Abetalipoproteinaemia, certain liver diseases.
- vi. **Burr cells (echinocytes):** These are small cells or cell fragments bearing one or few spines or spicules with regular distribution (unlike acanthocytes where spicules are seen all over RBC surface). Examples: Microangiopathic haemolytic anaemia, severe burns.

Structure or Content

- i. **Basophilic stippling (punctate basophilia):** It is characterized by presence within erythrocytes of irregular basophilic granules which vary from fine to coarse. Fine stippling is seen when there is increased red cell production and therefore increased polychromatophilia. Coarse basophilic stippling may be seen in lead poisoning, megaloblastic anaemia or pyrimidine-5-nucleotidase deficiency. This is attributed to an abnormal instability of the RNA in the young red cell.
- ii. **Pappenheimer bodies:** These are abnormal granules of iron found inside RBCs and stained by Wright stain and/or Giemsa stain. These bodies are a type of inclusion body formed by phagosomes that have engulfed excessive amounts of iron. They appear as dense, blue-purple granules within RBCs and are usually only one or two, located in the cell periphery. Examples: Sideroblastic anaemia, haemolytic anaemia, and sickle cell disease.

- iii. **Cabot rings:** These are ring shaped, figure of eight or loop-shaped structures. These rings are probably microtubules remaining from a mitotic spindle (due to defective erythropoiesis). Examples: Pernicious anaemia, lead poisoning
- iv. **Howell-Jolly bodies:** These are smooth, round remnants of nuclear chromatin. Single Howell-Jolly bodies may be seen in haemolytic anaemia, megaloblastic anaemia and after splenectomy. Multiple Howell-Jolly bodies in a single RBC usually indicate megaloblastic anaemia or defective erythropoiesis.
- v. **Rouleaux formation:** Rouleaux formation is the alignment of RBCs on one another so that they resemble stack of coins. Examples: Multiple myeloma, other paraproteinaemia (monoclonal gammopathy) and macroglobulinaemia.

White Blood Cells (WBCs)

Differential leucocyte count (DLC) (Fig. 2.11): The DLC is done on the basis of size, cytoplasm with or without granules and type of nucleus of WBCs. The WBC may be divided into:

- **Granulocytes** (WBC with cytoplasmic granules): Neutrophils, eosinophils and basophils.
- **Agranulocytes** (WBC without granules): Lymphocytes and monocytes.



Fig. 2.11: DLC counter (manual) for differential leucocyte count (DLC)

Total leucocyte count (TLC): Normal total leucocyte count in adult person is 4000–11000/mm³. Leukocytosis refers to TLC

more than 11000/mm³. Leucopenia less than 4000/mm³.

Neutrophils (Polymorphs) (Fig. 2.12)

This leucocyte averages 12 mm in diameter, they are smaller than monocytes and eosinophils and slightly larger than basophils. **Segmented neutrophil** has at least two of its lobes separated by a filament. **Band neutrophil** has either a U-shaped nucleus of uniform thickness or a strand of nuclear material thicker than a filament connecting the lobes (appearance of “telephonic receiver”).

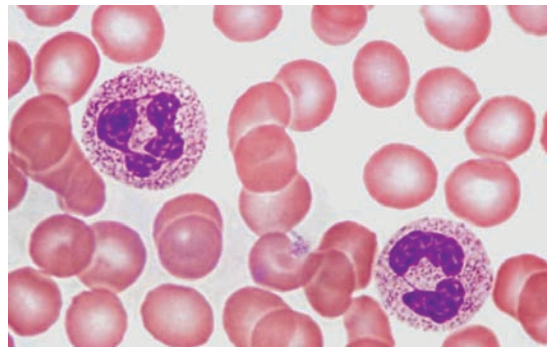


Fig. 2.12: Neutrophils

- The cytoplasm is filled up with tiny granules (0.2–0.3 mm) which stain tan to pink or orange with Romanowsky stains.
- Normal segmented neutrophils: 56% of leucocytes (DLC).
- Normal band neutrophils: 03% of leucocytes (up to 8% may be seen).
- Two lobes neutrophils: 10–30% of neutrophils.
- Three lobes neutrophils: 40–50% of neutrophils.
- Four lobes neutrophils: 10–20% of neutrophils.
- Five lobes neutrophils: ≤5% of neutrophils.
- In women, 2–3% of circulating neutrophils show an appendage at a terminal nuclear segment. This ‘drumstick’ is connected to the nucleus by a short stalk

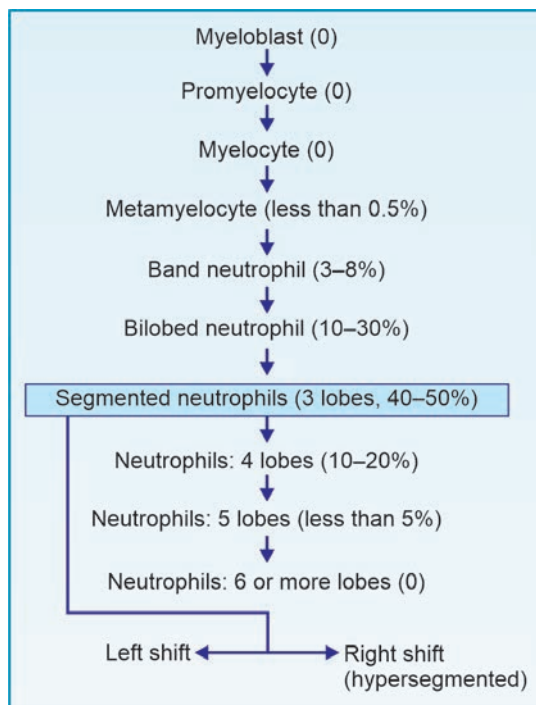
and is about 1.5 mm in diameter. It indicates the inactive X chromosome and corresponds to **Barr body**.

Hypersegmented neutrophils (Fig. 2.13): If the peripheral smear shows $\geq 5\%$ of neutrophils having 5 lobes or $\geq 1\%$ of neutrophils having 6 lobes, then the neutrophils are called hypersegmented.

Causes

1. Megaloblastic anaemia
2. Uraemia
3. Hydroxyurea treatment
4. Cytotoxic treatment especially with methotrexate treatment.

Stages of neutrophilic maturation



Segmentation of the nucleus of the neutrophil is a normal separation process. With the three-lobed neutrophil as a marker, shift to the left (less mature) or to the right (hypersegmented) can be understood. A left

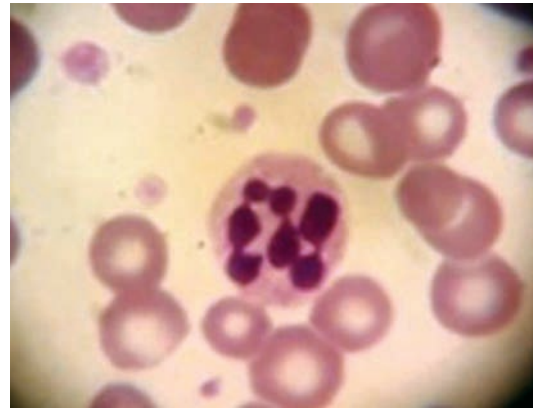


Fig. 2.13: Hypersegmented neutrophils (7 lobes) in megaloblastic anaemia

shift with band neutrophils, metamyelocytes and occasional myelocytes is common in sepsis and usually neutrophils contain toxic granules in cytoplasm. If myeloblast and promyelocytes are seen in peripheral blood, the causes may be leukaemia or leukoerythroblastic anaemia. Sometimes in pregnancy, a significant number of band forms is seen.

Arneth count: Neutrophils are divided in five groups according to number of lobe(s), it possesses:

1. Group I: One lobe
2. Group II: Two Lobes
3. Group III: Three lobes
4. Group IV: Four lobes
5. Group V: Five lobes.

One hundred neutrophils are counted in peripheral smear and number of each group of neutrophil is expressed as a percentage.

If there is increase in the group I and II neutrophils (as seen in sepsis) then there is a shift to left. Whereas, if there is more hypersegmented neutrophils (as seen in megaloblastic anaemia), then there is shift to right.

Arneth index: Percentage of neutrophils in groups I, II and $\frac{1}{2}$ of group III is about 60 (normal range 51–65).

Schilling count: In this count, all the granular leucocytes are divided into four groups and the number of each group is expressed as a percentage of the WBCs. The four groups are:

1. Myelocytes
2. Metamyelocytes
3. Band neutrophils
4. Segmented neutrophils

A dividing line is drawn (usually segmented neutrophils' number). A shift to left happens when the number in percent increases, to the left of the dividing line.

Morphologic alterations in neutrophils

Toxic granules: These are dark blue to purple cytoplasmic granules seen in neutrophils (also in metamyelocytes and band forms). Toxic granules are seen in severe bacterial infections and in other causes of inflammation or toxic conditions. These are myeloperoxidase positive and may be numerous or few in number. Toxic granules are azurophil granules that have retained their basophilic staining reaction by lack of maturation or have developed increased basophilia in mature neutrophils. Toxic granules like azurophilic granules seen in neutrophils with prolonged staining time or decreased pH of staining reaction.

Döhle bodies: Döhle inclusion bodies are small, round or oval pale blue-gray structure, usually found at the peripheral cytoplasm of neutrophil. They consist of decomposed ribosomes and endoplasmic reticulum. Originally they were described in scarlet fever, but they are seen in any other infections, in aplastic anaemia, following administration of toxic agents and in burns.

Cytoplasmic vacuoles: It usually indicates severe sepsis, when toxic granules are also present. Cytoplasmic vacuoles will develop as an artifacts with prolonged standing of the blood before smears are made.

May-Hegglin anomaly: Autosomal dominant disease in which pale blue inclusions resembling Döhle bodies are seen. But the

inclusions are larger and more prominent than Döhle bodies. Also, they are found in all leucocytes except lymphocytes.

Pelger-Huet anomaly: It is a benign inherited condition in which neutrophil nuclei fail to segment properly. Most of the neutrophils nuclei have two discrete equal-sized lobes connecting by a thin chromatin bridge. The chromatin is coarsely granular and cytoplasmic granular content is normal.

A similar type acquired morphological anomaly, known as pseudo-Pelger cells may be seen in acute myeloid leukaemia (AML). Here, the neutrophils are hypogranular and have irregular nuclear pattern.

Neutrophilia

Definition: When absolute neutrophil count $7500/\text{mm}^3$ or 72% of DLC.

Relative neutrophilia: It can be divided into primary (clonal) and secondary.

Primary neutrophilia: Myeloproliferative neoplasms (chronic myeloid leukaemia, acute myeloid leukaemia), neutrophilic leukaemia, hereditary neutrophilia.

Secondary neutrophilia: Localized acute infections (pneumonia, tonsillitis, meningitis, acute otitis media), systemic infection (e.g. septicemia), acute rheumatic fever, vasculitis, acute myocardial infarction, burns, leukoerythroblastic reaction.

Leukoerythroblastic reaction: The presence of normoblasts, tear drop cells and immature cells of neutrophilic series (promyelocytes, myelocytes, metamyelocytes, band forms) along with neutrophilia in the blood is known as leukoerythroblastic reaction. It often indicates space-occupying disturbances of the bone marrow such as myelofibrosis with myeloid metaplasia, metastatic carcinoma, leukaemias, multiple myelomas, Gaucher's disease, TB and other granulomatous diseases.

Neutropenia

Definition: When neutrophil count is $<43\%$ of leucocytes or an absolute neutrophil and band from $<1500/\text{mm}^3$.

Causes of neutropenia

1. Decreased bone marrow production of neutrophils: Myelodysplastic syndromes, chemotherapy, acute leukaemia, aplastic anaemia.
2. Increased bone marrow production but decreased survival of neutrophils: Hypersplenism, SLE, rheumatoid arthritis, autoimmune and isoimmune neutropenia.
3. Viral infections: Measles, influenza, infectious mononucleosis, HIV infection, hepatitis.
4. Bacterial infection: Miliary TB, overwhelming sepsis, typhoid and paratyphoid, brucellosis, tularaemia.
5. Drugs: Antibiotics (chloramphenicol, cephalosporin, vancomycin), sulfa drugs, antimalarials (chloroquine, quinine), antifungal agents (amphotericin B, flucytosine).

Agranulocytosis

Definition: It theoretically means total absence of granulocytes in the peripheral blood.

But severe granulocytopenia (neutrophils and bands $<500/\text{mm}^3$) also referred as agranulocytosis casually.

Causes: Peripheral destruction of polymorphs or neutrophils (often drugs related).

- i. Severe bone marrow failure.

A neutrophil and band count $<500/\text{mm}^3$ is high risk factor for sepsis, whereas a count $<200/\text{mm}^3$ leads to overwhelming bacterial infections.

Lymphocytes (Fig. 2.14)

At birth, in normal individuals, the absolute numbers of lymphocytes and T cells are highest and may represent 90% of all leucocytes. Thereafter, B cells begin to rise and T cell decreases. In adolescence and adulthood B cells (15%) and T cells (85%) stabilized and lymphocytes constitute 20–40% of all leucocytes. Normally, majority of circulating lymphocytes are small and $\leq 10\%$ are large.

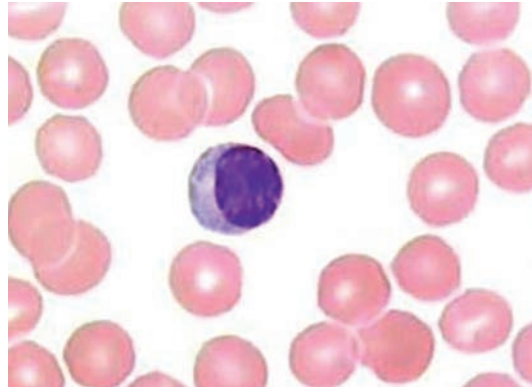


Fig. 2.14: Lymphocyte

The small lymphocytes have a thin rim cytoplasm occasionally containing scanty azurophilic granules. Nuclei are uniform in size (9 μm in diameter) which provides a useful guide for estimating red cell size (average 7–8 μm in diameter). The large lymphocytes (9–15 μm in diameter) have abundant pale blue cytoplasm containing azurophilic granules. Because of this, these are known as large granular lymphocytes (LGL) (Fig. 2.15) and basically they are activated B lymphocytes or NK (natural killer) cells. The nuclei of lymphocytes have homogeneous chromatin with some clumping at the periphery.

Türk cells: In bacterial and viral infections, transforming lymphocytes are present. These Türk cells are immunoblasts (10 μm in diameter) with a round nucleus and abundant deeply basophilic cytoplasm. Ultimately these cells will transform into

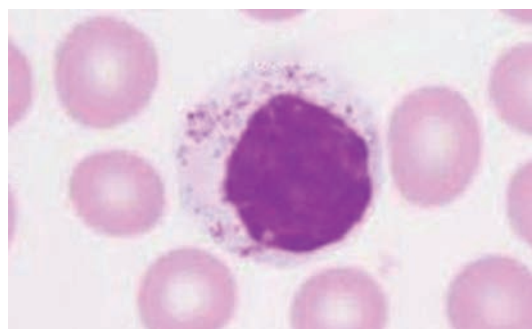


Fig. 2.15: Large granular lymphocyte

plasmacytoid lymphocytes or plasma cells. Occasionally, they may be seen in PBS.

Activated lymphocytes: These cells are seen in PBS in viral infections. These cells have slightly larger nuclei and more open chromatin (less dense) and abundant cytoplasm which may be irregular. Example: Infectious mononucleosis or glandular fever.

Lymphocytosis

Definition: It is defined as an absolute lymphocyte count $>4000/\text{mm}^3$ (or $>43\%$) in adults, $>7200/\text{mm}^3$ in adolescents and $>9000/\text{mm}^3$ in young children and infants.

Spurious lymphocytosis: When there is neutropenia with relative lymphocytosis, but normal absolute lymphocyte count (e.g. typhoid fever, thyrotoxicosis, agranulocytosis).

Causes of lymphocytosis

- i. *Viral causes:* Influenza, infectious hepatic viral infection, in various exanthemata like measles, mumps, chickenpox, rubella and infectious mononucleosis.
- ii. *Bacterial causes:* Enteric or typhoid fever, tuberculosis, pertussis (whooping cough), secondary syphilis, brucellosis.
- iii. *Protozoal causes:* Toxoplasmosis.
- iv. *Malignancy:* Chronic lymphocytic leukaemia (CLL), acute lymphoblastic leukaemia (ALL), prolymphocytic leukaemia, hairy cell leukaemia, large granular lymphocytic leukaemia, leukaemic phase of follicular, mantle cell and splenic marginal zone of lymphoma.
- v. *Miscellaneous:* Thyrotoxicosis, myasthenia gravis, hypopituitarism, hypersensitivity reaction, stress and drugs (efalizumab).

Lymphocytopenia

Definition: When lymphocyte count is $<1500/\text{mm}^3$ ($<18\%$) in adults, and $<3000/\text{mm}^3$ in children.

Causes: Corticosteroid therapy, Cushing syndrome, sarcoidosis, chemotherapy and

radiotherapy, neoplastic conditions especially Hodgkin lymphoma, epinephrine injection, few infections (e.g. acute and chronic retroviral infections of HIV, TB).

Monocytes (Fig. 2.16)

Monocytes are the largest of the circulating leucocytes; $15\text{--}18\text{ }\mu\text{m}$ in diameter and constitute 2–10% of leucocytes in PBS.

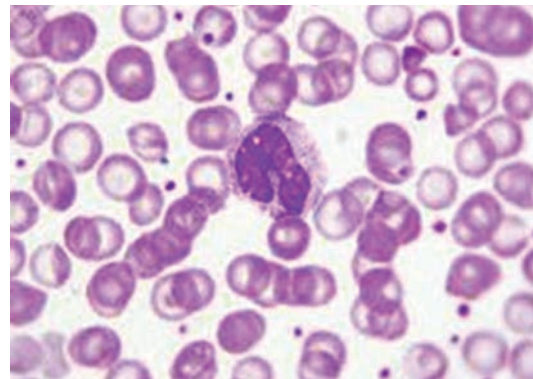


Fig. 2.16: Monocyte

Monocytes are characterized by a large, eccentrically placed nucleus which is stained less intensely than that of other leucocytes. Nuclear shape is variable but there is often a deep indentation giving a horseshoe or even bilobed appearance. The chromatin is finer and more evenly distributed compared to neutrophils. The abundant cytoplasm stains pale grayish-blue with Romanowsky stains. The cytoplasm contains numerous small pink-purple stained lysosomal granules and cytoplasmic vacuoles which may confer “frosted-glass” appearance. Monocytes are highly motile and phagocytic cells which are the precursors of macrophages or histiocytes (found in different tissues). Examples: Kupffer cells in liver, Langerhans cells of skin, osteoclast in bone and microglia in CNS.

Monocytosis

Definition: Absolute monocyte count is $>500/\text{mm}^3$ or $>12\%$ of DLC.

Causes

- i. Bacterial infections: TB, syphilis, brucellosis, bacterial endocarditis.
- ii. Protozoan infections: Malaria, kala-azar, trypanosomiasis.
- iii. Rickettsial infections: Typhus, Rocky Mountain spotted fever.
- iv. Malignancy: Acute monocytic or myelomonocytic leukaemia, chronic myelomonocytic leukaemia, myeloproliferative neoplasms, Hodgkin and non-Hodgkin lymphoma, multiple myeloma.
- v. Carcinomas: Ovary, breast, stomach.
- vi. Miscellaneous: Ulcerative colitis, Crohn's disease, sprue, sarcoidosis.

Eosinophils (Fig. 2.17)

Eosinophils are a little larger than neutrophils; 12–17 μm in diameter (average 13 μm). They usually have two lobes in the nuclei but may have three or four lobes also. The cytoplasm is packed with distinctive spherical gold/orange (eosinophilic) granules. They average 3% of the leucocytes in adults.

The most characteristic ultrastructural features of eosinophils are presence of large, ovoid, specific granules each containing an elongated crystalloid in the cytoplasm.

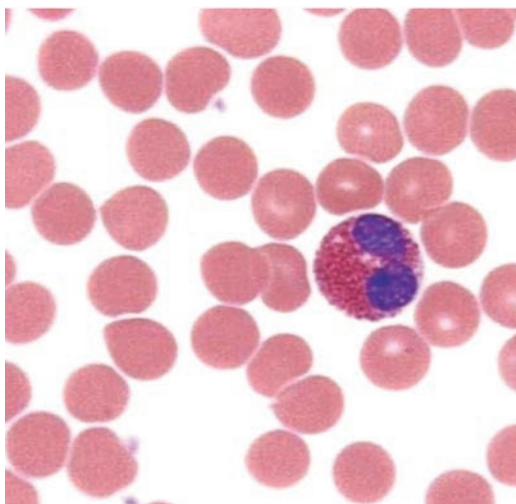


Fig. 2.17: Eosinophil

These specific granules are membrane bound and of uniform size and the matrix contains a variety of hydrolytic enzymes including histaminase. These crystalloids have a cubic lattice structure and consist of an extremely alkaline or basic protein, known as **major basic protein**.

In comparison to neutrophils, eosinophils are easily differentiated by the colour and size of the cytoplasmic granules. Eosinophilic granules are bright red with eosin and a more brick-red with Romanowsky stains. The cytoplasm is colourless. The nucleus stains less deeply compared to neutrophils and most eosinophils have two lobes or segments, rarely more than three.

Eosinophils are phagocytic cells but compared to neutrophils, they have greater oxidative capacity via the hexose monophosphate shunt. Eosinophils have particular phagocytic activity for antigen-antibody complexes.

Eosinophilia

Definition: Absolute eosinophil count $>600/\text{mm}^3$ or $>8\%$ of DLC (some use a cut-off value of $>>450/\text{mm}^3$).

Causes

- i. Allergic diseases: Bronchial asthma, seasonal rhinitis (hay fever).
- ii. Skin disorders: Atopic dermatitis, eczema, pemphigus eosinophilia.
- iii. Parasitic infestations: Trichinosis, tapeworm, cysticercosis, visceral larva migrans (due to roundworm) creeping eruption (due to hookworm), Löeffler's syndrome, pulmonary eosinophilia (due to roundworm), tropical pulmonary eosinophilia (due to hyperimmune reaction caused by microfilariae).
- iv. Infections: Scarlet fever, Echinococcus infection, early phase of *Pneumococcus pneumoniae*, fungal infections.
- v. Neoplastic disorders: Chronic eosinophilic leukaemia, myelomonocytic leukaemia with inversion, mastocytosis, T cell lymphoma, Hodgkin lymphoma.

vi. Drugs: Pilocarpine, digitalis, physostigmine, sulfonamide, etc.

Basophils (Fig. 2.18)

Basophils are the rarest (<1%) of the circulating leucocytes. The basophils are intermediate in a size between neutrophils and eosinophils. Like the eosinophils, basophil has a bilobed nucleus but this is usually obscured by numerous large, densely basophilic (deep blue) specific granules which are larger, but fewer in number than those of eosinophils. In few basophils, most of these granules may be missed as these granules are highly soluble in water and tend to be dissolved away during common blood smear preparation. When basophils are stained with the basic dye, like toluidine blue, the granules bind the basic dye and the dye changes colour to red. This phenomenon is called **metachromasia** and the granules are called metachromatic granules. The cytoplasmic granules of basophils and mast cells contain proteoglycans consisting of sulphated glycosaminoglycans linked to protein core, this accounts for their metachromatic staining characteristic.

Major function of basophils and mast cells is probably immunological response to

certain parasites and allergens. Release of histamine and other vasoactive mediators are responsible for the so-called immediate hypersensitivity (anaphylactoid) reaction, which is characteristic of allergic rhinitis (hay fever), urticaria, some forms of asthma and anaphylactic shock.

Basophilia

Definition: Basophil count is $>200/\text{mm}^3$ or $>2\%$ of DLC.

Causes

- i. Neoplastic conditions: Myeloproliferative syndromes (chronic myeloid leukaemia, myeloid metaplasia, polycythemia vera), acute basophilic leukaemia, Hodgkin lymphoma.
- ii. Infections: Chickenpox, small pox
- iii. Hypersensitivity states: Drugs, food, foreign protein injection
- iv. Others: Hypothyroidism, nephrotic syndrome, chronic haemolytic anaemia following splenectomy, chronic sinusitis and transient basophilia following irradiation.

Leukaemoid Reactions (Fig. 2.19)

Definition: A leucocyte count of $>50,000/\text{mm}^3$ in non-leukaemic condition.

The PBS (peripheral blood smear) shows an increase in and shift to the left of myeloid cells (band neutrophils, metamyelocytes, myelocytes, some promyelocytes and mye-

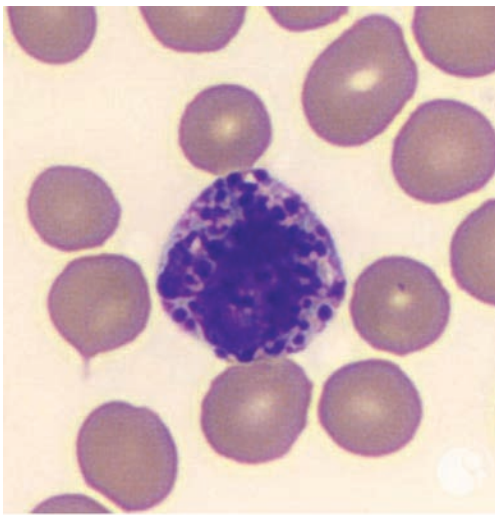


Fig. 2.18: Basophil

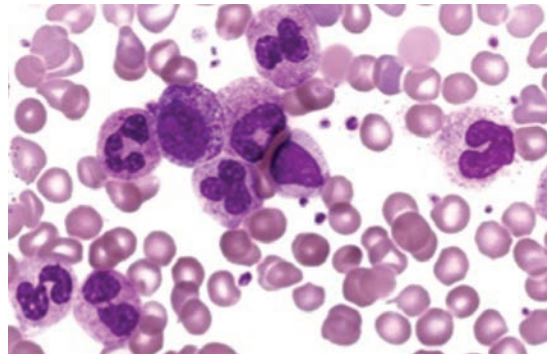


Fig. 2.19: Leukaemoid reactions (neutrophilic)

loblasts); and similar quantitative and quantitative changes in lymphocytes, or eosinophils or monocytes. Depending on the predominant cell, leukaemoid reactions may be neutrophilic (most common), lymphocytic, eosinophilic or monocytic.

Neutrophilic leukaemoid reactions: Excessive neutrophilia along with left shift of myeloid cells. It may occur in many situations like haemolysis, haemorrhage, Hodgkin lymphoma, myelofibrosis, malignancy with bone marrow involvement, severe burns, eclampsia, certain intoxications, and infections (especially tuberculosis).

Examination of PBS is more helpful than bone marrow examination. Increased primary granules (azurophilic granules) in the myeloid cells known as toxic granules, Döhle bodies and cytoplasmic vacuolization may be seen.

Lymphocytic leukaemoid reactions: Very high count of normal-appearing or mature lymphocytes may occur in measles, chickenpox, CMV, pertussis and in infectious mononucleosis. When atypical lymphocytes are many or immature lymphocytes (which may be seen in infectious mononucleosis) then the distinction from lymphocytic leukaemia may be difficult. In tuberculosis, normal-appearing or atypical lymphocytes may be found.

Examination of bone marrow is often helpful, because lymphocytes are minimally increased if at all in marrow, in contrast to PBS. If lymphocytes are increased in both PBS and marrow, then it is lymphocytic leukaemia (when fulfill other criteria).

Eosinophilic leukaemoid reactions: Blood cells as immature as eosinophilic myelocytes rarely appear in PBS in reactive eosinophilia, in which the TLC may exceed $50 \times 10^3/\text{mm}^3$. Eosinophilic leukaemoid reactions usually occur in children usually caused by parasitic infections. In adults, the idiopathic hypereosinophilic syndrome may be a cause.

Leukaemoid reaction is differentiated from leukaemia by

- i. Absence of hepatosplenomegaly, lymphadenopathy and haemorrhage.
- ii. Presence of blasts/immature cells <5% (in acute leukaemia, blasts $\geq 20\%$)
- iii. Presence of toxic granules and Döhle bodies in neutrophils (in leukaemia Döhle bodies are absent).
- iv. Presence of increased LAP (leucocyte alkaline phosphatase) score but in leukaemia LAP score is decreased.

Platelets (Thrombocytes) (Fig. 2.20)

Platelets (thrombocytes) are small, non-nucleated cells formed in the bone marrow from the cytoplasm of very large cells called megakaryocytes. In blood films from EDTA to blood and stained with Romanowsky stains, platelets are round to oval 2–4 μm in diameter. They are irregular in outline with fine red granules which may be scattered or centralized in the cell. A small number of larger platelets, up to 5 μm in diameter may be seen in normal persons.

Their number in circulating blood range from 150,000 to 400,000/ mm^3 . If the platelet count is normal, then on the average, one platelet is found per 10–30 RBCs. At 1000X or in oil immersion, this is equivalent to 7–20 platelets per oil immersion field.

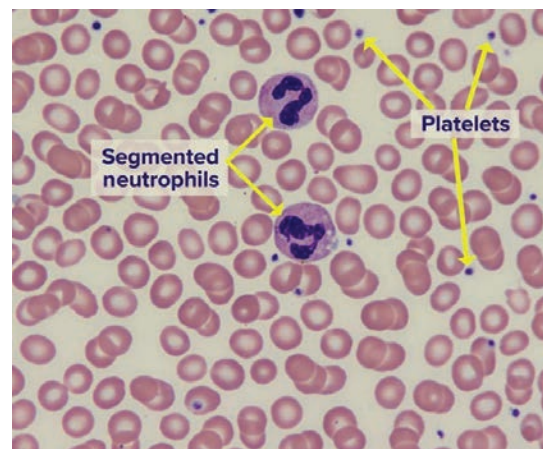


Fig. 2.20: Platelets

Platelet Functions

- i. They form plugs to occlude sites of vascular damage by adhering to collagenous tissue at the margin of the wound. Later the platelet plug is replaced by fibrin.
- ii. They promote clot formation by providing a surface for the assembly of coagulation protein complexes which are responsible for thrombin formation.
- iii. They secrete factors which are involved in vascular repair.

Thrombocytopenia

Thrombocytopenia refers to decrease in the number of platelets in peripheral blood below normal (1.5 lacs/mm^3). But practically a count below 1 lac/mm^3 is considered thrombocytopenia.

Causes**1. Increased destruction of platelets**

- a. Immune causes: Idiopathic thrombocytopenic purpura (ITP), infections (HIV, dengue, malaria), systemic lupus erythematosus, neonatal alloimmune purpura, post-transfusion purpura.
- b. Nonimmune causes: Thrombotic thrombocytopenic purpura (TTP), disseminated intravascular coagulation (DIC).

2. Decreased production of platelets

- a. Hereditary: Wiskott-Aldrich syndrome, Fanconi's anaemia.

- b. Acquired: Megaloblastic anaemia, aplastic anaemia, bone marrow infiltration (leukaemias, lymphomas, metastatic carcinomas), drugs (cytotoxic drugs, ethanol), radiation.

3. Increased sequestration: Hypersplenism**4. Dilutional thrombocytopenia: Massive blood transfusion.****Thrombocytosis (Thrombophilia)**

It refers to increase in the platelet count above normal (4 lacs/mm^3). Thrombocytosis may be primary or reactive (secondary). Primary thrombocytosis due to myeloproliferative disorders can be distinguished from reactive (secondary) thrombocytosis by the presence of leukocytosis, immature WBCs and nucleated RBCs in peripheral blood, defective platelet function (epinephrine-induced platelet aggregation) and splenomegaly in cases of primary thrombocytosis.

Causes of thrombocytosis

- i. Primary thrombocytosis: Polycythemia vera, chronic myeloid leukaemia, essential thrombocythaemia, idiopathic myelofibrosis.
- ii. Secondary thrombocytosis: Infections, chronic inflammatory diseases, trauma, haemorrhage, iron deficiency, splenectomy, malignancy.

VIVA VOCE

Q1. What are the characteristics of an ideal peripheral blood smear?

- Ans:**
- The smear should not be too thick or too thin.
 - It should occupy about central 2/3rds of the glass slide.
 - The smear should be tongue shaped and it should have straight lateral borders.

Q2. What is thick smear and when is it used?

- Ans:** Thick smear is prepared by spreading a large drop of blood on the slide approximately 2 cm in diameter and dry it. The dried smear is dipped (2–4 times) in tap water to dehaemoglobinize the RBCs and red-coloured solution comes out. The smear is fixed with methanol and stained with Romanowsky stain.

Thick smear is used for quick detection of malarial parasites and for screening of malaria.

Q3. What is fixation of smear and how is it done?

- Ans:** Fixation means fixation of blood cells and other things of smear to be fixed onto the slide.

So that, during subsequent staining and washing the smear do not wash off.

Usually acetone-free methyl alcohol is used for fixation. Acetone if present, it will wash away the nuclear stain and nuclear stain will be of poor quality. So, acetone-free methyl alcohol is used.

Q4. Why buffered water is used during blood smear staining? What is the ideal pH of buffered water?

- Ans:** Buffered water is used to maintain optimal pH during staining. If the pH of stain is acidic, it will cause poor nuclear stain and very reddish cytoplasm. On the other

hand, if the pH of stain is alkaline, the stained smear will be blue due to improper staining of cytoplasm.

The ideal pH for routine blood smear is 6.8 and for malarial detection is 7 to 7.2. At pH 6.8 (with the use of diluted phosphate buffer), the stained smear imparts a reddish hue to red cells and differential staining pattern of the granules present in granulocytes. To look, malarial parasites, a pH of 7.2 is recommended in order to detect Schuffner's dots of *Plasmodium vivax*, *ovale* and *malariae*. (Remember Maurer's dots in *P. falciparum* and Ziemann's dots in *P. malariae*.)

Q5. How platelet adequacy and platelet count are made on peripheral blood smear?

- Ans:** In normal healthy person, platelets are present in clumps as well as discretely. If there are platelet clumps (each clump containing ≥ 6 platelets), then platelet count is adequate. Usually 3–5 platelets are present per 100 RBCs. If there is < 3 platelet/100 RBCs, then platelet count is low and patient is probably suffering from thrombocytopenia.

As per **Henry's clinical diagnosis and management by laboratory's methods**, in stained film from EDTA to blood, there are 7–20 platelets/oil immersion field (1000X) or one platelet per 10–30 RBCs.

For rough estimation of platelet count, take average platelet count in 10 oil immersion field on a blood film, then multiply it by 15,000 which will give reasonably good platelet count (reference WHO laboratory book).

Some advocated that under oil immersion, normal person will have 10–25 platelets per oil field (average 20). Take average platelet count under oil immersion, then multiply by 20,000 if the average is < 10 , multiply by 15,000 if the average is ≥ 10 .

As per Gradwohl's clinical laboratory methods, the diagnosis is:

No. of platelets/oil immersion field	Estimated total platelet count
• Less than 1 platelet • Several platelets with occasional clumps • Over 25 platelets	• Decreased in number • Adequate in number • Increased in number

The number of platelets in 10 oil immersion fields multiplied by 2000 closely approximates the platelet count.

There are differences in opinion, but for practical reasons, take average platelet count in 10 oil immersion field on a blood film, then multiply it by 15,000 which will give reasonably good platelet count (reference WHO laboratory book).

Q6. What is platelet satellitism?

Ans: Platelet satellitism is phenomena in which platelets encircle neutrophils. It is usually seen when blood is collected in excess EDTA (though may be seen with other anticoagulants). It is caused by IgG or IgM antibodies that bind the CD16 antigen. CD16 is a low affinity Fc receptor, found on the surface of neutrophils, also present on NK cells, monocytes and macrophages. Satellitism around other leucocytes may be seen and some platelets may even be phagocytosed by neutrophils/WBCs.

Q7. What is "platelet dust"?

Ans: This was first described by Peter Wolf in 1967 which are basically microparticles and are not simply inert products of cellular debris. These microparticles are submicron vesicles shed from a variety of cells. These microparticles have roles in coagulation, cellular signaling, vascular injury and homeostasis. To date, the cell types reported to release microparticles either constitutively or when stimulated that include platelets, blood cells, endothelium, epithelium and many cancer cells.

Q8. What parasites can be identified in peripheral blood smear (PBS)?

Ans: i. Malaria: Most common parasite found in PBS.
 ii. Microfilaria of *Wuchereria bancrofti*
 iii. *Leishmania donovani* or LD bodies of kala-azar: Found free or inside monocytes.
 iv. *Trypanosoma cruzi*
 v. Others: Babesia, Brugia, Mansonella, etc. rarely.

Q9. What is the advantage of Leishman staining compared to Giemsa staining?

Ans: No separate fixation is needed as it contains methanol solvent within Leishman stain itself which will fix the smear. So, Leishman stain fixes and stains the smear simultaneously. Also, shorter time is required compared to Giemsa staining.

Leishman stain is a good alternative to Giemsa for malarial parasite detection. Leishman stain is superior for visualization of RBC and WBC morphology, which can be an advantage for the diagnosis of diseases involving RBCs and WBCs.

Q10. What is the advantage of Giemsa stain?

Ans: Giemsa stain is most complex Romanowsky stain and it contains maximum number of azo compounds. So, Giemsa stain provides maximum intermediate shades and better toning effect. Hence, it is good for microphotography. Also, Giemsa stain is best for malarial parasite detection.

Q11. What is the use of Field's stain and modified Field's stain?

Ans: Field stain was originally used to detect malarial parasite on thick smear. But it can also detect *H. pylori* on thin sections of stomach (paraffin section, 2–4 mm thickness). Also, it can be used as a quick Romanowsky stain for thin blood film and marrow smears. Modified Field's stain is recommended for rapid staining of proto-

zoans, such as *Acanthamoeba* and *Trichomonas* species.

Q12. How would you identify a well stained and good smear microscopically?

Ans: Ideally, the smear should be tongue shaped and after staining, a well stained blood smear will have pinkish tint at the stained portion and no staining on other portion of the slide.

Microscopically well stained smear should show following features:

- Red cells will have pinkish orange colour.
- Nuclei of white cells will be purplish blue.
- Neutrophilic granules should be violet pinkish or pale pink (neutral stain).
- Eosinophilic granules should be orange red (eosinophilic).
- Basophilic granules should be deep purple or bluish black (basophilic) and granules should overlap the nuclei.

- The nuclei of the lymphocytes are dark with condensed chromatin.
- The platelets are small, round, membrane bound, pinkish granular structures.

Q13. Why peripheral blood smear does not show schizonts of *P. falciparum*?

Ans: *Plasmodium falciparum* is associated with infected red cells expressing PfEMP (***Plasmodium falciparum* erythrocyte membrane protein**) leading to their attachment to the endothelial cells lining small blood vessels. PfEMP forms knobs on the RBC surface and binds to ligands on endothelial cells, including CD36, thrombospondin, VCAM-1, ICAM-1 and E-selectin. It also causes infected red cells to clump together (formation of rosette). All these block blood flow and sequestration of infected RBCs in the capillaries. So, schizonts of *P. falciparum* do not appear in PBS.

Marrow Puncture Needle and Examination of Bone Marrow

Bone marrow examination is an indispensable adjunct to the study of haematological diseases and sometimes the only way for a correct diagnosis. Apart from haematological disease, many other disorders can be diagnosed by bone marrow examination. It is estimated that in adults, weight of the marrow is 1300–1500 g.

Bone marrow or marrow can be obtained by needle aspiration, percutaneous trephine biopsy or surgical biopsy. Bone marrow aspiration (also called aspiration biopsy) is simple, safe and relatively painless. Aspiration can be performed on outdoor patients. On the contrary, trephine biopsy or surgical biopsy is not simple but can be performed on outdoor patients too.

The advantage of marrow aspiration is that, individual cells are perfectly preserved in the well-prepared marrow films and after staining subtle differences between cells can be recognized easily compared to trephine biopsy/surgical biopsy. But the disadvantage of aspiration is that the arrangement of the cells in the marrow and the relationship between different cells are more or less destroyed during aspiration. Also, in fibrosis (myelofibrosis) or in hypoplastic/aplastic anaemia, no marrow material except blood is aspirated (**called dry tap**).

The great advantage of trephine biopsy is that it can provide a perfect view of the

structure of relatively large pieces of bone marrow (perfect alignment of different cells in marrow biopsy). Also, morphological features of individual cells can be identified by making an imprint or a smear from the marrow tissue obtained.

HISTORY OF BONE MARROW EXAMINATION

- In 1905, the Italian physician Pianese reported bone marrow (BM) infiltration by the parasite Leishmania.
- In 1927, Anirkin, a Russian physician obtained BM from the sternum using a lumbar puncture needle.
- In 1931, Arjeff, introduced needles with a guard.
- In 1935, Klima and Rosegger developed BM needles with guards.
- In 1945, Vandenberghe and Blitstein were the first to use the iliac crest to obtain BM.
- In 1952, Bierman used the posterior iliac crest as the site for bone marrow aspiration and claimed to be very safe site.
- In 1958, McFarland and Dameshek described a trephination technique using the Vim-Silverman biopsy needle.

BONE MARROW ASPIRATION

The various sites for bone marrow aspiration are:

In children

- Tibia → superior medial surface of tibia, inferior to the medial to the tibial tuberosity. This site is favoured for newborn, infants and children <2 years of age.
- Posterior iliac crest and spine
- Calcaneum

In adults

- Iliac crest
- Anterior and posterior superior iliac spine
- Spinous processes of lumbar vertebra
- Sternum: It is no longer favoured though was popular in the past.

Most favoured site nowadays

The sternum should never be attempted in children. The preferred site for children of all ages are posterior superior iliac spine or iliac crest. Upper end of tibia is also a favourite site <2 years of age but caution should be taken as the weak tibia in children is vulnerable to fractures and lacerated injury to major blood vessels may occur in inexperienced hands.

Sternum though very popular site of aspiration in the past, but now is becoming obsolete. Unless the needle is correctly inserted, there is a risk of perforating the inner cortical layer and damaging the underlying large blood vessels and right atrium leading to medical emergencies.

Aspiration from iliac spine or iliac crest has the advantage that a large amount of marrow material can be aspirated and risk of injuring major blood vessels or organs is minimal compared to sternum. Also, unlike sternum, the patient cannot see what is happening or the medical procedure as the patient is lying on his/her side or lying prone. Multiple attempts can be made if necessary without making patient worried.

For aspiration, different needles used are:

1. **Salah bone marrow aspiration needle (Fig. 3.1):** It has three parts:

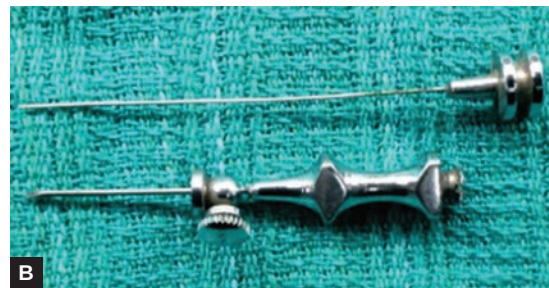


Fig. 3.1A and B: Salah bone marrow needle

- a. Trocar
- b. Cannula or stylet
- c. Adjustable side guard and screw

2. **Klima bone marrow aspiration needle (Fig. 3.2):** Here, there is no screw but there is a guard. But other two parts (trocar and the stylet) are there. Klima needle has the advantage as the guard has no chance of getting slipped and injuring underlying structures.

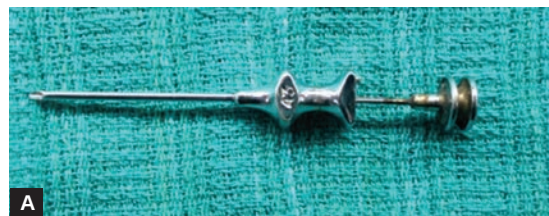


Fig. 3.2A and B: Klima bone marrow needle

- 3. Islam's bone marrow aspiration needle:** Here dome-shaped handle and T bar are intended to provide stability and control during aspiration.

Ideal bone marrow needle

- Should be stout
- 7–8 cm in length
- Adjustable guard
- Well-fitted stylet
- Edges well sharpened

Indications of Bone Marrow Aspiration

A. Diagnostic indications

1. Red cell disorders—megaloblastic anaemia, pure red cell aplasia
2. WBC disorders—leukaemia (acute and chronic), subleukaemic leukaemia
3. Platelet disorders—idiopathic thrombocytopenic purpura (ITP)
4. Myeloproliferative disorders and myelodysplastic syndromes
5. Storage disorders—Gaucher's disease, Neimann-Pick disorders
6. Assessment of iron stores (Perls' Prussian blue stain)
7. In evaluation of fever or pyrexia or pyrexia of unknown origin (PUO)
8. Detection of parasites—microfilaria, LD bodies of kala-azar (*Leishmania donovani*), malaria
9. Detection of LE cell in SLE patients
10. Metastatic deposits—different carcinomas
11. Staging of lymphoma (Hodgkin and non-Hodgkin)
12. Follow-up—therapy for leukaemia and lymphoma
13. Plasma cell dyscrasia—multiple myeloma
14. Bone marrow aspirated material as a source of culture to detect infective pathogens like TB, fungus, etc.
15. Granulomas in bone marrow: TB, histoplasmosis, sarcoidosis, lymphoma

16. Cytogenetic studies, molecular genetic studies, cytochemistry and flow cytometry.

B. Therapeutic indications: Bone marrow transplant.

BONE MARROW TREPHINE BIOPSY

Three types of needles are used:

1. Jamshidi trephine needle (Fig. 3.3A): Most popular and most commonly used
2. Islam's trephine needle (Fig. 3.3A)
3. Sacker-Nordin's bone marrow trephine biopsy.

Indications of Bone Marrow Biopsy

- Diagnosis of leukaemia and lymphoma
- Staging of different lymphomas
- Evaluating iron stores, fibrosis, granulomas, abscesses, metastases and vascular lesions
- Detection of metastatic deposit (carcinoma)

Contraindications of Bone Marrow Examination

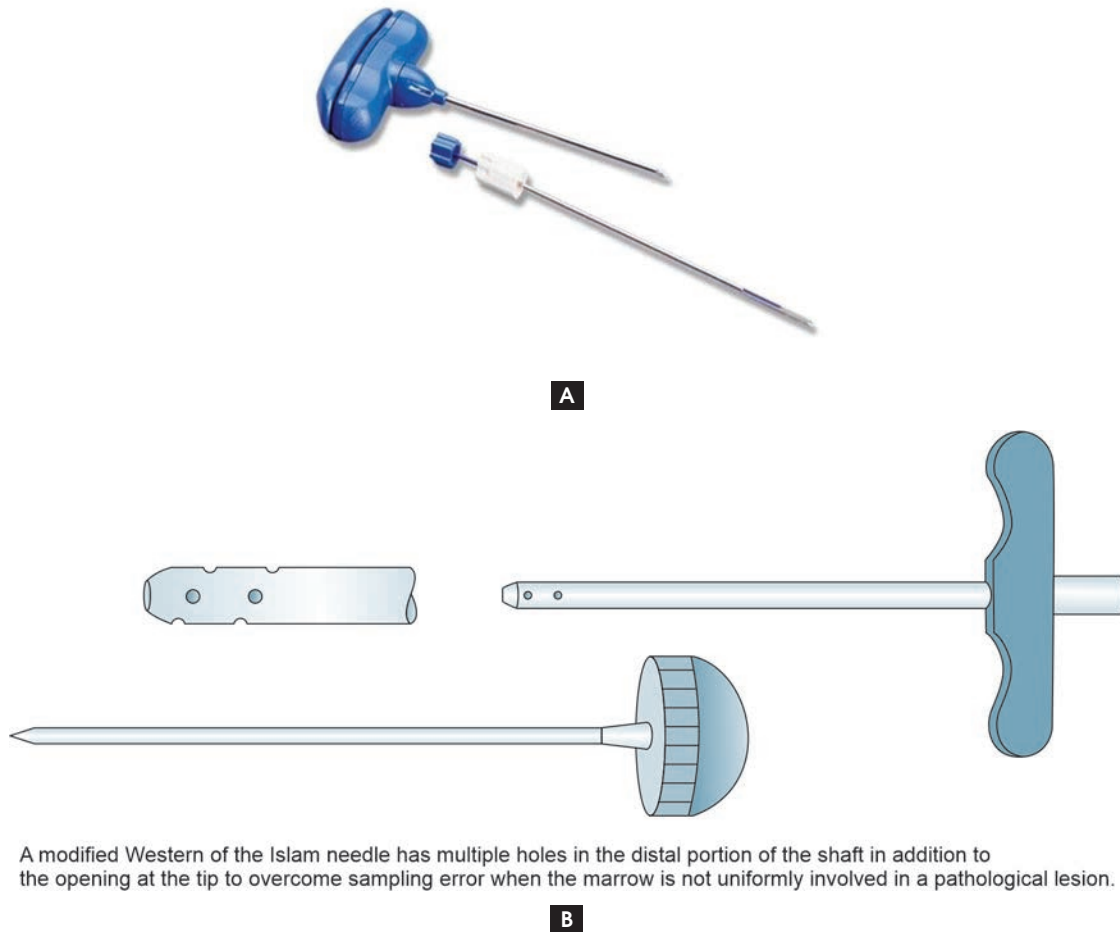
- Bleeding disorders or diathesis
- Haemophilia
- Infection at site of puncture.

Bone Marrow Aspiration Technique (Fig. 3.4)

Puncture of Ilium (Superior Iliac Spine and Iliac Crest)

The usual site for bone marrow aspiration or puncture is iliac crest in adults. Only needles designed for bone marrow aspiration should be used (Salah's needle or Klima's needle). They should be stout, about 7–8 cm in length with a well-fitted stylet and preferably with a guard (Klima's needle) (Fig. 3.5). The point of the needle and edge of the bevel must be sharp so that it can pierce bone easily. The skin of the chosen site should be cleaned with 70% alcohol (e.g. ethanol) or 0.5% chlorhexidine. Now follow the below mentioned steps:

1. Clean the area with 70% alcohol or 0.5% chlorhexidine as stated above.



A modified Western of the Islam needle has multiple holes in the distal portion of the shaft in addition to the opening at the tip to overcome sampling error when the marrow is not uniformly involved in a pathological lesion.

Fig. 3.3A and B: Jamshidi and Islam trephine needles

2. The skin, subcutaneous and periosteum overlying the site are infiltrated with a local anaesthetic (2% lignocaine) by a 2 ml syringe. Wait for 3–5 minutes.
3. With a boring movement (rotating clockwise and anticlockwise with some pressure but not full rotation). The needle is inserted to the bone. The bone is touched after passing through soft tissue (sensation of hard material as perceived by the person doing the technique). Further insertion as will give sudden release of resistance as if the needle has entered into an empty space.
4. Now the needle has entered into bone marrow space.
5. Take out the stylet.
6. With a well-fitted 2–5 ml syringe, take out 0.2–0.3 ml of marrow material.
7. Take out the needle and seal the puncture site.
8. Immediately prepare marrow films from the aspirated marrow materials.

Puncture of the Sternum

Usual site in sternum is manubrium sterni or the first or second pieces of the body of sternum.

If the manubrium sterni is chosen, the puncture site should be about 1 cm above the sternomandibular angle and slightly lateral to the midline.

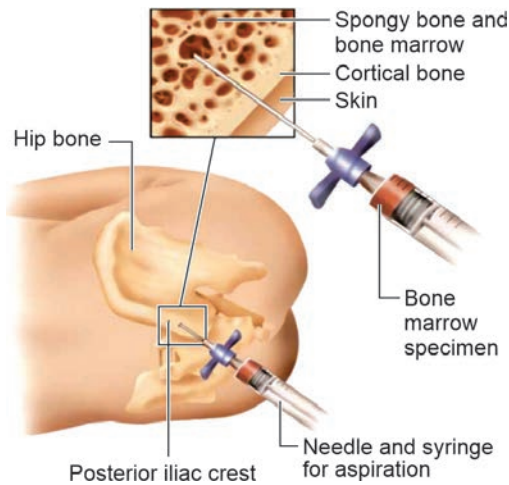


Fig. 3.4: Bone marrow aspiration

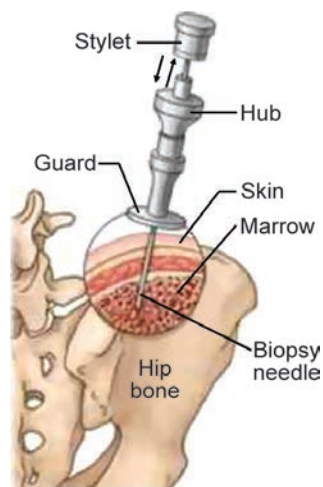


Fig. 3.5: Bone marrow aspiration by Klima's needle

If the body of sternum is chosen, the puncture site should be opposite, the second and third intercostal space slightly lateral to midline.

In case of sternal puncture, it is essential to use the guard. Clean the area as stated in puncture of ilium. Infiltrate lignocaine as stated previously. Now insert the needle with boring movement. After piercing the skin and subcutaneous tissues, when the needle touches the periosteum, adjust the guard on the needle so that the needle can be allowed to penetrate for about 5 mm further (not more than that to avoid risk of injury of

blood vessels and organs). Then aspiration is done as stated in puncture of ilium.

Preparation of Marrow Films

Transfer the aspirated material without delay, as it tends to clot quickly. One drop of marrow material is delivered onto each glass slide 1 cm from one end. Get rid of extra blood by quickly sucking off with a fine Pasteur pipette leaving the greyish marrow particles behind. Some advised to tilt the slide or place the slide on a slope so that the lighter blood come down over the slide and then remove it. After removing extra blood, marrow films are prepared by pushing a spreader (glass side) or by a coverslip. The marrow particles/fragments are dragged behind the spreader and leaves a trail behind them. The marrow film should be 3–5 cm length and not more than 2 cm in width.

Bone marrow imprints: One or more visible marrow particles/fragments are picked up with a capillary pipette, or by a toothpick and immediately transferred onto a slide and made to stick to it by a gentle smearing motion. The slide is air-dried rapidly by waving it.

Crush preparations: Marrow particles in a small drop of aspirate may be placed onto a slide near one end. Another slide is carefully placed over the first slide. Now crush the bone marrow particles by giving some pressure and prepared the smear of crushed marrow particles by pulling the slides apart in a direction parallel to their surface.

Confirmation of presence of bone marrow not only blood: Presence of sand like marrow particles and irregular holes of fat in the films give assurance of marrow and not just blood has been aspirated.

Fixation of marrow films and staining: Fix the marrow films and stain them with Romanowsky stain as for peripheral blood (refer to previous chapter).

- Leishman stain is used routinely
- Prussian blue staining may be done to demonstrate iron in haemosiderin deposit or in ferritin and assessment of iron status in body. Iron in haemoglobin is not stained.
- Few slides may be stained with PAS (periodic acid–Schiff) stain or May–Grünwald stain.

Reporting of Bone Marrow Films (Myelogram)

At least 300–1000 bone marrow cells (average 500 cells) should be examined. A myelogram report should include:

- General cellularity of marrow:** Whether this is hypercellular or hypocellular or normocellular. As a rough guide cellularity (haemopoietic cells) occupying <25% of the particle is considered hypocellular, whereas cellularity >75–80% is hypercellular. Some follow the simple formula as regards to age → $\text{cellularity}\% = (100 - \text{age})\%$. So, physiologically children have highest cellularity and elderly lowest cellularity.
- Myeloid–erythroid ratio (M:E ratio):** Leukocytes of all types and stages of maturation are counted together. Likewise, erythroblasts and normoblasts are counted together. A ratio is made. Normal M:E ratio is 1.2:1 to 5:1 (average 3:1). An increased M:E ratio (e.g. 6:1) may be found in erythroid hypoplasia, patients with infection or myeloproliferative disorders like CML. A decreased M:E ratio (<1.2:1) may be seen in normoblastic hyperplasia or in decreased leukopoiesis.
- Type of erythropoiesis:** Whether normoblastic, megaloblastic or dyserythropoietic.
- Type of leukopoiesis:** Myeloblast, promyelocyte, myelocyte, metamyelocyte, band neutrophil and mature neutrophils are present or not (Fig. 3.6). Also seen up to what stage of maturation is present.
- v. Number of lymphocytes, plasma cells, monocytes, etc.
- vi. **Megakaryocytes:** Number of megakaryocytes is estimated better in tissue sections than in marrow films. Under low power or 100X, an average of 1–3 megakaryocytes should be found in each low power field.
- vii. **Abnormal cells (if any):** Leukaemic cells, lymphoma cells or metastatic carcinoma.
- viii. **Parasites:** Microfilariae, malaria, LD bodies (kala-azar)
- ix. **Iron status:** Done by Perls'–Prussian blue stain. It is reported as negative or 1+ to 5+, storage iron is seen in macrophages (haemosiderin or ferritin). In normal adults it is 2+, whereas 3+ is slightly increased, 4+ is moderately increased and 5+ is markedly increased.

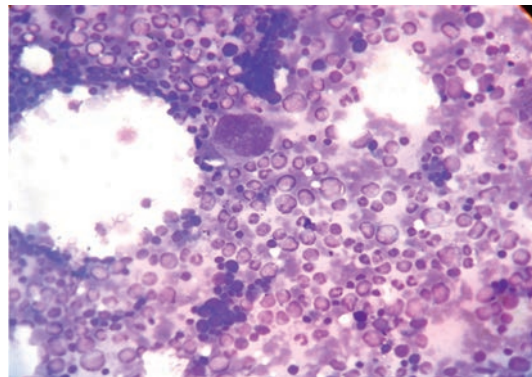


Fig. 3.6: Normal bone marrow shows trilineage haemopoietic cells (oil immersion, Leishman's stain, 1000X)

Summary of Bone Marrow Report

The report (myelogram) should include an estimate of cellularity, M:E ratio, statements about any cytological or maturation abnormalities, an estimate of the number of megakaryocytes, an estimate of the storage iron and proportion of sideroblasts and statement about any abnormal cell or other abnormal findings.

Remember, every bone marrow report (myelogram) (Table 3.1) should be interpreted

Table 3.1: Normal ranges of differential counts in bone marrow in adults (myelogram) in aspirated material

Type of cell	Range (%)
• Reticulum	0.1–2
• Myeloblasts	0.1–3.5
• Promyelocytes	0.5–5
• Myelocytes:	
– Neutrophilic	5–20
– Eosinophilic	0.1–3
– Basophilic	0–0.5
• Metamyelocytes	10–30
• Neutrophils including bands	7–25
• Eosinophil	0.2–3
• Basophil	0–0.5
• Monocytes	0–0.2
• Lymphocytes	5–20
• Megakaryocytes	0.1–0.5
• Plasma cells	0.1–3.5
• Proerythroblasts	0.5–5
• Early and intermediate normoblasts (basophilic and polychromatic)	2–20
• Late normoblast (pyknotic or orthochromatic)*	2–10

* The term pyknotic is preferred to orthochromatic as a description of most mature normoblasts or late normoblasts. But cells with fully haemoglobinized or mature cytoplasm (i.e. orthochromatic) is rarely seen in normal bone marrow.

along with a blood smear examination of the same patient. For bone marrow at least 500 cells and for blood smear at least 200 cells should be examined as differential count.

1. Jamshidi Needle (Fig. 3.7)

This needle has the advantages than Turkel and Bethel needles or needles of Vim-Silverman type (sometimes the specimen is crushed and its architecture altered). The Jamshidi needle should be inserted by to and fro rotation through approximately 90°. It should not be continuously rotated because this tends to distort and twist the core of marrow tissue, but once the needle is inserted up to desired length, the needle is rotated clockwise (few rotations) and anticlockwise (few rotations) without any downward pressure. This is done to cut the core tissue and put the tissue within needle.

2. Islam's Needle

This needle has the advantage over other needles, as it can provide long uniform core of marrow-containing bone spicules and there is no distortion of bone marrow architecture. These needles are usually performed

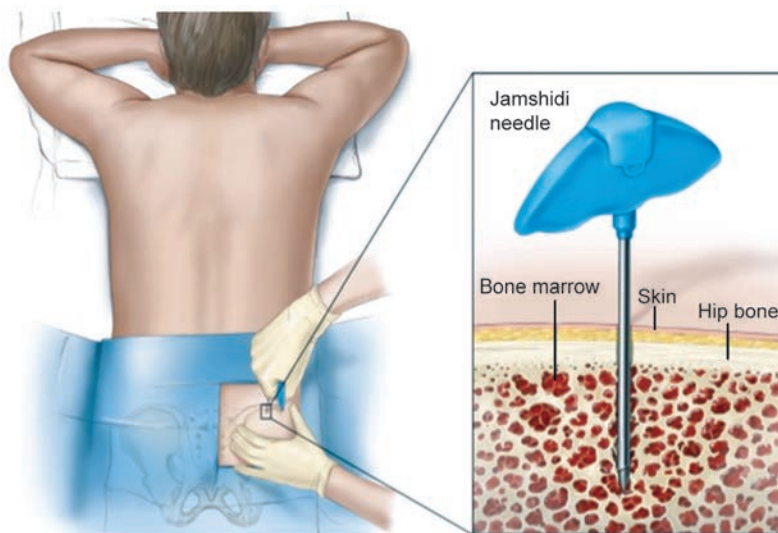


Fig. 3.7: Trepine biopsy by Jamshidi needle

at the anterior or posterior superior iliac spine. The posterior superior iliac spine provides core tissue which is longer and larger than other sites. A large trephine is sometimes of value as it provides sufficient bone marrow material for accurate diagnosis (Table 3.2).

Other trephine biopsy needles

The other trephine biopsy needles are: Westerman Jensen, drills and disposable needles.

Bore Size of Trephine Needles

Trephines have been developed with bore size of 4–5 mm and they can be safely inserted in iliac crest (but a small skin incision and local anaesthesia is required). Bore size of 2–3 mm is also good and provides good material.

Trephine Biopsy/Histological Sections

The needle biopsy and the clotted marrow particles or fragments are fixed in Zenker's acetic solution (5% glacial acetic acid; 95% Zenker) for 6–18 hours or in B-5 fixative for 1–2 hours. Prolonged time in fixation in either fixative will make the tissue brittle. The tissue is processed routinely for embedding in paraffin, cut at 4 mm and stained routinely with haematoxylin and eosin. Giemsa and PAS stains are also frequently used. But sometimes thinner sections are needed (1–3 mm). For this, plastic or resin embeddium medium (not paraffin) is required which enables to cut sections at 1–3 mm thickness.

Examination of Stained Sections

- Haematoxylin and eosin (H&E) stain (Fig. 3.8A and B): This is excellent for demonstrating the cellularity and pattern of the marrow and for revealing pathologic changes such as fibrosis or presence of granuloma or carcinoma.
- Romanowsky stain: Haemopoietic cells are better identified with this stain.
- Reticulin stain (silver impregnation stain): The bone marrow always contains

a small amount of glycoprotein matrix which is actually collagen and make supporting network. This collagen or connective tissue is known as reticulin or reticulin fibres. This reticulin can be stained by reticulin stain or silver impregnation stain (Flowchart 3.1).

- PAS stain: To demonstrate any parasite (intracellular or extracellular), myeloblast, lymphoblast, glycogen, etc.
- Myelofibrosis and myelosclerosis: Myelofibrosis refers to an increase in coarse fibres, whereas myelosclerosis refers to an increase in fine fibres. Both fibres are stained by reticulin stain. Coarse fibres predominate in myelofibrosis (chronic or idiopathic).

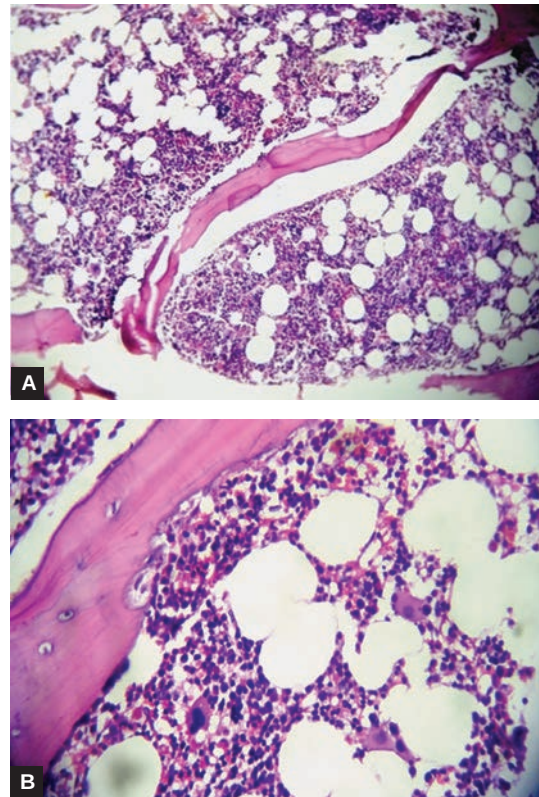
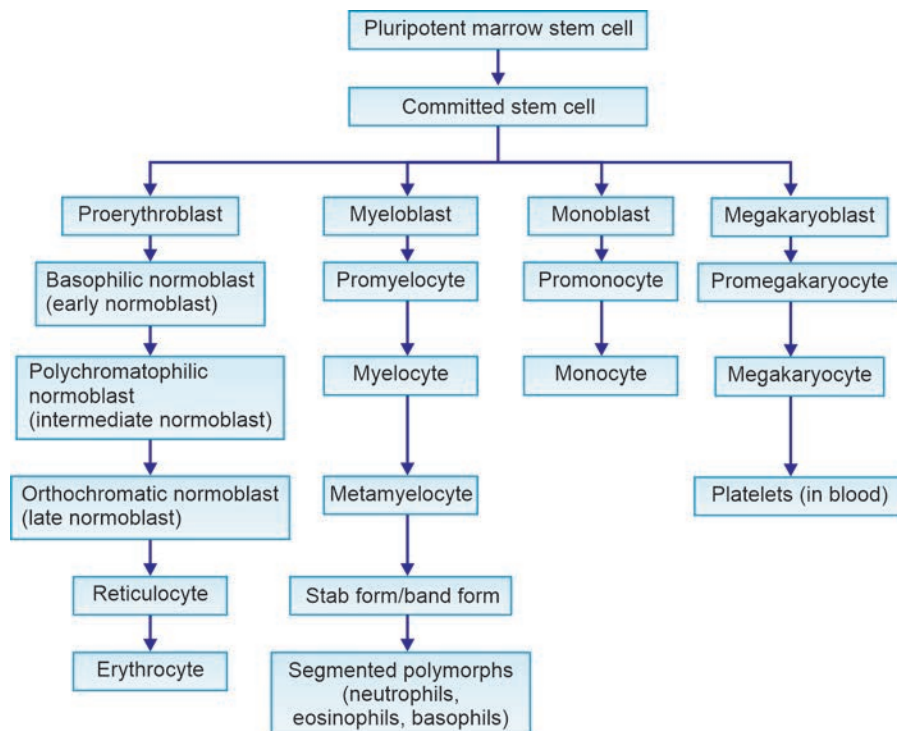


Fig. 3.8A and B: (A) Low power view of trephine biopsy shows marrow tissue, fat spaces and bony trabeculae; (B) High power view shows trilineage (erythroid, myeloid and megakaryocytic/thrombocytic cells) haematopoiesis including megakaryocytes

Table 3.2: Comparison of bone marrow aspiration and bone marrow biopsy

Parameter	Bone marrow aspiration	Bone marrow biopsy
1. Site	Iliac spine, sternum, tibia, spinous process of vertebra	Posterior superior iliac spine
2. Main indications	Suspected haematologic malignancies, unexplained cytopenias	Repeated dry tap, aplastic anaemia, myelofibrosis, lymphoma staging, focal lesions, hairy cell leukaemia
3. Needle used	Salah, Klima	Jamshidi
4. Information obtained	Morphology, cytochemistry, iron stain, flow cytometry (immunophenotyping), culture	Cellularity, architecture, fibrosis, focal lesions
5. Stains and special tests	Romanowsky stain, iron stain, cytochemistry, molecular genetics, flow cytometry (immunophenotyping), culture	H&E stain, reticulin stain, IHC (immunohistochemistry)
6. Reporting time	Same day	Up to 7 days

Flowchart 3.1: Pluripotent marrow stem cell and formation of haemopoietic cells

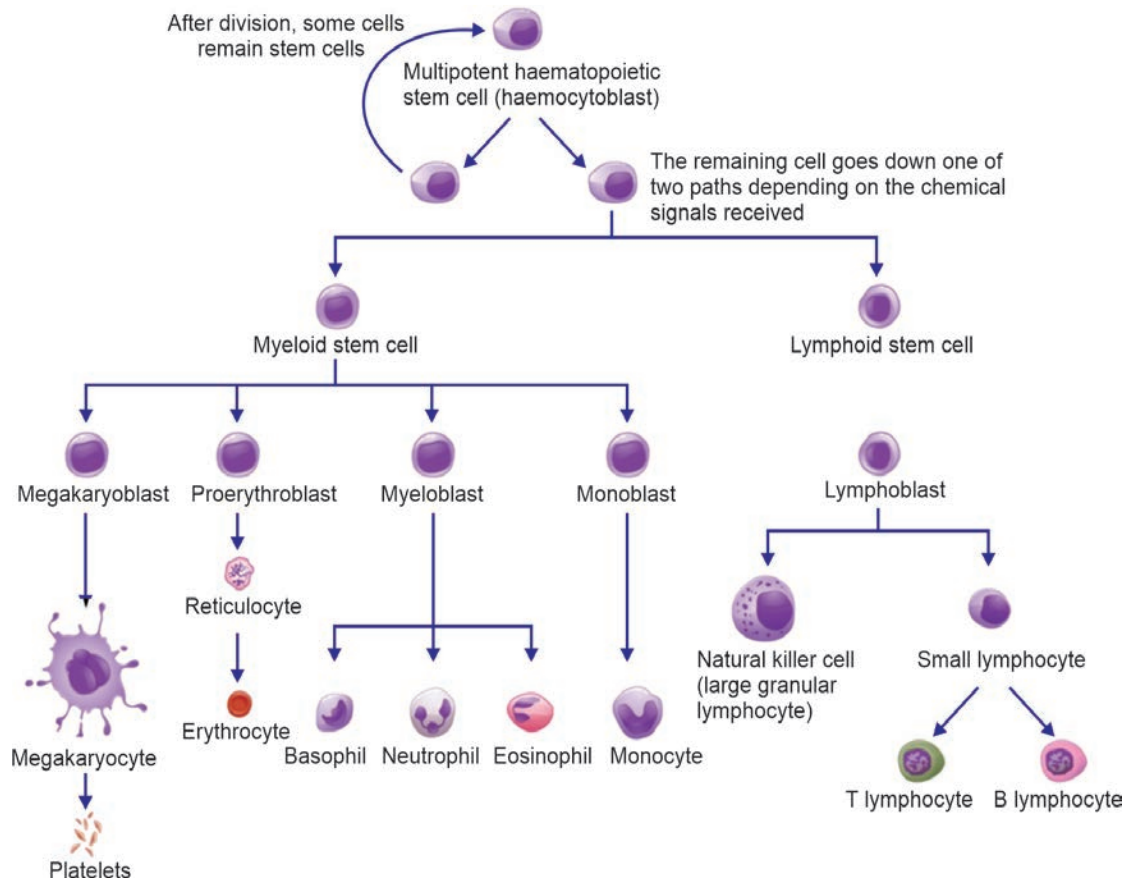


Fig. 3.9: Haematopoiesis of different blood cells in the bone marrow

- Increased reticulin fibres also occur in secondary carcinoma (metastatic deposit), osseous disorders like Paget's disease and hyperparathyroidism, in inflammatory reactions and in other myeloproliferative disorders (particularly lymphoproliferative disorders and proliferation of megakaryocytes) (Fig. 3.9).

Q1. What are the parts of bone marrow needle and how to identify?

Ans: A stout wide bore needle and guard. A stillete is there within the needle which prevents blockage of the needle when it penetrates the skin, soft tissue and bone chips. The guard allows penetration of the needle up to desired length and prevents damage to the underlying tissues/organs.

Q2. What are the differences between Salah's needle and Klima's needle? Which one is better?

Ans: Salah's needle it does not has side screw and guard is fixed to the body of needle itself by a spiral thread.

Q3. Which is the best site and why?

Ans: Posterior superior iliac spine is the preferred site because:

- Patient lies prone and cannot see the procedure (patient can see sternal puncture). So, patient is less apprehensive.
- The procedure is very safe as there is no underlying important structure (like substernal aorta) and risk of damaging vital organs is minimal.

Q4. What are the absolute indications for bone marrow aspiration examination?

Ans:

- Hypoplastic/aplastic anaemia
- Sideroblastic anaemia
- Megaloblastic anaemia
- Aleukaemic/subleukaemic leukaemia
- Assessment of remission during chemotherapy of acute leukaemia
- Kala-azar (acute)
- ITP (Idiopathic thrombocytopenic purpura)
- Multiple myeloma

Q5. What are the relative indications for bone marrow aspiration?

Ans:

- Chronic kala-azar
- Before starting chemotherapy for acute leukaemia
- Staging of lymphoma (Hodgkin and non-Hodgkin lymphoma).

Q6. What is dry tap and what are the causes?

Ans: When only blood and no marrow material is aspirated then it is called 'dry tap'. Common causes are aplastic anaemia, myelofibrosis, and faulty technique.

Q7. How would you confirm that marrow has been aspirated?

Ans:

- Macroscopically, by looking at the granularly. When bone marrow smear is touched the surface is irregular and sand-like feeling due to presence of marrow particles. Marrow particles look glistening.
- Microscopically, by looking at the megakaryocytes and fat spaces (in-between marrow cells) under microscope. Marrow particles are dragged during smear preparation, and trail of cells is left behind.

Q8. What are the indications of trephine or bone marrow biopsy?

Ans: All the indications of bone marrow aspiration and 'dry tap' during aspirations (myelofibrosis, myelosclerosis and aplastic anaemia).

Bone marrow biopsy removes a core of bone to evaluate both bone and surrounding tissue (marrow tissue). Also associated cells, protein deposits or inflammatory processes can be assessed. It shows the relationship of the cells to

each other or to the bone or the cells' precise location in relation to the bone. Bone marrow infiltration by tumour cells or cancer cells can also be examined which helps in staging in many tumours like Hodgkin and non-Hodgkin lymphomas.

Q9. What are the contraindications of bone marrow aspiration?

Ans: Haemophilia A, haemophilia B, bleeding diathesis and infection at the site of puncture.

Q10. What are the routine stains and special stains for bone marrow smear examination?

Ans: Routine stains: Leishman's stain, Giemsa stain and MGG.

Special stains

- i. Perls'-Prussian blue stain: To detect iron status. Absent in iron deficiency anaemia, increased in haemochromatosis, haemosiderosis, refractory sideroblastic anaemia and decreased in aplastic anaemia and pernicious anaemia.
- ii. Reticulin stain for myelofibrosis, polycythaemia vera and myeloproliferative disorders (increased in all).
- iii. PAS stain, myeloperoxidase stain and Sudan black stain: Differentiate between ALL (acute lymphoblastic leukaemia) and AML (acute myeloblastic leukaemia).

Total Count of WBC, RBC and Platelets

For total count of blood cells (RBCs, WBCs and platelets) and haemocytometers are used. There are a number of different haemocytometers in the market and each of them has a different grid (device containing horizontal and perpendicular lines) as well as different recommended uses. Different haemocytometers are:

- Neubauer/improved or modified Neubauer chamber
- Louis-Charles Malassez device: French histologist and anatomist (1842–1909) who first invented haemocytometer.

- Burkner chamber
- Thoma chamber (Fig. 4.1)
- Fuchs-Rosenthal counting chamber: Used mainly for total eosinophil count.

But most popular and most frequently used haemocytometer nowadays is **improved or modified Neubauer chamber**. Most haemocytometers are manufactured from crystal glass and generally measured 30×70 mm with a thickness of 4 mm. Two vertical lines are ground from the glass to define the cell counting area and the double cell counting chambers have a ground out 'H' shape.



Fig. 4.1: Thoma counting chamber

OLD VS. IMPROVED NEUBAUER CHAMBER

- In the old chamber, in the central area, there are 16 large squares each having 16 small squares. But in the improved chamber there are 25 large squares, each containing 16 small squares.
- The triple lines, dividing the central area is not very close in old chamber. But in improved chamber, the triple lines are very close.
- The space occupied by the triple lines in old Neubauer chamber being used to produce extra large space.
- In old chamber, the gap between triple lines was wide and the rectangular space between them looks as similar as the

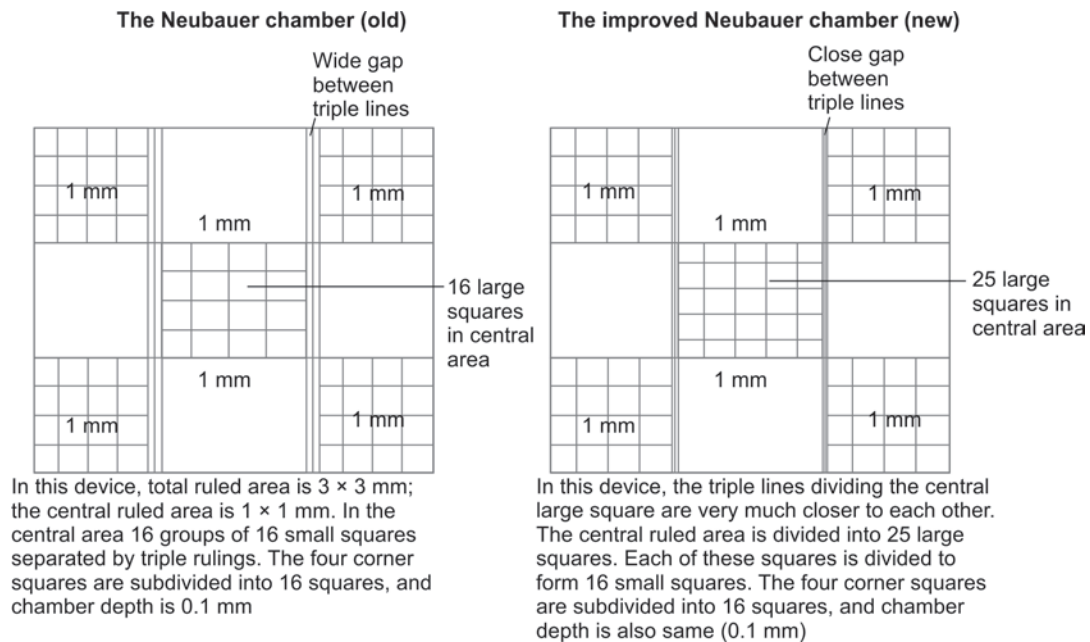


Fig. 4.2: Old and improved Neubauer chamber

squares in which the cells are to be counted. So, it makes the count very difficult and chances of error was very high.

- In old Neubauer chamber, the separating lines were very dull and some time it was very difficult to recognize them. But in the improved chamber, the separating lines are vivid and clear (Fig. 4.2).
- By dividing, central space in 25 large squares, the RBC and platelet count have become easier.

Parts of Haemocytometer (Improved Neubauer's Chamber)

(Figs 4.3, 4.4 and 4.6)

- A counting chamber
- A WBC pipette
- A RBC pipette
- A thick coverslip.

Counting Chamber (Fig. 4.5)

The improved Neubauer's chamber has two ruled stages separated by a small gutter (a shallow trough/channel beneath the edge of

a roof). The two ruled stages again are separated from two ridges by gutters, one on each side. The surface of these two ruled stages is 0.1 mm above the surface of the stage. So, when cover glass is kept on the platform of this counting chamber, the space between the bottom of the coverslip and the base of the grooved area becomes 0.1 mm in depth.

There are two chamber stages, one above and one below, which is separated by gutter. Each stage has a ruled area measuring 3×3 mm which is divided into 9 squares. This squares measuring 1×1 mm each. The four corner squares are divided into 16 small squares ($1/4 \times 1/4$ mm each). The four corner areas (A, B, C and D) are used in Fig. 4.6 for counting WBCs.

The central ruled square area (1×1 mm) is divided into 25 (5×5) small squares in the improved Neubauer's chamber. Each small square is again subdivided into 16 (4×4) smaller squares. Area of each small square is $1/5 \times 1/5$ mm or $1/25$ mm². For counting

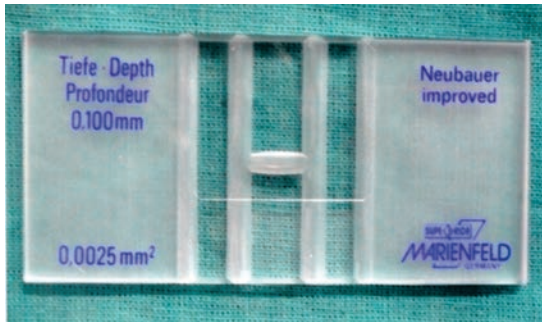


Fig. 4.3: Improved Neubauer's chamber

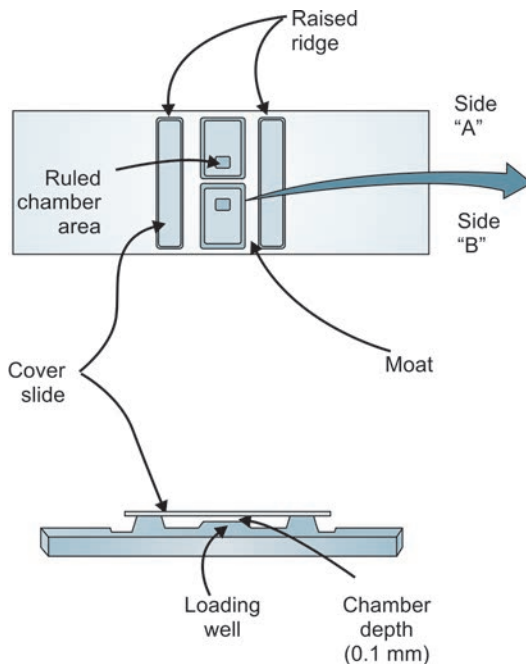
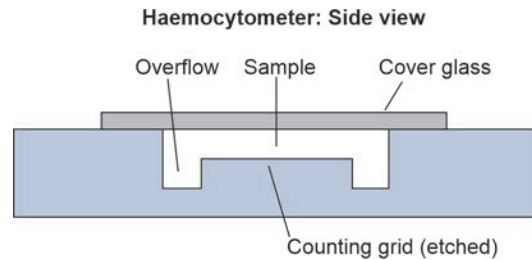


Fig. 4.4: Schematic diagram of using improved Neubauer's chamber

RBCs, this central ruled area is used. Usually four corners and one central square ruled area (blackened in picture) are used total RBC count. This area is also used for platelet count and sperm count from seminal fluid.

WBC Pipette (Fig. 4.7)

The pipette is marked 0.5, 1 below the bulb and 11 above the bulb. There is a white-coloured bead inside the bulb.

Uses

- Total WBC count of blood (TLC).
- Total cell count from different body fluids like pleural fluid, CSF (cerebrospinal fluid, peritoneal fluid).
- Total platelet count, if the platelet count is very low.
- Total sperm count if the sperm count is low.

Blood or body fluid or seminal fluid is taken by WBC pipette and four corners of the haemocytometer are changed for cell counting.

RBC Pipette (Fig. 4.8)

The pipette is marked 0.5, 1 below the bulb and 101 above the bulb. There is a red-coloured bead inside the bulb.

Uses

- Total RBC count of blood
- Total platelet count of blood
- Total sperm count from seminal fluid
- Total WBC count in case of leukaemias when there is very high WBC count and counting in the four corners may be problematic.

Blood or body fluid or seminal fluid is taken by RBC pipette and central ruled area of the haemocytometer is charged for cell counting.

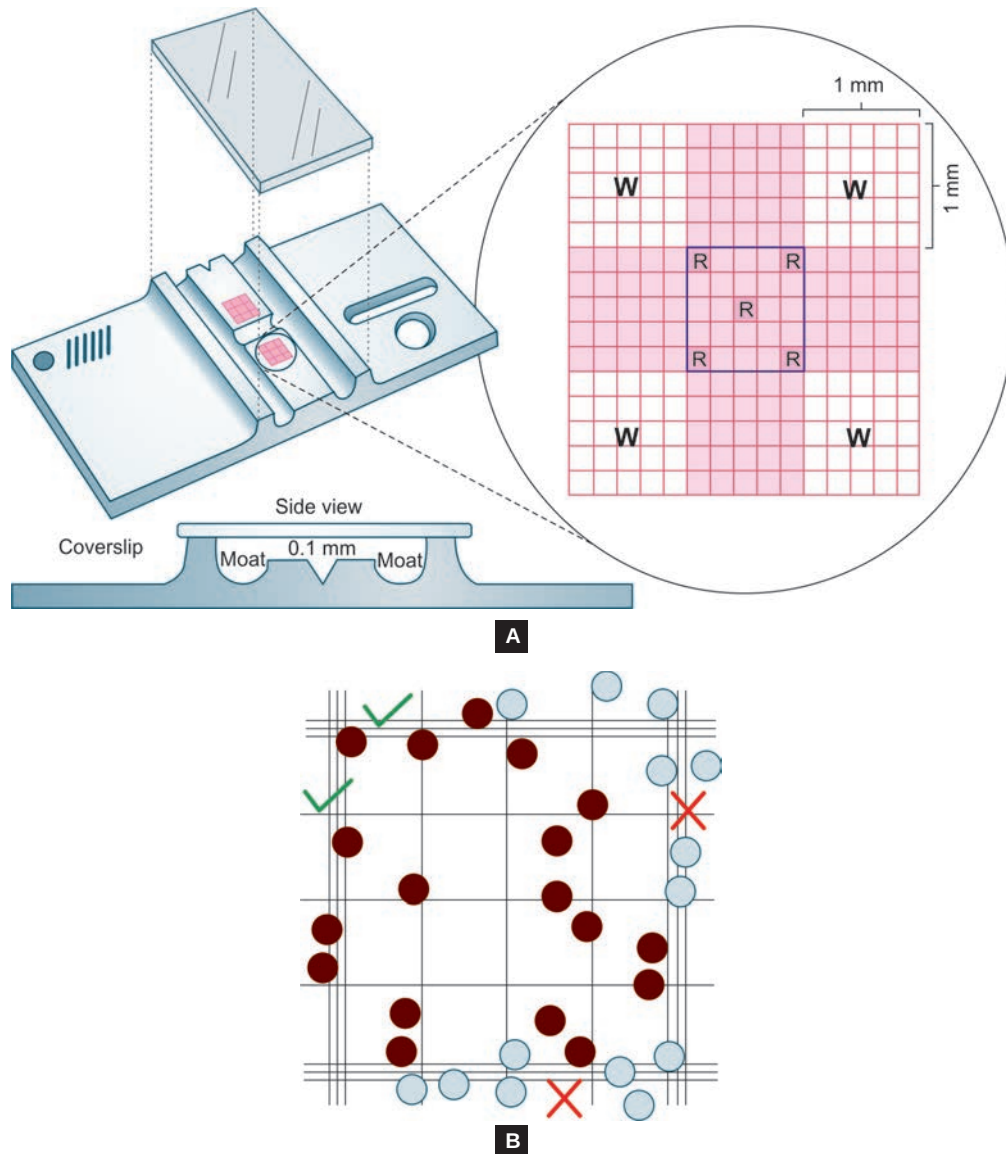


Fig. 4.5A and B: (A) Counting of WBC and RBC in the chamber; (B) Right way of counting cells. Cells on the upper and left triple lines are counted (black-coloured circles) while on right and lower triple lines are not counted (white-coloured circles)

Thick Coverslip (Fig. 4.9)

A specially made coverslip which has very smooth surface and even thickness of 0.3 mm or 0.4 mm or 0.5 mm is used. Commonly, coverslip with a thickness of 0.4 mm is used. Two sizes of coverslip are available in the market. One is 16 × 22 mm coverslip which is used for single ruled haemocytometer. The

other coverslip is 22 × 23 mm which is used for double-ruled counting chamber.

Differences between RBC pipette and WBC pipette are given in Table 4.1.

White Blood Cell Count (Manual Method)

Blood is collected up to mark 0.5 of WBC pipette, either from EDTA or oxalate mixed

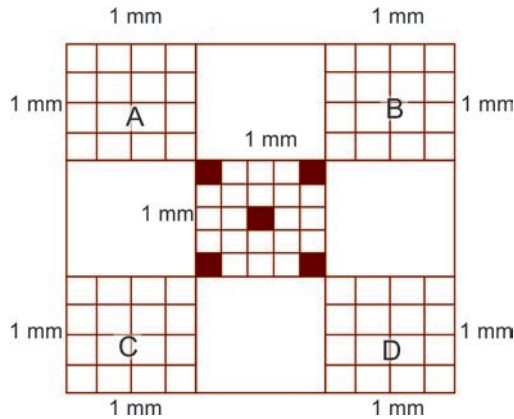


Fig. 4.6: Counting chamber in improved Neubauer's chamber

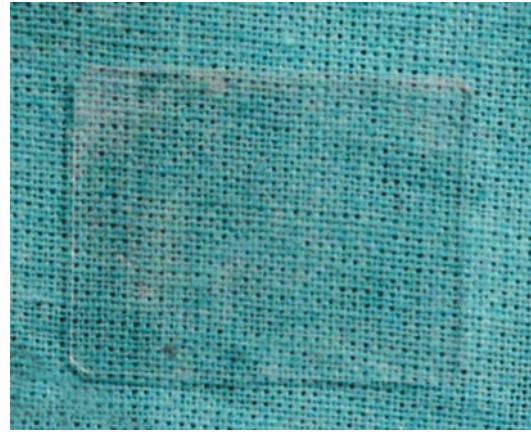


Fig. 4.9: Thick coverslip



Fig. 4.7: WBC pipette



Fig. 4.8: RBC pipette

(anticoagulated) venous blood or after finger prick (fresh blood without anticoagulant). WBC diluting fluid is then sucked up to the mark 11 above in the WBC pipette. Well mixed the blood and WBC fluid in the

Table 4.1: Differences between RBC pipette and WBC pipette

1. It has a red bead	1. It has a white bead
2. It has graduations up to mark 101	2. It has graduations up to mark 11
3. Size of bulb is larger	3. Size of bulb is smaller
4. Size of lumen is smaller	4. Size of lumen is larger

pipette by rotating the pipette between fingers by rotation. Then keep it over the table for few minutes (5–10 minutes). The red cells are lysed by the diluting fluid (acetic acid) but the leukocytes remain intact. Nuclei of the WBCs stain deep violet black by methylene blue or gentian violet stains.

Composition of WBC Diluting Fluid

- Glacial acetic acid: 2 ml
 - Distilled water: 100 ml
 - Aqueous methylene blue Solution (0.3%, w/v): 10 drops*
- Or
- 1% aqueous solution of gentian violet: 1 ml.

* Aqueous methylene blue solution is prepared by dissolving 0.3 gm of methylene blue in 100 ml of distilled water. Filter it before use. Alternatively, gentian violet (1 ml of 1% solution, w/v) can also be used instead of methylene blue (0.3%, w/v).

Dilution of Blood

If the blood is taken up to 0.5 mark, then the dilution is 1 in 20. Because the mark 11 above the bulb is to indicate the total volume of the marked portion of WBC pipette. Firstly, the blood is sucked (up to 0.5 mark), then the WBC fluid is taken. So, blood moves up and is diluted in the bulb and above up to mark 11 (from mark 1 to mark 11), whereas the long bar in the pipette contains only WBC fluid (up to mark 1). So, 10 volumes (11–1 = 10) contain 0.5 ml volume of blood. Hence, the dilution is 1 in 20 or 1:20.

Charging (Filling) the Improved Neubauer's Chamber

- Chamber and coverslip should be clean and dry.
- Hold the WBC pipette slightly inclined and pressure is released slowly with finger so that a small volume of WBC fluid mixed blood is allowed in the chamber under coverslip. This will be accomplished by the capillary action. Blood outside the pipette is wiped with tissue paper or cotton.
- The WBCs are allowed to settle for 2–3 minutes, so that they can be seen in the same plane of focus.

Counting of Leukocytes

WBC count is made under high power (40X) objective; but low power (10X) objective is used to focus them. The WBCs in each of the four large (1×1 mm) corner squares (ruled area) are counted, each of this large square has 16 small squares or division. Only WBCs within the squares are counted and cells lying on lines of any two adjacent sides (top and right, or bottom and left) are included in total WBC count.

The volume of each large square = $1 \times 1 \times 1/10$ mm (depth)

So, the volume of four large squares = $1 \times 1 \times 1/10 \times 4$ mm³ or cu mm = $2/5$ mm³

Let the leukocyte count (WBC) in four large squares is N.

Then, $2/5$ mm³ of volume of blood contains leukocytes = $N \times \text{dilution factor}$

Or, $2/5$ mm³ blood contains leukocytes = $N \times 20$

Or 1 mm³ blood contains leukocytes = $N \times 20 \times 5/2 = N \times 50$.

Note

- As many leukocytes as possible should be counted; a reasonable and practical figure is to count 100 cells. If 400 cells are counted then chances of error will as low as 5%.
- Causes of error: These include mistaking clumped red cell debris or debris or dirt for leukocytes or clumped leukocytes. Clumped leukocytes are usually seen in blood with several hours storage or in heparinized blood (>25 IU/ml of blood).
- Filing or charging defects leading to error:
 - Chamber area incompletely filled
 - Air bubble anywhere in the chamber area
 - Any dirt or debris in the chamber (unclean or moist chamber).
 - Overflow of the counting and ruled area.
- Nowadays, bulb pipettes are not recommended because they are easily broken. The volumes of blood used are unnecessarily small and the WBC pipette is difficult to handle. Particularly charging the chamber with pipette is very difficult and needs experience. So, 20 µl micropipette (or Pasteur pipette) is used which is easy to handle.

Red Blood Cell Count (Manual Method)

Blood is collected up to the mark 0.5 in the RBC pipette, either from EDTA or oxalated mixed (anticoagulated) venous blood or after finger prick capillary blood. Wipe tip clean. RBC diluting fluid is then sucked up to the mark 101 in the RBC pipette. Clean the tip of pipette again. It is well mixed and shaken for 2–3 minutes. First few drops are discarded and then counting chamber

(central ruled area) is charged. It is then kept in a moist chamber or in a Petri dish with wet filter paper and allowed to settle the RBCs for 10–15 minutes; so that they can be seen in the same plane of focus.

Composition of RBC Fluid

- Formalin (40% formaldehyde): 1 ml
- Trisodium citrate: 3.13 g
- Distilled water: 100 ml
- Tinge of eosin (optional)

Formalin acts as a preservative and prevents undesirable growth of microorganism/fungus in the diluting fluid. Eosin tinge is added to distinct the RBCs quickly but not to stain RBCs.

Alternatively, Hayem's fluid may be used (costlier than previous diluting fluid).

Composition of Hayem's Fluid

- Sodium chloride (NaCl): 1 g
- Sodium sulphate: 5 g
- Mercuric chloride: 0.5 g
- Distilled water: 100 ml

Remember the RBC diluting fluid should be isotonic so that RBCs are not lysed. Normal saline can be used if there is no diluting fluid available RBCs and in emergency. But normal saline cause crenation of RBCs and may form rouleaux. Hence, not recommended for use.

Counting of RBCs

Red cells are counted in the central ruled area of the improved Neubauer chamber. In the central large square (1×1 mm) there are 25 small squares ($1/5 \times 1/5$ mm). Four corners and central small squares are counted for RBCs. Let the RBC count in 5 small squares is N.

$$\begin{aligned}\text{Volume of 5 small squares} &= 1/5 \times 1/5 \times 1/10 \times 5 \\ &\quad (\text{depth is } 0.1 \text{ mm or } 1/10 \text{ mm}) \\ &= 1/50 \text{ cu mm or mm}^3\end{aligned}$$

So, $1/50 \text{ mm}^3$ blood contains RBCs

$$= N \times \text{dilution factor}$$

$$= N \times 200 \text{ (dilution of blood in RBC diluting fluid)}$$

Then, 1 mm^3 blood contains RBCs

$$= N \times 200 \times 50/1 = N \times 10,000$$

Note

- Like WBC pipette, RBC pipettes are also not used nowadays. Instead micropipette or Pasteur pipettes are used.
- Error in counting RBC may be due to filing or charging defects (vide WBC count).
- Errors may also be due to inaccurate apparatus or due to technical errors (bad technique in collecting blood, insufficient mixing of blood specimen).

Platelet Count (Manual Method)

The diluent consists of 1% ammonium oxalate. Not more than 500 ml should be made at a time. The solution should be filtered through a micropipette filter (0.22 mm) and kept at 4°C .

Method: Use venous blood preferably for platelet counts. Finger pricks may cause clumping of platelets. Fill the blood and diluent as described for RBC count and using RBC pipette. Charge the chamber (central ruled area). Keep the haemocytometer in a moist chamber. Wait for 20–30 minutes to settle down the small platelets.

Using high power (40X) objective with reduced condenser aperture, count the platelets in the same squares as indicated the RBC counting (five small squares). Suppose total platelet count is N. Then as for RBC counting.

So, $1/50 \text{ mm}^3$ blood contains platelets

$$= N \times \text{dilution factor}$$

$$= N \times 200$$

Then, 1 mm^3 blood contains platelets

$$= N \times 200 \times 50/1 = N \times 10,000$$

Note

- If platelet count is low, then WBC pipette may be used (dilution 1:20) and four large corner squares are counted for platelets like WBCs ($N \times 50$).
- Charged haemocytometer is kept inside a moist chamber or it can be put in a Petri dish with a moistened or wet filter paper. The moist environment does not allow evaporation of platelet fluid. Adequate time (20–30 minutes) is given to settle down the platelets.
- Platelets under high power objective (light microscope), are small (but not minute) highly refractile particles. Platelets appear bluish and must be distinguished from debris.

ELECTRONIC METHOD FOR BLOOD CELL COUNT

Total WBC count along with other routine haematologic investigations are often done by using electronic instrument like 'Coulter counter' (Fig. 4.10A and B).

The Coulter analyzers utilize the electrical impedance with low-frequency electro-magnetic current, high frequency electro-

magnetic current, laser light scattering, light scattering and absorbance depending on the device used. The compact semiautomated Coulter system provides only the basic WBC count, RBC count, haemoglobin level, haematocrit value. On the other hand, the Coulter MAXM, ONYX, STKR and STKS use positive identification bar code system and close vial sampling. Eighteen to twenty parameters may be assessed, including WBC count, RBC count, platelet count, haemoglobin level, haematocrit value, MCV, MCH, RDW (red cell distribution width) and MPV (mean platelet volume).

Rapid performance, minimal or no technical error, elimination of visual error and biasness, more accurate and precise results are advantages of electronic system over manual or haemocytometer method. In many laboratories, use of manual methods by haemocytometer has become obsolete. Only if the result is very very low or very very high, then it is used (with more dilution for very very high count) as it may be outside the range of the electronic machine.



Fig. 4.10A and B: Automated blood cell counters

Q1. How to identify WBC pipette?

Ans: By marks 0.5 and 1 below the bulb and mark 11 above the bulb. Also, a white bead inside the bulb.

Q2. What is the use of WBC pipette?

Ans:

- Used for total WBC count
- Sperm count in semen
- Eosinophil count
- Also may be used to count cells in any fluid where the count is more ($>1000/\mu\text{l}$), like cell count in CSF, pleural/ascitic fluid.
- RBC count in severe anaemia where RBC count is low.

Q3. How to identify RBC pipette?

Ans: By marks 0.5 and 1 below the bulb and mark 101 above the bulb. Also, a red-coloured bead inside the bulb.

Q4. What is the use of RBC pipette?

Ans:

- Used for total RBC count
- For total platelet count
- For sperm count in semen
- High TLC count

Q5. What is the electronic method for counting WBC? What are the advantages and disadvantages?

Ans: Electronic counter is based on aperture impedance method, or light scattering technology, or both. In this method, particles (WBCs) passing through a chamber in single file scatter the light and convert by a chamber in single file scatter the light and convert by a detector into pulses proportionate to the size of the cells, which are counted electronically. During counting of WBCs, a lysate is used to lyse RBCs.

Advantages

- Easy and rapid method
- High level of precision

- Very large number of cells is counted quickly

- Time saving method

Disadvantages

- The instrument is costly, so beyond the scope of small size laboratory.
- Calibration to be done at regular interval, otherwise there will be error.
- Normoblasts (nucleated RBCs) are counted as leukocytes
- Clumps of platelets are also falsely counted as WBCs.

Q6. What is electronic method of counting platelets? Is it advantageous or disadvantageous?

Ans: The principal is electrical impedance like counting RBCs.

Platelet counting by electronic method is disadvantageous.

Disadvantages

- Debris and fragments of blood cells (small size) are counted as platelets.
- Howell-Jolly bodies and Heinz bodies are also counted as platelets.
- Equipment is costly and calibration error may occur.

Q7. How do you differentiate between platelets and dust particles?

Ans:

- Platelets are refractile
- Platelets are stained light blue when stained by brilliant cresyl blue. Dust particles cannot be stained.
- Platelets have brownian movements but dust particles lack it.

Q8. Which is the best method for absolute eosinophil count (AEC)? What is the composition of diluting fluid for eosinophil count?

Ans: The best method is the automated counter which detects eosinophil peroxidase during

counting. Other methods are Fuchs-Rosenthal counting chamber or hemocytometer or using Dunger's solution. Other dilution fluids are Randolph's diluting fluid, Pilot's stain.

i. Dunger's solution:

- Acid dye, e.g. eosin or phloxine, 0.1% aqueous solution
- Water to lyse RBCs and rupture leukocytes

ii. Randolph's fluid:

- Stock solution A (methylene blue, propylene glycol, distilled water)
- Stock solution B (phloxine, propylene glycol, distilled water)
- Working solution is prepared from above stocks 1:1 volume

iii. Pilot's stain:

- Propylene glycol (50 ml), distilled water 40 ml, phloxine 0.1 aqueous solution, 10 ml; sodium carbonate 1% aqueous solution, 1 ml.

Q9. Why special coverslip not the ordinary one is used while doing various counts by Neubauer chamber?

Ans: Neubauer chamber and its coverslip (thicker than the ordinary coverslip) are so designed that after charging the chamber a fixed/desired amount of fluid is present in-between the ruled chamber and overlying coverslip. And the depth of fluid 0.1 mm (prerequisite for calculation). The ordinary coverslip is thin and light. Fluid depth is not 0.1 mm, so cell counts become

erroneous. Moreover, coverslip of Neubauer chamber is uniformly flat while ordinary cover glass is not.

Q10. How do you clean Hb, WBC and RBC pipettes?

Ans: These pipettes are filled with distilled water and cleaned by blowing it out twice. Then, acetone is sucked into the pipette and blown it out. Acetone removes residual water within pipette and dries it completely. When pipette is completely dry, the bead with the bulb moves/rolls freely but if it is wet, bead does not roll freely.

Q11. How sperm count is done in Neubauer chamber?

Ans: Semen diluting fluid (sodium bicarbonate 5 g, neutral formalin 1 ml, and distilled water to make 100 ml). Semen may be diluted in a test tube (0.1 ml semen + 1.9 ml diluting fluid) or in a WBC pipette (draw semen to the mark 0.5 and diluting fluid to the mark 11) and mix well. After charging improved Neubauer chamber, wait for 2–3 minutes to settle down the sperms.

Calculation: Count spermatozoa in 2 mm^3 (two large squares at two corners) and multiply by 100,000 or 1 lakh. It gives the number of spermatozoa per ml of semen (unlike in terms of per mm^3 of WBC count).

Normal range: 60–150 million/ml. Count below 20 million/ml is considered abnormal.

Erythrocyte Sedimentation Rate (ESR) and Packed Cell Volume (PCV)

ERYTHROCYTE SEDIMENTATION RATE

The erythrocyte sedimentation rate (ESR) is the rate at which red blood cells sediment in a period of one hour.

It is also known as sedimentation rate (sed rate).

Brief History of ESR

This test was invented by the Polish pathologist Edmund Biernacki in 1897. In some regions of the world, the test continues to be referred to as Biernacki's reaction. In the year 1918, the Swedish pathologist Robert Sanno Fahraeus along with Alf Vilhelm Albertsson Westergren described the same and is eponymously remembered as Fahraeus-Westergren test. But in the UK, it is usually termed Westergren test which became popular throughout world.

Mechanism of Erythrocyte Sedimentation

The ESR or rate of fall of red cells is governed by the balance between pro-sedimentation factors, mainly fibrinogen and those factors resisting sedimentation, e.g. the negative charge of the erythrocyte (zeta potential). The decreased zeta potential promotes rouleaux formation and hence raised ESR.

ESR depends upon the difference in specific gravity between red cells and plasma. Also,

it is greatly influenced by the extent to which RBCs form rouleaux, which sediment stacks of RBCs than single cell. Many other factors are also responsible which include the ratio of RBCs to plasma (i.e. PCV), the plasma viscosity, the bore of the tube, dilution, if any of the blood, the verticality of ESR tube, etc.

The very important factor is rouleaux formation and red cell clumping. This is mainly controlled by the plasma concentration of fibrinogen and other acute phase proteins/reactants, e.g. haptoglobin, ceruloplasmin, α_1 -antitrypsin and C reactive protein (CRP). Rouleaux formation is also enhanced by increased concentration of plasma immunoglobulin. On the contrary, it is retarded by higher concentration of albumin and test done with defibrinated blood (≤ 1 mm/hr), which removes fibrinogen. In anaemia, there may be quantitative deficiency of RBCs, so ratio of RBCs to plasma is altered which results more rouleaux formation and increased ESR.

Methods of ESR Estimation

- i. Westergren method
- ii. Wintrobe method
- iii. Landau method: Not accurate, used in the past in children with limited supply of blood. The method uses capillary blood from heel, toe or fingertip.

iv. Electronic method by automated analyzers.

The first two methods are commonly used methods which are done by manual technique. Of these two, Westergren method is most popular and commonly used method.

Westergren Method using Westergren Pipette (Fig. 5.1)

Though the equipment is sometimes called Westergren tube, it should be called pipette (more scientific term) as it is open at both ends.

Westergren pipette is a slender, thick-walled pipette. It is 300 mm long (i.e. 30 cm or 12 inches) of which lower 200 mm is graduated (markings) and upper 100 mm part is ungraduated. The inner diameter of pipette should not be less than 2.5 mm. The capacity of the tube is about 1 ml.



Fig. 5.1: Westergren pipette

Anticoagulant used: 3.2% trisodium citrate solution (no other anticoagulant with this method), 109 mmol/L (32 g/L, $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$). EDTA mixed blood is used in modified Westergren method.

Blood and anticoagulant ratio: For this test 2 ml of venous blood is mixed with 0.5 ml of sodium citrate anticoagulant. So, the blood and anticoagulant ratio is 4:1.

Method or procedure: 0.5 ml of 3.2% sodium citrate solution is taken in a test tube. Then, 2 ml of venous blood (usually from antecubital vein) is mixed with the anticoagulant immediately with the help of a syringe. The sample is well mixed.

The anticoagulated blood is drawn up in to Westergren pipette up to 200 mm mark with the help of a teat or mechanical device (mouth suction is avoided). The pipette is

now set exactly vertical in a Westergren stand (Fig. 5.2). A spring clip, pressing on the top and rubber piece at the lower end hold the pipette in the Westergren stand or rack. Now the pipette in the rack which is vertically placed is kept at room temperature without vibration and exposure to sunlight.

Modified Westergren method: It produces the same results but uses EDTA blood rather than citrate as an anticoagulant. In this method, 2 ml of EDTA blood is diluted with 0.5 ml of 0.85% sodium chloride or with 0.5 ml of 3.8% sodium citrate. Precision is poor when EDTA blood is undiluted.

This method has the advantage that same EDTA-mixed blood may be used for other haematologic studies.

Recording result: The RBCs begin to settle down and a clear plasma zone is formed above the settled RBCs. The upper level of the RBC column is read (zero mark is upside and 200 mark is downside) at the end of one hour.

The measurement in mm is ESR (Westergren, 1 hour).

Previously it was thought that mean average ESR per hour (total duration 2 hours) is more accurate.

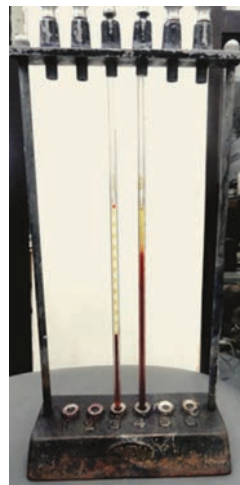


Fig. 5.2: Westergren or ESR stand (rack)

Mean ESR = (1st hour result + $\frac{1}{2}$ of 2 hours result) $\div$ 2

But later on, concept of mean ESR disregarded because result after 1 hour gives more accurate result.

Normal range of ESR: The ESR gradually increases with age. Westergren's original upper limit of normal (10 mm/hour for men and 20 mm/hour for women) seems to be low. According to studies by scientists, upper limits of reference values in this method should be as follows:

	Men	Women
i. Below age 50 years	0–15 mm/hr	20 mm/hr
ii. Above age 50 years	0–20 mm/hr	30 mm/hr
iii. Above age 85 years	0–30 mm/hr	42 mm/hr

But in Indian context, acceptable normal range for ESR is

- Men: 4–14 mm/hr
- Women: 6–20 mm/hr
- Newborn: 0–2 mm/hr
- Newborn to puberty: 3–13 mm/hr

Wintrobe's Method

Instrument: Wintrobe's tube is a special, thick-walled glass tube 11 cm long with an internal diameter of 3 mm and the bottom 10 cm are graduated. It has flat inner base. It is calibrated at 1 mm intervals to 100 mm and holds about 1 ml of blood. Graduations are from zero (top) to hundred (bottom) for ESR and zero (bottom) to hundred (top) for PCV.

Anticoagulant: Venous blood is mixed with EDTA (preferred), or double oxalate or heparin.

Methods

1. 2 ml venous blood is collected and mixed with anticoagulant (EDTA) in the vial immediately.

2. Wintrobe tube is filled with this blood till zero mark on top with the help of a Pasteur pipette.

3. The tube is now kept vertically over stand for one hour and is noted by measuring the free plasmatic zone above (descending orders, 0 marks on top and 100 marks on bottom).

Normal range: Men 0–9 mm/hr, women 2–20/hr.

Stages of Sedimentation (ESR)

Stage 1. Rouleaux formation: In this stage (first 15 minutes) red cells form rouleaux and minimum sedimentation occurs.

Stage 2. Formation of fine threads by proteins: During this stage (second 15 minutes) fibrinogen and globulin in plasma form network by forming fine threads. The rouleaux of red cells are trapped within this network and becomes heavier. So, they begin to fall (settling) rapidly.

Stage 3. Rapid fall of protein network: In this phase (third 15 minutes), red cell mass and protein network fall rapidly.

Stage 4. Packing of red cells: In the last 15 minutes, the sedimented red cell mass—protein undergoes packing at the bottom of pipette.

MODIFIED WESTERGREN METHOD

Disposable ESR Pipette (Westergren Method)

A disposable ESR pipette offers a highly accurate, risk free, easy, efficient and safe method for performing *Westergren's ESR determination* directly in a 12 × 75 mm/13 × 75 mm non-vacuum/vacuum blood collection tube (Fig. 5.3A and B).

Disposable ESR pipette is made of clear polystyrene, which gives a clear visibility of the blood for determining ESR. A biodegradable vacuum plug at the bottom,



Fig. 5.3A and B: ESR estimation with disposable plastic pipettes (modified Westergren method)

which creates pressure on the blood in the 12×75 mm non-vacuum or vacuum blood collection tubes and thus the blood raises up in the pipette till the zero level mark, which acts as a barrier that stops hazardous substances (blood) from escaping through the top of the pipette.

Advantages of a Disposable ESR Pipette

1. A single time use of the pipette.
2. It avoids mouth pipetting and ensures user safety.
3. Can be used with almost all tubes like 12×75 mm/ 13×75 mm Non-vacuum/vacuum tubes.
4. Highly accurate, reproducible, risk free, easy, efficient and safe method of conducting an ESR.
5. Fibrous barrier protects user from hazardous aerosols.
6. Speed of application.

Procedure

1. Collect 1.6 ml of whole blood in 0.4 ml of sodium citrate 3.8% solution or take 1.6 ml of EDTA anti-coagulated whole blood in 0.4 ml of saline (use 12×75 mm non-vacuum blood collection tubes with 0.4 ml of sodium citrate 3.8% solution).

2. Mix the blood gently.
3. Gently insert the lower end of pipette bearing the vacuum plug in to the blood. Collect ion of tube and using continuous force, push the pipette down to the bottom of the blood collection tube.
4. The blood will automatically rise into the pipette and stop at the "zero" mark.
5. Place the assembly (tube + pipette absolutely vertical on a suitable stand (example rack for ESR pipette). And allow the blood cells to sediment without disturbing it for 60 minutes.
6. At the end of 60 minutes the numerical results are read in millimeters directly from the imprinted scale on the pipette.

Errors Faced while Using a Disposable Pipette

1. If the blood collection tube is cracked
2. If the ESR pipette has not been placed absolutely vertical
3. If the blood collection tube is not gently mixed
4. If the proportion of the blood and sodium citrate/EDTA is improper
5. If the disposable ESR pipette is reused.

VACUETTE® ESR BLOOD COLLECTION TUBES

Instruction for Use

Intended Use

VACUETTE® ESR tubes are used for the collection and transport of venous blood for blood sedimentation rate testing. ESR measurements refer to the Westergren method.

Product Description

VACUETTE® ESR tubes are plastic tubes with a pre-defined vacuum for exact draw volume. They are fitted with colour-coded VACUETTE® Safety Cap (13/75 mm tube) and Brom Butyl Caoutchouc Cap (9/120 mm tube). The tubes, additive concentrations, volume of liquid additives, and their permitted tolerances, as well as the blood-to-additive ratio are in accordance with the requirements and recommendations of the international standard ISO 6710 "Single-use containers for venous blood specimen collection".

The VACUETTE® ESR tubes contain a 3.2 % buffered tri-sodium citrate solution (0.109 mol/L). The mixing ratio is 1 part citrate solution to 4 parts blood. Tube interiors are sterile.

Storage

Store tubes at 4–25°C (40–77°F).

Note

Avoid exposure to direct sunlight. Exceeding the maximum recommended storage temperature may lead to impairment of the tube quality (i.e. vacuum loss, drying out of liquid additives, colouring, etc.)

Handling**Closed VACUETTE® ESR System (Fig. 5.4)**

Equipment required for ESR measurements:

- A 9/120 mm, graduated, plastic tube with a citrate solution. Draw volume 1.5 ml and 2.75 ml.
- A 9/120 mm glass tube with a citrate solution. Draw volumes of 1.6 ml or 2.9 ml are available.
- ESR rack with scale suitable for 1.5 ml/1.6 ml tubes, respectively ESR rack with scale suitable for 2.75 ml/2.9 ml tubes.

Procedures

After blood sampling and also before starting the ESR measurement, gently invert the tube 5–10 times to obtain the correct mixture. Use of a rotating mixer is recommended.

Note

It is recommended to do the determination within the first 4 hours when stored at room temperature. If longer storage is required, keep the specimen at the refrigerator (maximum 24 hours). Note that the sample must be brought to room temperature before use.

1. Place 1.5 ml, 1.6 ml or 2.75 ml, 2.9 ml tube into the corresponding rack vertically. Align the 0 mark at top of scale with the bottom of the meniscus of the blood at the blood-air interface.

For the 1.5 ml/1.6 ml VACUETTE® ESR tube set timer for 30 minutes. The ESR



Fig. 5.4A and B: VACUETTE® ESR system

rack suitable for 1.5 ml/1.6 ml tubes delivers only the 1 hour Westergren value after 30 minutes reading time.

For the 2.75 ml or 2.9 ml ESR tube set timer for 60 minutes. The ESR rack for 2.9 ml tubes delivers the 1 hour and if required 2 hour Westergren value after 120 minutes reading time.

2. Discard VACUETTE® ESR tubes without opening.

Note

The conversion scale becomes highly compressed above Westergren values of 100 mm and ESR readings above this level should be repeated using the classic Westergren method if precise values are required.

1.5 ml and 1.6 ml tubes can be used with the following VACUETTE® ESR instruments: SRT 10/II, SRS 20/II, SRS 100/II.

The instrumentation allows for 1 hour Westergren results after 15 minutes or 30 minutes.

(For further information contact Greiner Bio-One or see "VACUETTE® Automated ESR Systems Brochure")

Open VACUETTE® ESR System

The system consists of 3 parts:

1. A 13/75 mm plastic tube with a citrate solution.
2. A graduated pipette with rubber adapter.
3. ESR rack without any scale.

Procedure

After blood sampling and also before starting the ESR measurement gently invert the tube 5–10 times to obtain the correct mixture. Use of a rotating mixer is recommended.

Note

It is recommended to do the determination within the first 4 hours when stored at room temperature. If longer storage is required, keep the specimen at the refrigerator (maximum 24 hours). Note that the sample must be brought to room temperature before use

1. Remove the cap of the tube.
2. Insert the pipette into the opened tube and the blood will fill automatically to the zero-line of the pipette.

Note

If there is a bubble in the column of the pipette, the determination is not valid!

1. Place tube and pipette into the suitable rack. Tube and pipette must be in a vertical position.
2. After 60 and if required 120 minutes, read level between settled erythrocytes and the supernatant plasma from pipette.
3. Afterwards dispose of the tube and pipette together in a suitable biohazard disposal container.

Disposal

1. The general hygiene guidelines and legal regulations for the proper disposal of infectious material should be considered and followed.
2. Disposable gloves prevent the risk of infection.
3. Contaminated or filled blood collection tubes must be disposed of in suitable biohazard disposal containers, which can then be autoclaved and incinerated afterwards.
4. Contaminated ESR pipette and VACUETTE® tubes must be disposed of together in suitable biohazard disposal containers for infectious material.

Disposal should take place in an appropriate incineration facility or through autoclaving (steam sterilisation).

Use of ESR

1. Diagnosis

- a. **Marked elevation:** Multiple myeloma, macroglobulinaemia, tuberculosis, hyperfibrinogenemia, myocardial infarction, temporal arthritis, rheumatoid arthritis, chronic kidney disease, SLE, inflammatory bowel disease, polymyalgia rheumatica.

- b. Moderate evaluation:** Chronic infection (chronic osteomyelitis, chronic lung abscess, chronic bronchiectasis), rheumatoid arthritis, neoplasms (Hodgkin lymphoma, carcinomatosis, leukaemia), infective endocarditis, physiological (pregnancy), drugs (oral contraceptives, methyldopa, dextran, vitamin A, theophylline).
- 2. Disease severity assessment:** ESR is a component of PCDAI (Paediatric Crohn's Disease Activity Index), an index for assessment of severity of inflammatory bowel disease in children.
- 3. Monitoring response to therapy:** ESR has limited role to monitor the response to therapy in certain inflammatory disease such as rheumatoid arthritis, polymyalgia rheumatica and temporal arthritis. In Hodgkin lymphoma, ESR can be used as a crude measure to response. Also, it is used to define one of the several possible adverse prognostic factors in staging of Hodgkin lymphoma.
- Heparin alters the membrane zeta potential of RBCs and cannot be used as an anticoagulant.
 - Tilting the pipette accelerates the ESR. The RBCs aggregate along the lower side, whereas the plasma rises along the upper side. Subsequently, the retarding of influence of the rising plasma becomes less effective. An angle of 3° from vertical position, may accelerate the ESR by as much as 30%.
 - Plasma factors: An accelerated ESR is seen in elevated levels of fibrinogen and to a lesser extent of globulins. Albumin retards ESR. High rise of plasma viscosity also retards ESR. Cholesterol increases and lecithin decreases ESR.
 - The test should be done within two hours. If the blood is stored for more than two hours, ESR will increase.
 - If blood is kept in refrigerator, ESR is highly increased. So, refrigerated blood should be allowed to return to normal room temperature before the test started.
 - Temperature of the environment: The ideal temperature for the test is $20-25^\circ\text{C}$. Increase in temperature is directly proportional to increased ESR.
 - Bubbles left in the pipette, when the blood is filled, will affect ESR. The cleanliness of pipette is also important.
 - Haemolysis may modify ESR.

Causes of Slow or Decreased ESR

- Polycythaemia vera
- Sick cell anaemia, spherocytosis, poikilocytosis
- Congestive heart failure
- Stages of severe dehydration like cholera, acute gastroenteritis
- Infections: Typhoid and undulant fever, trichinosis, malarial paroxysm, pertusis.
- Allergic states
- Drugs: Aspirin, cortisone, quinine.

In case of sickle cell anaemia, spherocytosis or poikilocytosis, there are abnormal red cells. These abnormalities of RBCs prevent rouleaux formation. Hence, decreased ESR.

Sources of Error and other Interfering Factors

- If the concentration of anticoagulant is higher than recommend the ESR may be elevated.
- Provide more rapid results
- Use small sample volumes
- Save technician time
- Provide increased safety because the need for sample manipulation is decreased.

Different Automated Methods of ESR

(Fig. 5.5A and B)

- Ves-Matic
- ESR STAT-PLUS
- SEDIMAT
- Zeta sedimentation

Advantages of Automated Methods

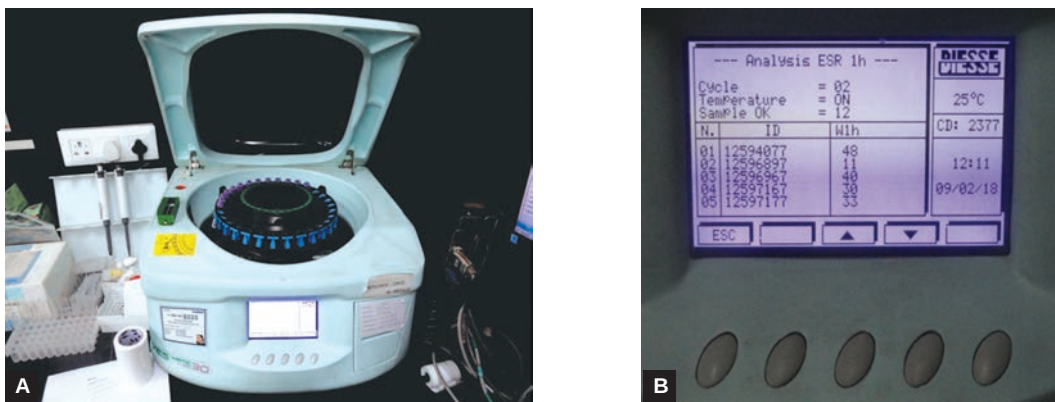


Fig. 5.5A and B: (A) Automated ESR machine; (B) Recording of ESR result

Zeta Sedimentation Rate (ZSR)

EDTA mixed blood (0.2 ml) is filled in a special capillary tube and is centrifuged in special apparatus (zeta fuge, Coulter electronics) for four times, each for 45 seconds. The capillary tube is mechanically rotated at 180° and centrifugation is done in reverse direction at every 45 seconds for four times. The red cell rouleaux develops better and travel down the capillary tube by alternate compaction and dispersion.

Result of ZSR: ZSR is expressed in terms of percentage.

Normal range in adults 40–50%. Rise in ZSR indicates rise in ESR.

Zeta crit: It is the ratio of the height of red cells to the total height of blood column.

Advantages of ZSR

- Requires small amount of blood (0.2 ml)
- No dilution is required.
- Eliminates the effect of anaemia.
- It is more sensitive than Westergren's method of ESR estimation.
- It requires minimum time.

Micro-ESR Method

Barrett (1980) described this micro-ESR method using 0.2 ml of blood to fill a plastic disposable tube 230 mm long with 1 mm inner diameter or internal bore. Both venous

and capillary blood are suitable for this method. The tube filled with blood is kept vertically on a stand and the result is read after one hour.

This method has more utility in paediatric patients.

WINTROBE'S HAEMATOCRIT (PACKED CELL VOLUME)

The term 'haematocrit' theoretically means blood separation. Wintrobe haematocrit tube is mainly used for measurement of packed cell volume (PCV).

Definition of PCV

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

Two methods are employed for measurement of PCV.

1. Macro-method using Wintrobe tube.
2. Micro-method using capillary tube.

1. Macro-method—Wintrobe's Tube (Fig. 5.6)

It is a spherical, thick-walled glass tube 11 cm long and has an internal diameter of 2.5 mm with flat inner base. The tube is calibrated at 1–100 mm intervals and holds about 1 ml of blood. The markings on the tube are in



Fig. 5.6: Wintrobe's tube

reversed directions. Ascending marking is used for determination of PCV and descending marking is used for determination of ESR.

Uses

1. Wintrobe's tube is primarily used for determination of packed red cell volume (PCV) of blood.
2. Also it can be used for determination of ESR especially for anaemia correction with the help of correction curve.
3. Buffy coat smear preparation for demonstration of LE cell and staining with Leishman stain in diagnosis of SLE.
4. Abnormal or blast cells in aleukaemic leukaemia.

Blood and anticoagulant: Venous blood anticoagulated with double oxalate powder, EDTA powder or heparin.

Method of PCV Determination (Wintrobe Tube)

1. 2 ml venous blood is taken and immediately mixed with anticoagulant. Mix well by shaking.
2. The Wintrobe tube is filled with anticoagulated blood with the help of a long Pasteur pipette from the bottom up to mark '0' or '10' above.
3. The tube is then centrifuged at 3000 r.p.m. for 30 minutes.
4. The packed cell volume (PCV) is measured by noting the upper level of column of packed red cells by the markings in ascending order. PCV is expressed as percentage of the total volume of blood.

Zones Separated after Centrifugation

1. The layer of packed red cells or PCV is lower most which is usually 45 to 50%.
2. An intermediate thin layer comprises WBCs and platelets. It is above the lower most layer (red cells). The grey-coloured layer is known as buffy coat. Normally, this layer is 2 to 3%. Buffy coat layer is increased in leukaemia and severe degree of leukocytosis.
3. Upper most layer of plasma: This straw-coloured layer is above buffy coat layer and composed of free plasma. This layer may be pink in haemolysis, yellow in jaundice, and colourless in iron deficiency anaemia.

Normal Range of PCV

- Men: $45 \pm 5\%$, i.e. 40 to 50%
- Women: $41 \pm 5\%$, i.e. 36 to 46%
- At birth: 44 to 62%
- One year infant: 35% (approximate)
- 10 years: 37.5% (approximate)

Increased PCV: Polycythaemia, severe degree of dehydration, cholera, acute gastroenteritis.

Decreased PCV: Anaemia (usually less than 30%)

Sources of Error of PCV

- Inadequate duration and speed of centrifugation.
- Inadequate mixing of blood.
- Excess anticoagulant.
- Irregularity of the bore of Wintrobe tube.
- Trapping of leukocyte—platelet clumps in the tube will result defective red cell packing.

2. Micro-method—Capillary Tube Method for PCV

Nongraduated capillary tube (75 mm in length and about 1 mm internal diameter) is

rinsed with heparin solution (1:2000 heparin or 1 in 1000 dilution). Well dry this heparinised capillary tube at 56°C and stored.

When the test is done a capillary tube (haematocrit) is filled up with blood (finger prick or EDTA mixed venous blood) and the empty end is sealed with a micro-burner. The tube is filled up $\frac{1}{2}$ to $\frac{2}{3}$ of its length (not the entire length). The tube is then fitted on the micro-haematocrit centrifuge and centrifuged at 12000 r.p.m. for 5 minutes with the sealed end away from the centre.

Calculation of result: Then the capillary tube is taken out and PCB is calculated with the help of a millimetre rule commercially available.

Normal range of PCV (Table 5.1)

- Male: $47 \pm 7\%$
- Female: $43 \pm 5\%$

Advantages of Micro-method

- Time requirement for centrifugation is short.

Table 5.1: Normal levels of packed cell volume (PCV) and haemoglobin

Age and sex	Packed cell volume (PCV) %	Haemoglobin (g/dl)
• Adult male	40–50	13–17
• Adult female (nonpregnant)	38–45	12–15
• Adult female (pregnant)	36–42	11–14
• Children, 6–12 years	37–46	11.5–15.5
• Children, 6 months to 6 years	36–42	11–14
• Infants, 2–6 months	32–42	9.5–14
• Newborns	44–60	13.6–19.6

- Small amount of blood is required to fill the capillary tube.
- Cost effective and easy to work.

Generally, PCV% is three times that of Hb g/dl or Hb%. So, if a person has haemoglobin of 15 g/dl, his PCV will be $15 \times 3 = 45\%$ (approx) (Fig. 5.7).

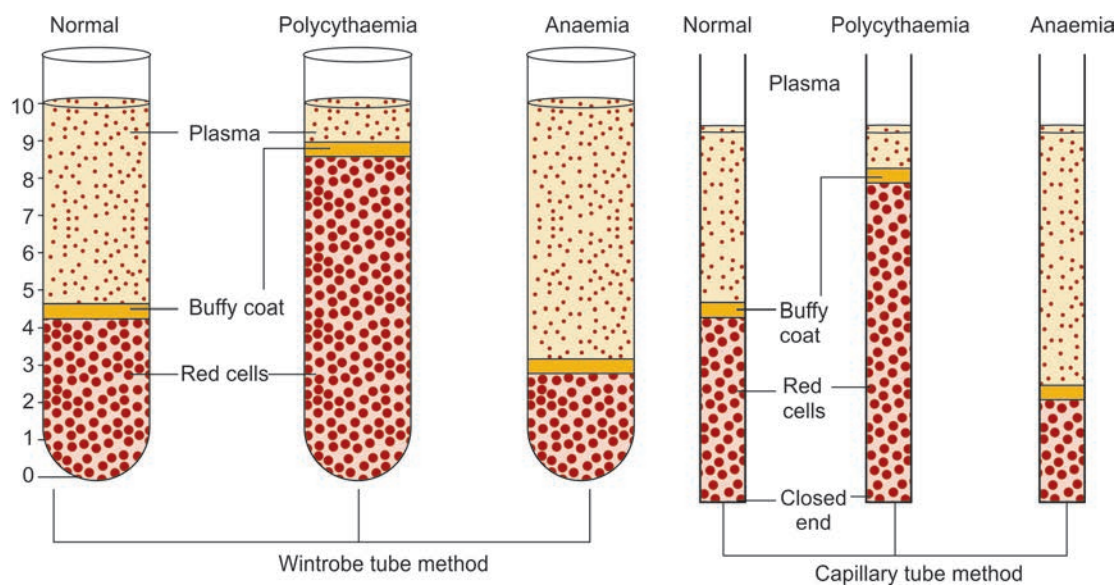


Fig. 5.7: Packed cell volume (PCV) shows comparison of normal, polycythaemia and anaemic blood samples by two different methods (Wintrobe and capillary tube)

Q1. Why ESR is raised in anaemia?

- Ans:** i. In anaemia there is low erythrocyte mass compared to plasma. This change in the ratio of erythrocyte to plasma favours rouleaux formation and quicker sedimentation.
- ii. Microcytes sediment more slowly and macrocytes somewhat more quickly compared to normocytes. The sedimentation of RBCs is directly proportional of RBC aggregates and inversely proportional to the RBC surface area. The microcytes have lower surface area to volume ratio.

Q2. Why ESR is low in sickle cell anaemia and spherocytosis?

- Ans:** Red cells with abnormal or irregular shape (poikilocytosis) hamper rouleaux formation. In sickle cell anaemia, the RBCs are abnormal in shape (sickle or crescentic in shape). So, ESR becomes low because of slower rouleaux formation.
- In spherocytosis, because of the spherical shape (normal biconcave shape) RBCs have more surface area. As ESR is inversely proportional to the RBC surface, this increase in surface area causes decreased ESR.

Q3. How ESR can be used to monitor prognosis of disease?

- Ans:** ESR can be used to see the response to treatment in some diseases like tuberculosis, rheumatoid arthritis, polymyalgia rheumatica and temporal arteritis. If these diseases respond to treatment, the ESR tends to be lower over time.
- In Hodgkin's disease, ESR, of less than 10 mm in first hour indicates good prognosis while ESR of more than 60 mm in first hour indicates poor prognosis.

Q4. Compare Westergren and Wintrobe methods of ESR as far as advantages and disadvantages are concerned.

- Ans:** Westergren method is more sensitive when ESR is high. Because ESR in this method has three phases with a longer second phase, so sinking of RBCs occurs better in a larger tube add longer second phase gives more accurate result when ESR is high.
- But in Wintrobe's tube sinking of RBCs occurs quickly and packing is fast because it has a shorter tube length. So, Wintrobe's method is more sensitive when ESR is low.

Q5. Why Westergren's method is preferred to Wintrobe's method while estimating ESR?

- Ans:** i. When ESR becomes high Westergren method gives more accurate result.
- ii. It is more sensitive because the pipette is longer and there are more markings (graduations).

Q6. What are advantages of Wintrobe's method?

- Ans:** In this method, ESR is estimated first and then the Wintrobe tube is centrifuged to get PCV. Moreover, the colour of plasma gives clues to certain diseases. Yellow plasma indicates jaundice, red-coloured plasma indicates haemoglobinaemia (intravascular haemolysis) and white in hyperlipidaemia (chyle).

Q7. What is automated ESR method?

- Ans:** The blood was drawn into special MONOSED vacutainers of Monitor 100® (1.6 ml, 120 mm long, 6 mm diameter) with 1.28 ml of automatic draw containing 0.32 ml of 3.2% sodium citrate. The blood citrate mix reaches up to a maximum length of 60 mm from the

bottom of the tube. After proper mixing, the samples were immediately transferred to the analyzer. The ESR reading is taken through a 45 mm high window, 2 mm above the maximum sample level. The Monitor 100® has the advantage of giving the result of 100 samples in 30 minutes (equivalent to 1 hour Westergren reading) and 60 minutes (equivalent to 2 hours Westergren reading). The machine Monitor 100® supplied by Electra Lab, Italy. Marked discrepancy in the ESR result was noted for high ESR values when compared between manual and automated methods. But it was not seen for normal ESR values. So, a correction factor to be applied when ESR is very high for this automated method.

Q8. What are the length and diameter of Wintrobe's tube? What amount of blood it can hold?

Ans: The tube has length of 110 mm or 11 cm and internal diameter of 3 mm. It is graduated at 1 mm intervals and marked 0 to 100 mm (10 cm) from above downward and also from below upwards. The tube can hold about 1 ml of blood.

Q9. How PCV is used to determine red cell indices?

- Ans:** i. Mean corpuscular volume (MCV)
It is the average volume of RBC and is calculated from red cell count and haematocrit volume
$$MCV = PCV \text{ in L/L} \div RBC \text{ count/L}$$
(normal value is either 85 ± 8 fl or 77–93 fl)
- ii. Mean corpuscular haemoglobin (MCH)
It is the content by weight of haemoglobin of average red cell.
$$MCH = Hb/L \div RBC \text{ count/L}$$
(normal range is either 29.5 ± 2.5 pg or 27–32 pg)
- iii. Mean corpuscular haemoglobin concentration (MCHC)
It is the average of haemoglobin concentration and haematocrit value which is expressed in terms of PCV (0.45 deciliter normally).

$$MCHC = Hb/dl \div PCV \text{ in L/L}$$
(normal range is either 32.5 ± 2.5 g/dl or 30–35 g/dl).

As MCHC is independent of RBC count and size, it is considered to have greater clinical significance as compared to other red cell indices. It is low in iron deficiency anaemia but usually normal in macrocytic anaemia.

Clinical significance of red cell indices

- In iron deficiency anaemia and thalassaemia, MCV, MCH and MCHC are reduced.
- In anaemia due to acute blood loss and haemolytic anaemias, MCV and MCH are usually within normal limits.
- In megaloblastic anaemia, MCV and MCH are high but MCHC is usually normal. This is because the amount of haemoglobin increases proportionately with the increase in cell size. Hence, MCHC remains normal though MCV and MCH are high.

Q10. What are the values of red cell indices (absolute values) when there is both iron and folate deficiencies?

Ans: Anaemia is macrocytic and hypochromic. So, MCV is high, MCH is low or normal and MCHC is low.

Q11. Why ESR is highly raised in multiple myeloma?

- Ans:** i. In multiple myeloma, there is increased level of abnormal proteins, known as paraproteins. This abnormal monoclonal antibodies or M proteins can coat red blood cells and interfere with normal electrical charge on the RBC that causes RBC to repel each other. Hence, RBCs stick to one another in linear arrays, a finding referred to as rouleaux formation. So, ESR is highly raised.
- ii. In multiple myeloma, associated anemia is also present with decreased RBC mass compared to plasma. This change in ratio of RBC to plasma favors stacking of RBC and raised ESR.

Haemoglobin Estimation

INTRODUCTION

Hemoglobin (American) or haemoglobin (British); abbreviated as Hb or Hgb, is the iron-containing oxygen-transporting **metal-loprotein** in the red blood cells of all vertebrate animals as well as the tissue of some invertebrates. It carries oxygen in the blood from respiratory organs (lungs) to other parts of the body or tissues. In the tissues, Hb releases oxygen to permit aerobic respiration which provides energy by metabolic process.

In mammals including humans, the protein component in red blood cell is about 96% by dry weight and approximately 35% when water is included. Hb has an oxygen carrying capacity of 1.34 ml O₂ per gram. The mammalian Hb can bind up to four oxygen molecules. Hb also transports other gases like carbon dioxide (CO₂) in respiratory system, some of which form carbaminohaemoglobin in which CO₂ is bound to globin protein thiol group. It can also bind nitric oxide (NO) to a globin protein thiol group.

Apart from red cells, Hb is also found in the A9 dopaminergic neurons of the substantia nigra, macrophages, alveolar cells and mesangial cells of kidney. In these tissues, haemoglobin does not act as oxygen carrier rather it acts as an anti-oxidant and a regulator of iron-metabolism.

SYNTHESIS OF HAEMOGLOBIN

It is synthesized in a complex series of steps. The heme part is synthesized in mitochondria and in the cytosol of immature RBCs. The globin protein is synthesized by ribosomes in the cytosol of RBC. Production of Hb continues in the cells (RBCs) throughout its early development from the proerythroblast to the reticulocyte in bone marrow. At this stage, the nucleus of mammalian red cells is lost but not in birds and in some other species. Even after losing the nucleus in mammalian RBCs, residual ribosomal RNA allows further synthesis of haemoglobin until the reticulocytes loses its RNA as soon it enters the vasculature. This haemoglobin-synthetic RNA has reticulated appearance and hence it is named **reticulocyte**.

HAEMOGLOBIN DERIVATIVES

Methaemoglobin (Hi)

Methaemoglobin is a derivative of haemoglobin in which the ferrous iron of haemoglobin is oxidized to the ferric state which results in the inability of Hi to combine reversibly with oxygen. The polypeptide chains are not altered.

Up to 1.5% of total haemoglobin may be methaemoglobin in normal person. Increased in the Hi the blood will cause

cyanosis and functional anaemia. Cyanosis becomes evident when the methaemoglobin concentration is 1.5 g Hi/dl or 10% of total haemoglobin in blood. Five abnormal haemoglobins have been identified in humans, whose principal consequence is asymptomatic cyanosis as a result of methaemoglobinemia. These are known as haemoglobin M (HbM) disease.

But most cases of methaemoglobinemia are secondary or acquired mainly due to drugs. These drugs are nitrates, nitrites, quinones, chlorates, sulfonamides, phenacetin, aniline dyes, etc.

Sulphaemoglobin (SHb)

Sulphaemoglobin is a mixture of oxidized, partially denatured forms of haemoglobin which is formed during oxidative haemolysis. During oxidation of haemoglobin, sulphur from source is incorporated into the heme rings of haemoglobin. It results in the formation of green-coloured hemochrome, known as sulphaemoglobin (SHb). Further oxidation of this sulphaemoglobin will result in the denaturation and precipitation of haemoglobin known as **Heinz bodies**. Unlike methaemoglobin, sulphaemoglobin cannot be reduced back to haemoglobin (irreversible) and it remains within the cells until they break down.

Sulphaemoglobin (SHb) has been reported after receiving drugs like sulfonamide, phenacetin, acetanilid, in patients with severe constipation or bacteremia due to *Clostridium perfringens*. Normal person may have <1% sulphaemoglobin. Sulphaemoglobinemia results in cyanosis but is usually asymptomatic.

Carboxyhaemoglobin (HbCO)

Haemoglobin can bind with carbon monoxide (CO) with an affinity 210 times greater than that for oxygen. Carbon monoxide can bind with Hb even its concentration in the air is very low (0.02–0.04%). In this situation, HbCO or carboxyhaemoglobin will be formed.

Endogenous CO is produced during degradation of heme to bilirubin, usually accounts for 0.5% of carboxyhaemoglobin.

Carboxyhaemoglobin cannot bind to and carry oxygen. Also increased concentration of HbCO, shifts the Hb–oxygen dissociation curve towards left resulting in tissue anoxia.

Increased carboxyhaemoglobin is formed due to acute carbon monoxide poisoning. The chief sources of CO are illuminating gas, gasoline motors, gas heaters and tobacco smoking. Chronic exposure through tobacco/cigarette smoking may lead to chronic increase of HbCO and left shift of oxygen dissociation curve. Hence, smokers tend to have higher haematocrit/PCV (increased red cell mass) and may have polycythemia too.

NORMAL HAEMOGLOBIN TYPES IN HUMAN

Human haemoglobin is formed of two pairs of globin chains to each of which is attached one molecule of haem (Fig. 6.1). There are six different types of globin chains, designated by the Greek letters α , β , γ , δ , ϵ , and ζ . The composition of a haemoglobin is specified by a formula such as $\alpha_2\beta_2$ (adult haemoglobin) which indicates a tetramer containing two α chains (one pair) and two β chains (one pair). The α chain is directed by two α genes, α_1 and α_2 which are present on chromosome 11. The γ chain is directed by two genes like α chains, these are $G\gamma$ and $A\gamma$, which are also present on chromosome 11.

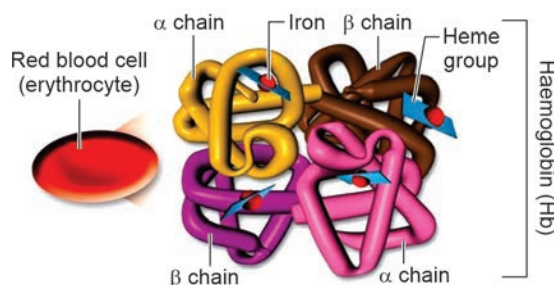


Fig. 6.1: Structure of haemoglobin

In the first three months of embryonic development, when blood cells are produced in the yolk sac, embryonic haemoglobins such as Hb Gower 1 ($\zeta_2 \epsilon_2$), Hb Gower 2 ($\alpha_2 \epsilon_2$) and Hb Portland ($\zeta_2 \gamma_2$) are produced. As erythropoiesis shifts to the liver and spleen, the foetal haemoglobin, (HbF) ($\alpha_2 \gamma_2$) appears. When erythropoiesis shifts to the bone marrow during the first year of life, the adult haemoglobins (HbA) ($\alpha_2 \beta_2$) and HbA₂ ($\alpha_2 \delta_2$) begin to be produced.

In the embryo

- Gower 1 ($\zeta_2 \epsilon_2$)
- Gower 2 ($\alpha_2 \epsilon_2$)
- Haemoglobin Portland 1 ($\zeta_2 \gamma_2$)
- Haemoglobin Portland 2 ($\zeta_2 \beta_2$)

In the foetus

Haemoglobin F ($\alpha_2 \gamma_2$)

After birth

- **Haemoglobin A ($\alpha_2 \beta_2$)**, the most common Hb (95%)
- **Haemoglobin A₂ ($\alpha_2 \delta_2$)**: δ chain synthesis begins late in the third trimester and in adults, it has normal range of 1–3.5%.
- **Haemoglobin F ($\alpha_2 \gamma_2$)**: It is present in large amount at birth (65–95%) but in adults, it is trace in amount (<1%).

Hb Variants in Different Diseases

- **Haemoglobin H (β_4)**: A variant form of haemoglobin, formed by tetramer of β chains, which may be present in variants of a thalassaemia.
- **Haemoglobin Bart (γ_4)**: A variant of haemoglobin, formed by tetramer of γ chains, which may be present in α -thalassaemia.
- **Haemoglobin S ($\alpha_2 \beta_2^S$)**: Hb found in sickle cell disease, here glutamine is replaced by valine in β_6 position.
- **Haemoglobin C ($\alpha_2 \beta_2^C$)**: Here, there is substitution of glutamic acid by lysine molecule in β_6 position.
- **Haemoglobin SC disease**: A compound heterozygous form with one sickle gene and another encoding haemoglobin C.
- **Haemoglobin AS disease**: A heterozygous form causing sickle cell trait with

one adult gene and one sickle cell disease gene.

- **Haemoglobin D-Punjab ($\alpha_2 \beta_2^D$)**: The disease produces mild anaemia.
- **Haemoglobin E ($\alpha_2 \beta_2^E$)**: Another variant due to a variation in the β chain gene. This variant causes a mild chronic haemolytic anaemia.
- **β -Thalassaemia major**: Moderate to high increase of HbF and normal to slight increase of HbA₂.
- **β -Thalassaemia minor**: Moderate to high rise of HbA₂, normal or slight increase in HbF.
- **Hb Bart-hydrops foetalis (α -thalassaemia)**: Haemoglobin Bart (γ_4) is 80–90%, some HbH and Hb Portland. Usually HbA, HbA₂ or HbF absent.

DIFFERENT METHODS OF HAEMOGLOBIN ESTIMATION

1. Photocolorimetric method: Cyanomethaemoglobin method or Drabkin's method
2. Acid haematin method or Sahli method
3. Alkaline-haematin method
4. Oxyhaemoglobin (HbO₂) method
5. Tallqvist method
6. Copper sulphate method
7. Lovibond comparator method
8. HemoCue method
9. Chemical (iron content) method
10. Van Slyke's oxygen capacity method or gasometric method
11. Automated analyzer method
12. Spectroscopic method
13. Haemoglobin colour scale method
14. Haldane method.

Of these methods, cyanomethaemoglobin (HiCN) method or Drabkin's method is most widely used and reliable method. Acid haematin method or Sahli's method used in the past mainly in the small laboratories, now become obsolete as it is less accurate. With the invent of automated analyzer, other

Table 6.1: Different methods of haemoglobin estimation and principle of the method

Method	Principle of the method
1. Colorimetric method A. Visual colorimetric method i. Sahli's method or acid haematin method ii. Alkaline haematin method B. Photocolorimetric or photoelectric method i. Cyanomethaemoglobin (HiCN) method ii. Automated analyzer iii. Haldane method iv. Oxyhaemoglobin method v. HemoCue method 2. Physical method 3. Gasometric method 4. Chemical method	These methods are based on measuring the colour of haemoglobin of the test compared to standard. This is done either by naked eye (visually) or by photocolorimetry . The optical density of a coloured solution is directly proportional to the concentration of the coloured material in the solution Specific gravity Oxygen combining capacity of haemoglobin Iron content of the haemoglobin

methods are being replaced in modern laboratories as it is accurate, reliable and fast (Table 6.1).

Acid Haematin or Sahli's Method (Fig. 6.2)

This method of Hb estimation is not very accurate because different forms of haemoglobins are not converted to acid haematin, and the brown colour which develops is unstable and begins to fade almost immediately after it has its peak. Also, there is subjective biasness of colour matching. It is done in some rural areas where colorimeter is not available.

Principle

Haemoglobin is converted into acid haematin by adding N/10 hydrochloric acid. The acid haematin solution is further diluted with the acid until its colour (brown) matches exactly that of the permanent standard (brown glass reference block) of the comparator block.

But the acid haematin formed in this method is a colloidal suspension and it can not be read in colorimeter which requires optically clear solution.

**Fig. 6.2:** Reagents and equipment for Sahli's method

Reagents and Equipment

1. Hydrochloric acid solution (N/10 or 0.1 N)
2. **Haemoglobinometer pipette or Sahli's pipette:** This is a slender special pipette with a single mark of 0.02 ml or 20 cu mm.
3. **Haemoglobinometer tube (calibrated tube):** This tube has markings on ascending order on both sides. On one side it shows grams per 100 ml (left side) and other side it shows percentage (right side). Presently used Hellige's tubes are square shaped, it has 14.5 g as 100%.
4. **Sahli's haemoglobinometer or comparator box:** It is used to match the colour

of acid haematin formed in the haemoglobinometer tube with that of colour standard in the comparator box.

5. **Glass rod (stirrer):** For mixing the blood in the square/round tube.

6. **Distilled water**

Procedure

The graduated haemoglobinometer tube is filled to the lowest mark, i.e. till 20 mark with N/10 HCl (0.1 N). Now, with the help of haemoglobinometer pipette or Sahli's pipette draw 20 cu mm of blood. This blood can be EDTA or double oxalate mixed or it can be from finger prick (capillary blood). This blood is immediately mixed with the N/10 HCl present in the haemoglobinometer tube. Mix them well by shaking the tube well. The tube is then allowed to stand with in the comparator box and keep it for 10–30 minutes (at least 10 minutes) for full conversion of the haemoglobin into acid haematin (development of brown colour).

Distilled water is then added drop by drop and stirred with a stirrer (glass rod) till the colour matches well with the fixed colour (reference) in the comparator box. The matching or comparison should be done only in natural day light.

Reading: The mark matching with the upper level of diluted acid haematin indicates the level of haemoglobin and is expressed in terms of g/dl.

Note

- After 10 minutes, 95% colour develops (conversion of haemoglobin to acid haematin) and after 20 minutes 98% colour develops.
- This method does not estimate caboxyhaemoglobin, methaemoglobin, foetal haemoglobin and sulfhaemoglobin.
- Non-haemoglobin substances like protein, lipid or cell stroma may affect the colour of blood diluted with N/10 HCl. They may interfere with the converted acid haematin and hence the result.

Advantage of the Method

It is easy, simple and cheap method.

Disadvantages of the Method

- i. It is not an accurate method.
- ii. It cannot measures all type of haemoglobins like caboxyhaemoglobin, methaemoglobin, foetal haemoglobin and sulfhaemoglobin as it cannot convert these haemoglobins into acid haematin.
- iii. As it is a visual method chances of error is high because of subjective variation.

Sources of Errors

- i. Improper collection of blood (venipuncture technique, finger prick).
- ii. Improper mixing of blood with anti-coagulant (EDTA).
- iii. Delay in taking results. The brown colour of acid haematin is unstable and the colour fades away with time.

Cyanomethaemoglobin Method (by Colorimetric or Spectrophotometric Method)

As already described, this is the most widely used method of haemoglobin estimation in the world. Also, as a best quality control this method is internationally recommended.

Principle

Blood is mixed with potassium cyanide and potassium ferricyanide mixture. Potassium ferricyanide oxidises haemoglobin to methaemoglobin (Hi). Then potassium cyanide provides cyanide ions (CN^-) to it, and it is converted to cyanomethaemoglobin (HiCN). This cyanomethaemoglobin has a broad absorption, maximum at a wavelength of 540 nm. The absorption of the solution is measured in a photoelectric colorimeter or spectrophotometer using 540 nm wavelength or yellow-green filter with that of a standard HiCN solution.

Remember, in this method haemoglobin, methaemoglobin and carboxyhaemoglobin can be measured but not the sulfhaemoglobin

(sulphaemoglobin can be measured by spectroscopic method or photoelectric method at 620 nm wavelength and alkaline haematin method).

Original Drabkin's solution

- Potassium cyanide (KCN): 50 mg
- Potassium ferricyanide [$K_3Fe(CN)_6$]: 200 mg
- Sodium bicarbonate ($NaHCO_3$): 1 g
- Distilled water: 1000 ml.

Modified Drabkin's solution (as recommended by International Committee for Standardization in Haematology or ICSH)

- Potassium cyanide: 50 mg
- Potassium ferricyanide: 200 mg
- Potassium dihydrogen phosphate: 140 mg
- Non-ionic detergent: 1 ml (like Nonidet P40 from Sigma, Triton X-100 or Saponic 218)
- Distilled water: 1000 ml.

The original Drabkin's solution had a pH of 8.6. But the modified Drabkin's solution has a pH of 9.6. This modified solution is less likely to cause turbidity from precipitation of plasma proteins. Also it takes shorter conversion time (3–5 minutes) compared to original Drabkin's solution (15–25 minutes).

Reference solution: It can form to the international specifications and is available commercially. It contains 550–850 mg of haemoglobin/litre and it is dispensed in 10 ml sealed ampoules.

Procedure

20 cu mm (20 μ l) of blood is collected (EDTA or oxalate mixed venous blood or capillary blood after finger prick) with the help of haemoglobinometer pipette. This blood is then mixed with 4 ml of Drabkin's solution in a test tube. Invert the test tubes several times for proper mixing. So, dilution of blood is 201 times (dilution factor is 201).

Allow the mixture to stand at room temperature for 15–25 minutes (minimum 15 minutes) for complete development of

cyanomethaemoglobin (HiCN). Test solution is compared with the standard and reagent blank in a colorimeter or spectrophotometer.

Calculation

Hb concentration of the test

= optical density of test solution / optical density of standard solution $\times$ **concentration standard (mg/100 ml) $\times$ dilution factor/1000.**

Note

If 20 cu mm (0.02 ml) of blood is mixed with 4 ml of Drabkin's solution, then dilution factor is 200, whereas if 20 cu mm (0.02 ml) of blood is mixed with 5 ml of Drabkin's solution, then dilution factor is 250.

Advantages

1. The test result is accurate, so, ICSH recommends it.
2. It measures different forms of haemoglobin (except sulphaemoglobin).
3. The standard solution is commercially available as per international specification (International Committee for Standardization in Hematology or ICSH). So, the test can be easily standardized.
4. Cyanomethaemoglobin reagent (also called Drabkin's solution) is very stable.

Disadvantages

1. The use potassium cyanide (KCN) in the preparation of Drabkin's solution is a potential health hazard. However, Drabkin's solution contains only 50 mg of KCN/litre, so it is relatively safe. To produce serious health problem/poisoning, someone has to swallow 600–1000 mg of it (12–20 liters of solution).
2. It takes longer time (15–25 minutes) for complete conversion of cyanomethaemoglobin (HiCN). Modified Drabkin's solution takes shorter time (3–5 minutes).
3. If the blood contains carboxyhaemoglobin (HbCO), this rate of conversion is more slower.

Note

- To avoid cyanide (KCN), lauryl sulphate has been proposed as it has similar properties to HiCN.
- Dilution factor is 200 if 20 μ l blood is mixed with 4 ml of Drabkin's solution (i.e. 1:200). But when 20 μ l blood is mixed with 5 ml of Drabkin's solution (i.e. 1:250), then dilution factor is 250.

Other Methods of Hb Estimation

1. **Oxyhaemoglobin method:** This is the quickest and simplest method for use with a photoelectric colorimeter. The haemoglobin is converted to oxyhaemoglobin (HbO₂) with the help of liquor ammonia (ammonium hydroxide) solution and is read in 540 nm wavelength or with yellow-green filter. The reliability of this method is not affected by a moderate rise in plasma bilirubin. But the result is not satisfactory in presence of carboxyhaemoglobin, methaemoglobin or sulphaemoglobin.

2. **Alkaline haematin method:** There are two methods which follow alkaline haematin method: (a) The standard method using Gibson and Harrison's standard, (b) the acid alkali method.

It is a useful ancillary method under special circumstances as it gives a true estimate of total haemoglobin even if methaemoglobin, carboxyhaemoglobin or sulphaemoglobin is present. Plasma proteins and lipids have a little effect on the development of colour. Fetal haemoglobin (HbF) and Bart haemoglobin (γ_4) cannot be estimated by this method as they are resistant to alkali denaturation normally. But this problem can be overcome by heating the solution containing HbF or γ_4 in a boiling water for 4–5 minutes.

3. **Spectroscopic method:** For qualitative and quantitative estimation of methaemoglobin and sulphaemoglobin; spectroscopic examination of blood is very reliable.
4. **Chemical (iron content) method:** Normally iron content of haemoglobin is

0.347%. Hb estimation of blood can be done by estimating total blood iron and dividing it by 3.47. This method is no longer in use.

5. **van Slyke's oxygen capacity method or gasometric method (Fig. 6.3):** It is an indirect method which estimates the amount of haemoglobin from the amount of oxygen it absorbs with the use of van Slyke's apparatus. This method is very complicated.

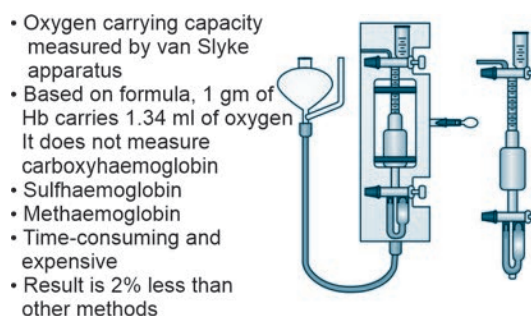


Fig. 6.3: Gasometric method

6. **Specific gravity method:** Normal specific gravity of human average in men is 1.057 and in women is 1.053. By calculating specific gravity of an unknown sample, its haemoglobin content is estimated. This method is very rapid and uncomplicated. It is used to screen the potential blood donors for anaemia.

7. **WHO haemoglobin colour scale method:** The haemoglobin colour scale (HCS) is a simple, rapid and cheap method for Hb estimation with a finger prick sample. It has been developed for use in resource-poor settings when there is no laboratory. The method relies on comparing the colour of a drop of blood sample absorbed onto a filter paper with standard colour on a laminated card, varying from pink to dark red. These colours correspond to haemoglobin levels of 4, 6, 8, 10, 12, and 14 g/dl. Intermediate shades can be identified, allowing haemoglobin levels to be judged to 1 g/dl. This test is used for mass screening of anaemia and has been adopted by World Health Organization (WHO).

8. HemoCue method: In this method, the reaction in the microcuvette is a modified azide methaemoglobin reaction. The microcuvette contains three reagents in dried forms (sodium deoxycholate, sodium nitrite, sodium azide) which convert Hb into azide methaemoglobin (HiCN). Single purpose derived photometer (colorimeter) uses double wavelength filters (570 nm and 880 nm) and measures Hb.

It is WHO approved method for Hb estimation among blood donors.

Normal/Reference Range of Haemoglobin

- Adult males: 13–17 g/dl
- Adult females (nonpregnant): 12.0–15.0 g/dl
- Adult females (pregnant): 11.0–14.0 g/dl
- Child: 6 months–12 years: 11.5–15.5 g/dl
- Children: 6 months–6 years: 11.0–14.0 g/dl
- Infants: 2–6 months: 9.5–14.0 g/dl
- Newborns: 13.6–19.6 g/dl

Decreased in Hb concentration: Hb is decreased in all anaemias, in most causes as a

consequence of another disease or a deficiency (folate, vit B₁₂, iron).

Increased in Hb concentration

- Hb is increased as a physiologic response to high altitude due to low oxygen tension or in advanced lung or cardiac disease.
- Certain myeloproliferative neoplasms, especially polycythaemia vera (when Hb in men is >16.5 g/dl and in women is 16 g/dl or haematocrit >49% in men and >48% in women).

Limitations of Accurate Hb Estimation

(Table 6.2)

- Errors arise from improper venipuncture or finger prick that may induce haemoconcentration.
- During sample preparation in manual methods, dilution mistakes may occur, or there may be sample turbidity due to improperly lysed RBCs during processing by automated counters which affect the accuracy of the results.
- Hyperlipidaemia, dehydration, marked leukocytosis or high plasma protein result in erroneous result.

Table 6.2: Advantages and disadvantages of different methods of haemoglobin estimation

Name of the method	Advantages	Disadvantages
1. Sahli method	• Simple • Cheap	• Inaccurate results • Inter-observer variability • Colour developed (acid haematin) is unstable • No International Standard
2. Cyanomethaemoglobin method	• Stable compound (HiCN) gives accurate measurement • International Standard available	• Turbidity due to other factors may cause inaccurate results • Time consuming • Reagent cyanide is toxic
3. Tallqvist method	• Simple and rapid • Inexpensive and portable • Reagents and electricity not required	• Results affected by size, thickness of blood spot, temperature, lighting and humidity • Only supplied filter paper can be used which is limited quantity
4. Copper sulphate method	• Inexpensive • Simple and rapid • Electricity not required	• Inaccurate • Requires fresh solutions • Only ranges of Hb levels are obtained not exact figure • Proper disposal of standard solutions

Contd.

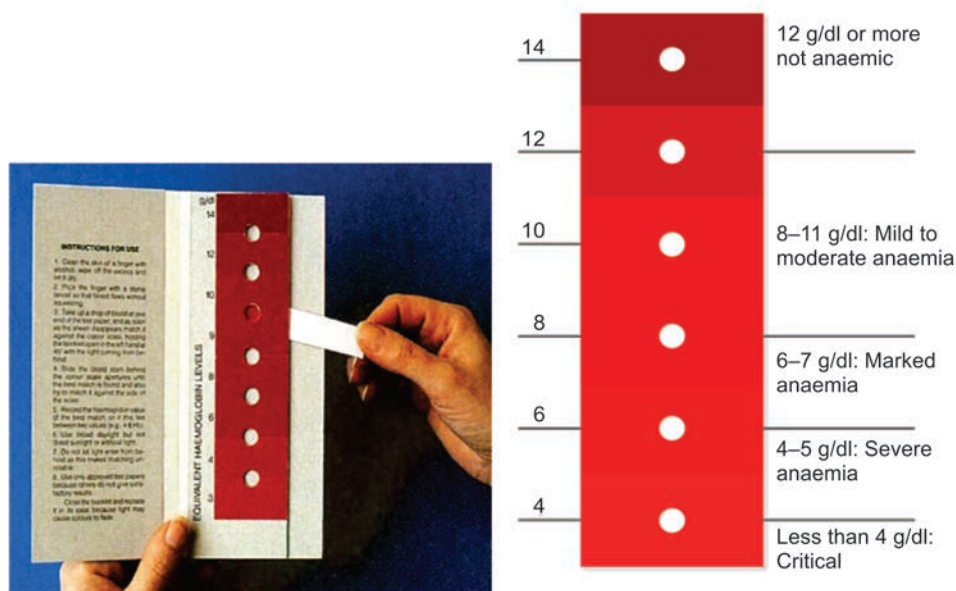
Table 6.2: Advantages and disadvantages of different methods of haemoglobin estimation (Contd.)

Name of the method	Advantages	Disadvantages
5. Haemoglobin colour scale method adapted by WHO	- Simple and portable - Cheap - Electricity not required	Inter-observer biasness
6. Lovibond comparator method	- Rapid and simple - Useful for routine screening - Electricity not required	- Expensive - Requires precise dilution - Requires large drop of blood - Subjective interpretation
7. HemoCue	- Simple and portable - Rapid and immediate result - Accurate and reliable - Easy to perform - Battery operated	Expensive as it uses disposable cuvettes
8. Automated analyzer	- Accurate - Reliable	- Expensive - Small laboratories or rural setups cannot afford

Haemoglobin Colour Scale (HCS)**Method** (Fig. 6.4)

The haemoglobin colour scale is a simple, rapid and cheap method for Hb estimation with a finger prick sample. It has been developed for use in resource-poor settings when there is no laboratory. The method relies on comparing the colour of a drop of

blood sample, absorbed onto a filter paper with standard colours on a laminated card, varying from pink to dark red. These colours correspond to haemoglobin levels of 4, 6, 8, 10, 12 and 14 g/dl. Intermediate shades can be identified, allowing haemoglobin levels to be judged to 1 g/dl. This test has been adopted by World Health Organization (WHO).

**Fig. 6.4:** Haemoglobin colour scale (HCS)

Q1. Which is better and practical method for haemoglobin estimation and why?

Ans: In laboratories, where there is no auto-analyzer, cyanomethaemoglobin estimation is most widely used method for haemoglobin estimation. Because it has several advantages over other methods:

- Cyanomethaemoglobin is a stable compound and colour does not fade easily. But in other methods like Sahli's (acid haematin) method, colour begins to fade after ten minutes. So, in acid haematin method, lower haemoglobin level will be obtained if reading is delayed.
- Almost all haemoglobin except sulphaemoglobin is converted to cyanomethaemoglobin. So, this method can calculate almost all haemoglobins.
- This is a colorimetric method, so personal error like matching colour as in Sahli's method is absent.
- A stable reference standard is available.
- The test result is accurate, hence, ICSH (International Committee for Standardization in Haematology) recommends it.

However, automated analyzer method is best as it is reliable, accurate and fastest.

Q2. What are the different uses of haemoglobin (Hb) pipette?

Ans: It uses 0.02 ml (20 mm^3) of blood (or fluid). So, Hb pipette can be used wherever different dilutions are required:

- For RBC count: 4 ml RBC fluid + 0.02 ml blood (dilution 1:200).
- For total WBC count: 0.4 ml Türk's/WBC fluid + 0.02 ml blood in a small test tube to mix it (dilution 1:20).
- For platelet count: 0.4 ml platelet count fluid + 0.02 ml blood (dilution 1:20).

- For eosinophil count: 0.2 ml of Dunger's fluid + 0.02 ml blood (dilutions 1:20).
- It can be used for body fluid cell count, sperm count, etc. also.

Q3. Why is N/10 HCl used in Sahli's method, not N/5 or N/15 HCl?

Ans: The Sahli's method (acid haematin method) has been standardized by using N/10 HCl. Brown colour of the comparator glass is equivalent to the colour of acid haematin produced by using N/10 HCl in a standard blood sample which contains 14.8 g% Hb. Test sample is compared against this standard sample.

Q4. In automated cell counter, what chemical reagent is used to detect Hb?

Ans: Sodium lauryl sulphate (SLS) is used which converts all Hb into detectable chromogen rapidly.

Q5. What are the physiological variations of Hb concentration?

- Ans:**
- Splenic contractions may occur after strenuous exercise which pumps more blood in the circulation as spleen is a reservoir of blood. So, hemoconcentration and falsely raised Hb occurs. Even anticipation of venipuncture (blood) collection may cause splenic contraction as observed by Brown A et al.
 - Hb raises as increase with altitude.
 - Hb value highest in the morning and lowest in the evening.

Q6. What are the conditions where Hb is falsely raised?

- Ans:**
- When blood is drawn for Hb estimation during intravenous blood transfusion or iron containing drugs.
 - After burns, acute diarrhoea, severe dehydration due to hemoconcentration.

Q7. In which diseases, Hb level is raised?

- Ans:**
- Polycythaemia vera
 - COPD (chronic obstructive pulmonary disease)
 - Emphysema.
 - Renal cell carcinoma due to ectopic productions of erythropoietin
 - Congenital heart disease in adults
 - Smoking for long duration may cause smoker's polycythaemia due to formation of carboxyhaemoglobin.

Q8. What are the conditions where Hb is falsely decreased or spurious/pseudo-anaemia?

- Ans:**
- In 3rd trimester of pregnancy, due to increase in plasma volume, Hb concentration falls (1–2 g/dl).
 - Splenomegaly due to pooling of red cells in spleen.
 - Congestive heart failure due to fluid retention.
 - Multiple myeloma/paraproteinaemia.
 - During hypervolaemia (intravenous fluid infusion), Hb level falls.

Q9. Why should the stirrer not taken out of haemoglobin meter tube?

- Ans:** If stirrer is taken out of tube immediately small amount of acid haematin (formed during reaction) sticking to the stirrer (glass rod) is lost which will give lower Hb result. So, the stirrer should be lifted from the solution in the upper part of the tube when reading is taken.

Q10. In Sahli's method, why are flat comparator glasses and square tube are preferred?

- Ans:** It is easier to compare flat surface rather curvature or round surfaces. So, square tube containing acid haematin can be easily compared with flat brown surface of comparator box. Error due to curvature can be avoided.

Q11. What is mild, moderate and severe anaemia?

- Ans:** When Hb value is in between lower limit of normal range to 10.0 g/dl, it is called mild anaemia. When Hb value is 7.0 to 10.0 g/dl, it is called moderate anaemia and when Hb value is <7.0 g/dl, then it is called severe anaemia.

Q12. Which conditions affect Hb estimation /value?**Ans: Hb level is decreased in:**

- Anaemia, recumbent position (5–10%), excess squeezing during finger puncture, presence of clots in the sample, inadequate mixing of blood with anticoagulant and "spurious anaemia."
- Causes of "spurious" or "pseudo" anaemia are fluid retention in congestive heart failure (CCF), rise in plasma proteins in paraproteinemias, increased plasma volume in 3rd trimester of pregnancy and pooling of RCBs in splenomegaly.

Hb level is increased in:

- Following strenuous exercise in dehydration (hemoconcentration), at high altitudes, in polycythaemia and prolonged application of tourniquet during venipuncture.

Q13. How Hb is estimated in automated analyzer/cell counter?

- Ans:**
- First, RBCs are lysed by the lysate and the color is matched against the inbuilt standard color and the Hb value is obtained.
 - Then Hb estimation is performed directly by a modification of cyanmethaemoglobin method. Most of the analyzers use a cyanide free biodegradable reagent.
 - Hb estimation is accurate, provided these analyzers are frequently standardized. But the standards are expensive.

Bleeding Time (BT), Clotting Time (CT), Prothrombin Time (PT) and APTT

BLEEDING TIME

Definition: The time required for complete stoppage of free flow blood from a deep puncture wound on the skin is known as bleeding time.

Principle: A standard incision is made on the skin and the total time the incision bleeds (starting of bleeding to end of bleeding) is measured. Cessation of bleeding indicates the formation of hemostatic plugs which are dependent on an adequate number of platelets and on the ability of the platelets to adhere to the subendothelium and to form aggregates.

Standardized Template Method for BT

Materials

- Sphygmomanometer
- Cleansing swabs
- Template bleeding time device (commercially available)
- Filter paper (1 mm thick)
- Stopwatch

Method: Place a sphygmomanometer cuff around the patient's arm above the elbow. Inflate the cuff to 40 mm Hg pressure and keep this pressure throughout the entire test. Clean the ventral (volar) aspect of the forearm with 70% ethanol. Choose an area of forearm skin (cleaned) that is devoid of visible

superficial veins. Press a sterile metal template with a linear slit 7–8 mm long firmly against the skin aligned along the long axis of the arm and use a scalpel blade with a guard (the tip of the blade should protrude 1 mm through the template slit). Then make an incision 6 mm long and 1 mm deep.

Modifications of the template and blade which make two simultaneous cuts with a spring mechanism are also available commercially.

Now, blot off the blood exuding from the cut with filter paper at 15 seconds interval. Do not contact the wound during this procedure. When bleeding has ceased, carefully oppose the edges of the incision and apply an adhesive strip to lessen the rise of keloid formation and an ugly scar.

Normal range: 2.5–9.5 minutes.

Ivy's Method for BT

This test is almost similar to the previous standardized template method. But instead of a standardized incision, two punctures, 5–10 cm apart are made in quick succession using a disposable (or No. 11 surgical blade). The punctures should have cutting depth of 2.5 mm and width of just 1 mm is suitable. When the bleeding has ceased, a sterile adhesive strip is placed on the wounds.

Normal range: 2–7 minutes.

Duke Method for BT

The ear lobule (or heel of an infant) is cleaned with 70% alcohol and allowed to dry. A 3 mm deep stab wound is made with the help of disposable lancet in the margin of ear lobe (or heel). Stopwatch is started when the wound starts to bleed. The flowing blood is soaked by a filter paper lightly and gently at 15 seconds interval. When the wound stops to bleed, the stopwatch is pressed to stop. The time is noted as bleeding time.

Normal range: 2–5 minutes

Interpretation of the Test (BT)

- Bleeding time 1–9 minutes: Normal
- Bleeding time 9–15 minutes: Platelet dysfunction
- Bleeding time >15 minutes: Critical, test must be discontinued and pressure should be applied to the wound to stop bleeding.
- Bleeding time may be prolonged due to low platelet count or thrombocytopenia ($<100,000/\text{mm}^3$). When platelet counts are low, expected bleeding time can be predicted with the following formula:

$$\text{Bleeding time (BT)} = \frac{30.5 \times \text{platelet count}/\text{mm}^3}{3850}$$

A bleeding time longer than that expected calculated time from number of platelet alone (using above formula), may be due to defective platelet function in addition to platelet number.

- The Ivy's method and standardized template method are better to evaluate bleeding time.

Causes of Prolonged Bleeding Time

1. **Thrombocytopenia (platelet count $<100,000/\text{mm}^3$):** It may be primary (essential) or secondary. When the platelet count is $<50,000/\text{mm}^3$, patient may have very long bleeding time and the bleeding may be difficult to arrest. So, bleeding time is contraindicated in this situation.

2. **Defective or qualitative abnormality of platelets:** They may be congenital such as thrombasthenia, storage pool defects or acquired; due to drugs, the presence of paraprotein (multiple myeloma) or platelet abnormalities as in myelodysplastic syndromes (MDS).
3. **von Willebrand's disease:** There is defective platelet adherence to the sub-endothelium as there is absence/defective von Willebrand factor.
4. **Vascular abnormalities:** As found in Ehlers-Danlos syndrome, or in pseudo-xanthoma elasticum.
5. **Deficiency of clotting factors:** Occasionally severe deficiency of factor V or XI or afibrinogenaemia may cause prolonged bleeding time.
6. **Others:** Severe liver disease, leukaemia, DIC, aplastic anaemia.

Interfering Factors

- The normal range may vary if the puncture wound is not of standard depth and width.
- Ingestion of certain drug before the test will cause prolonged BT. Examples: Aspirin, dextran, streptokinase, mithramycin.
- Heavy alcohol consumption may cause prolonged BT.
- Touching the incision, during the test will break of haemostatic plugs or fibrin strands that will lead to prolonged BT.

CLOTTING TIME OR COAGULATION TIME

Definition: Coagulation time is the time required for a whole blood sample to coagulate *in vitro* under standard conditions.

Principle of the test: Coagulation or clotting time (CT) measures all three stages of coagulation (intrinsic pathway, extrinsic pathway and common pathway) (Fig. 7.1) as a whole but it is more sensitive to intrinsic pathway defects. Utmost care should be taken so that thromboplastin (tissue factor) does not enter into the blood sample as a

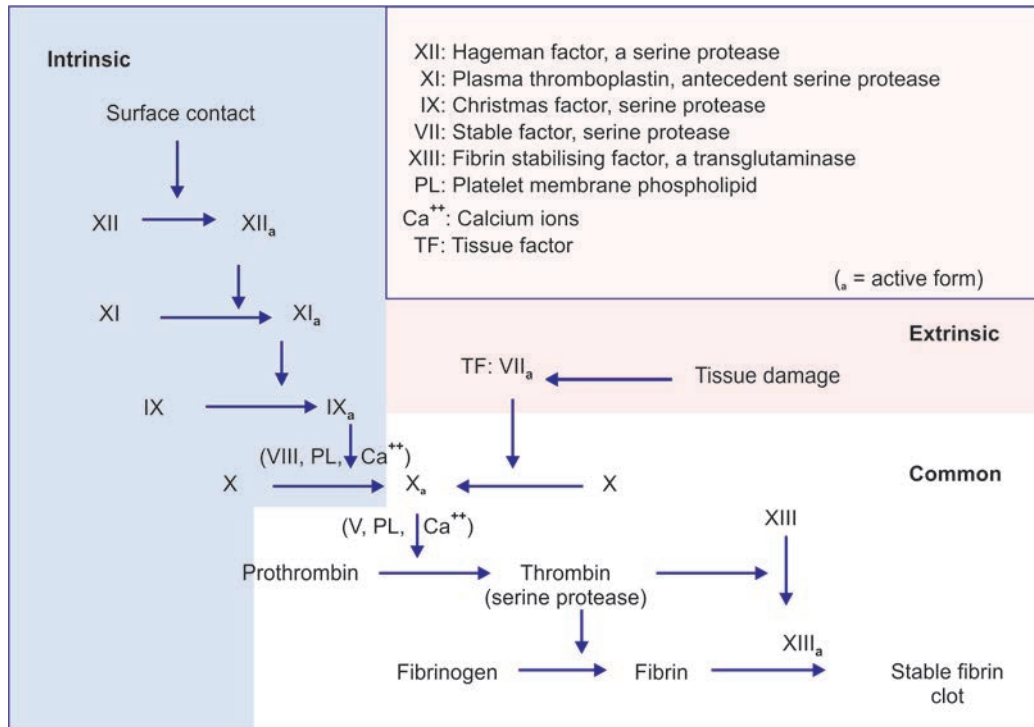


Fig. 7.1: The three pathways that makeup the classical blood coagulation pathway

very small amount will cause shortened CT. It is usually not affected by mild to moderate deficiency of platelets, because for normal coagulation (clotting) only very small number of platelets are required.

Two methods are done for CT

1. Lee and White method
2. Capillary tube method of Wright

Lee and White Method (Fig. 7.2)

Requirements

- Stopwatch
- Equipment for collection of blood
- Clean, dry glass test tubes (10 × 75 mm), three in number
- Water bath (37°C), preferable

Methods

- Make a clear venipuncture with as little trauma to the connective tissue between skin and vein as possible.

- Draw 3–5 ml of blood by a disposable plastic syringe or siliconized dry glass syringe.
- After detaching the needle, deliver 1 ml of blood in each of the three test tubes (10 × 75 mm).
- Place all the 3 test tubes containing blood in a stand so that they remain upright and undisturbed at room temperature. If water bath is available, then place the tubes at 37°C.
- Check the coagulation by tilting the test tubes or by gentle tipping. The first test tube is gently tilted every minute while other test tubes are examined every 30 seconds. When the blood samples are clotted (test tubes can be inverted without blood running down the edge of the tubes).
- The average of the clotting time in three test tubes gives the result.

Normal range: 5–11 minutes at 37°C

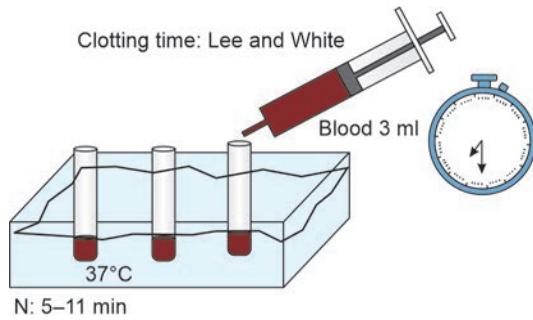


Fig. 7.2: Lee and White method of clotting time

Note

- Rise of temperature will speed up the coagulation process, so the test should be done at a particular temperature.
- If the test is done at room temperature (without water bath) then the time will vary with regard to temperature and time of tipping. If someone waits for 10 minutes before starting to tip, then normal CT value may go as high as 20–25 minutes. Whereas if someone waits for 5 minutes before starting to tip, range will be 8–18 minutes.
- Vigorous agitation of the test tubes will significantly shorten coagulation time (CT). So, one should tip the tubes very gently to see if the blood has clotted (no movement of blood).

Capillary Tube Methods of Wright

Blood is collected from a clean finger prick (aseptic precautions) in 4–5 fine capillary tubes. The tubes are sealed at both ends by flame (alternatively by chemical plasticine) and the capillary tubes are kept in a water bath at 37°C.

After one minute, one end of the tube is broken gently and the breakage of tube is repeated every 30 seconds until a thin line of unbroken coagulum is stretched between the two broken ends.

Normal range: 6–10 minutes (if test is done in room temperature the CT will be longer; not recommended).

Prolonged Coagulation Time

- Haemophilia A** (deficiency of factor VIII. and **haemophilia B** or Christmas disease (deficiency of factor IX).
- Anticoagulant therapy** (hyperheparinemia or warfarin therapy).
- Hypoprothrombinaemia:** Seen in cirrhosis of liver, obstructive jaundice, vit K deficiency, malignancy of liver.
- Fibrinogen deficiency:** When fibrinogen level falls below 50 mg/dl in blood.
- von Willebrand's disease:**

PROTHROMBIN TIME (PT)

Definition: Prothrombin time is the time required for clotting of citrated plasma (platelet poor) in a glass test tube after the addition of calcium chloride and thromboplastin (tissue factor) (Fig. 7.3).

Reagents

- Patients control plasma samples:** Patients control plasma samples (preferably platelet poor) are prepared from whole blood and citrate anti-coagulant in a ratio of 9:1.

- Sodium citrate (3.2%) solution: 0.5 ml
- Whole blood: 4.5 ml

Whole blood and sodium citrate (3.8%) are mixed well gently. Then **platelet-poor plasma (PPP)** is prepared by centrifugation at 2000 g for 15 minutes at 4°C (approximately 4000 r.p.m./minute). It should be kept at room temperature for prothrombin time assay. The test should be done within 2 hours of collection.

- Thromboplastins:** Thromboplastins are tissue extracts obtained from different species and different organs. Majority of thromboplastins now in use are extracts of rabbit brain or lung. It is now commercially available as powder.
- Calcium chloride (CaCl₂):** 0.025 mol/litre. The test reagent is prepared fresh before use.

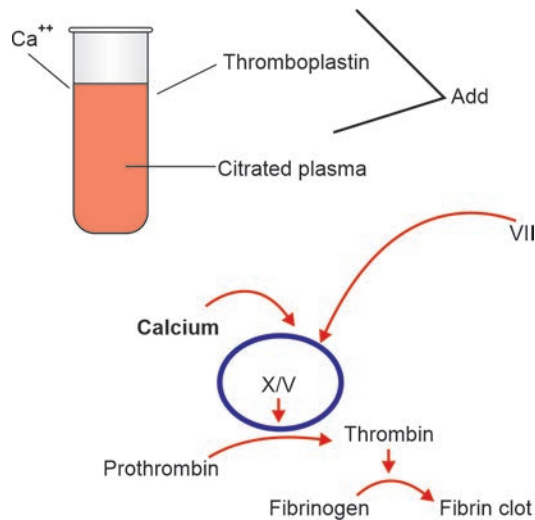


Fig. 7.3: Schematic diagram of prothrombin time (PT)

Points to Remember

- Tissue thromboplastin serves two functions. It activates extrinsic pathway and provides phospholipid surface for certain coagulation reactions.
- Calcium chloride (CaCl_2) supplies calcium ions or Ca^{2+} which bind vitamin K dependent factors (II, VII, IX and X) to phospholipid.

Methods (Fig. 7.3)

1. Put 0.1 ml of plasma into a glass test tube placed in a water bath and add 0.1 ml of thromboplastin.
2. Wait for 1–3 minutes to allow the mixture to warm at 37°C .
3. Then add 0.1 ml of warmed CaCl_2 (previously kept at water bath in a separate test tube) to that mixture in the glass test tube.
Mix well the contents and start the stop watch.
4. The test tube is continuously tilted within the water bath and look for clot formation.
5. As soon as the fibrin clot is formed, the watch is stopped and the time is recorded.

Normal values: 11–16 seconds (when rabbit thromboplastin is used)

International Normalized Ratio (INR)

The result of prothrombin time (PT) in seconds which is performed on a normal individual will vary according to the type of analytical system employed. This is due to the variations between different types and batches of manufacturer's tissue factor used in the reagent to perform the test. The **international normalized ratio (INR)** was devised to standardize the test results. Each manufacturer assigns an **ISI (international sensitivity index)** value for any tissue factor they manufacture. The ISI value is usually between 0.94–1.4 for more sensitive and 2.0–3.0 for less sensitive thromboplastins.

The INR is the ratio of a patient's prothrombin time to a normal (control) sample, raised to the power of the ISI value for the analytical system being used.

For example, a ratio of 2.5 (patient's PT to control PT) using a thromboplastin with ISI of 1.4, then INR be calculated using this formula:

$\text{INR} = 2.5^{(1.4)} = 3.61$ (normal INR = 1.0 ratio).

Prothrombin index =

$$\frac{\text{PT of control plasma}}{\text{PT of patient's plasma}} \times 100\%$$

Suppose PT of control plasma and patient's plasma are 12 seconds and 16 seconds respectively, then prothrombin index would be $12/16 \times 100\% = 75\%$.

Cause of Prolonged Prothrombin Time (PT)

1. **Oral anticoagulant therapy:** Oral anticoagulants like warfarin interfere with the carboxylation of vitamin K-dependent factors. PT is the standard test for monitoring oral anticoagulant therapy.

But INR is preferred to monitor patients on anticoagulant therapy. For all other uses, the use of PT is encouraged over INR. The recommended range for INR during most indications for oral anticoagulants is 2–3, or 2.5–3.5 for patients with mechanical heart valves.

2. **Vitamin K deficiency:** PT is useful test to detect vitamin K deficiency. It measures three vitamin K-dependent factors (II, VII and X) out of four (II, VII, IX and X).
3. **Liver disease,** particularly obstructive jaundice, cirrhosis, malignancy of liver.
4. **Inherited deficiency of extrinsic or common pathway coagulation factor(s):** Deficiency of VII, X, V, II or I.
5. **Others:** Post-partum hypofibrinogenaemia, DIC.

ACTIVATED PARTIAL THROMBOPLASTIN TIME (APTT)

Synonym: Partial thromboplastin time with kaolin (PTTK) and kaolin cephalin clotting time (KCCT). Also known as partial thromboplastin time (PTT).

Principle: This test measures the clotting time of plasma after the activation of contact factors but without added tissue thromboplastin, and so it evaluates the overall efficiency of the intrinsic pathway. To standardize the activation of contact factors, the plasma is first pre-incubated with kaolin. A standardized phospholipid is provided to allow the test to be performed on platelet-poor plasma.

Reagents

1. **Platelet-poor plasma:** Both from the patient and control as described in PT.
2. **Kaolin:** 5 g/litre or 0.5 g% in barbitone buffered saline pH is 7.4. A few glass beads are added to aid resuspension. This suspension is stable at room temperature. In place of kaolin, other insoluble surface active substances such as elagic acid or celite can also be used.
3. **Phospholipid:** Commercially available lyophilised reagent and working solution is prepared as per direction. This reagent must be sensitive to detect deficiencies of factors VII, C, IX and XI, at concentration of 20–25 IU/dl.
4. **Calcium chloride (CaCl₂):** 0.025 mol/litre.

Methods

1. Mix equal volume of the phospholipid reagent and the kaolin suspension (0.5 ml each) and leave in a glass tube in water bath at 37°C.
2. Place 0.1 ml of control and patient's (test) plasma in two separate glass tubes. Add 0.2 ml of prewarmed kaolin phospholipid solution to these tubes. Mix the contents well and gently. Start the stopwatch immediately.
3. Keep it in the water bath at 37°C and wait for 10 minutes, with occasional shaking.
4. At exactly 10 minutes, add 0.1 ml of prewarmed CaCl₂ and start a second stopwatch.
5. The tubes are tilted back and forth in front of a good light source and watch for appearance of fibrin clot. The watch is stopped when the clot forms and the time is recorded. The time taken by the mixture to clot is the APTT (Fig. 7.4).

Normal range: 30–40 seconds.

Causes of Prolonged APTT (Fig. 7.5)

- **Inherited deficiency** of factor VIII and factor IX. Also prolonged in inherited deficiencies of other coagulation factors in intrinsic pathway and common pathway.
- **Circulating inhibitors:** Inhibitors may be of two types—specific and nonspecific.

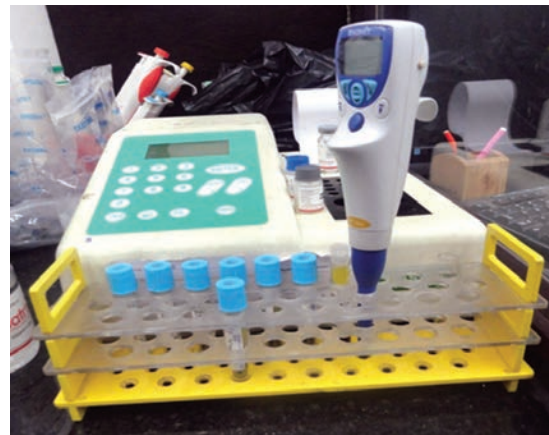


Fig. 7.4: Coagulometer (coagulation analyzer) to determine PT and APTT

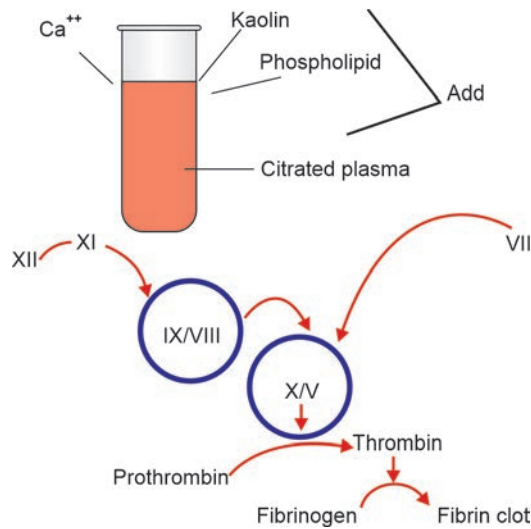


Fig. 7.5: Schematic diagram of activated partial thromboplastin time (APTT)

Specific inhibitors are directed against specific coagulation factors. The most common specific inhibitor is antibody against factor VII. **Non-specific inhibitors** are antibodies that are not directed against specific coagulation factors but block the interaction of clotting factors, e.g. lupus inhibitors.

- Liver disease
- Disseminated intravascular coagulation (DIC)
- Heparin administration: Heparin accelerates the action of antithrombin and inhibits thrombin and factors Xa, XIa and IXa.
- Massive transfusion with stored blood
- A circulating anticoagulant.

Causes of Shortened APTT

- Thrombosis
- Pregnancy

Points to Remember (Fig. 7.6)

- **Bleeding time (BT)**
 - i. It is the time taken for a standard skin puncture to stop bleeding
 - ii. This test examines the ability of blood vessels to constrict and platelets to form a hemostatic plug.
- **Clotting time or coagulation time (CT)**
 - i. It is the time required for a whole blood sample to coagulate *in vitro* under standard conditions.
 - ii. It measures all three stages of coagulation (intrinsic pathway, extrinsic pathway and common pathway).
- **Prothrombin time (PT)**
 - i. It is the time required for clotting of platelet-poor citrated plasma in a glass tube after the addition of thromboplastin (tissue factor) and calcium chloride.
 - ii. The prothrombin time along with its derived measures of prothrombin ratio (PR) and international normalized ratio (INR) are assays evaluating the extrinsic pathway of coagulation.
 - iii. The PT may be used along with APTT as the starting points for investigating excessive bleeding or clotting disorders.
- **Activated partial thromboplastin time (APTT)**
 - i. It is used to monitor the functioning of the intrinsic and the common coagulation pathways.
 - ii. A relatively rare cause of prolonged APTT is presence of antibodies against coagulation plasma factors/proteins. These are known as inhibitors. Some of the causes are: Autoimmune diseases, pregnancy, dermatologic conditions, malignancies (prostate cancer, lymphoma), haemophilia A and B, patients receiving clotting factors to control their bleeding disorders.
- Glass syringe should not be used for blood collection in coagulation studies since it activates coagulation.
- Coagulation studies are carried out within 2 hours of collection of blood sample.

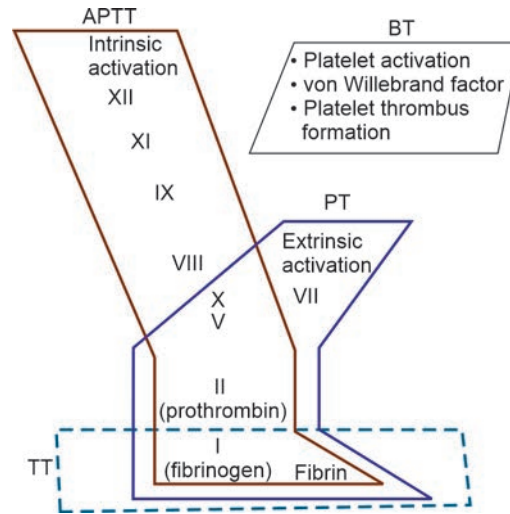


Fig. 7.6: Basic test for haemostasis

BT: Bleeding time, APTT: Activated partial thromboplastin time, PT: Prothrombin time, TT: Thrombin time

VIVA VOCE

Q1. What are intrinsic and extrinsic pathways of coagulation and why are they named so?

Ans: The clotting system can be activated by two pathways—either by intrinsic pathway or by extrinsic pathway. The intrinsic pathway is activated by exposing factor XII to any thrombogenic surface (as for example, glass surfaces or other negatively charged surfaces). On the other hand, extrinsic pathway requires exogenous triggering agent (which was originally provided by tissue extract or tissue thromboplastin prepared from animal tissue rich in tissue factor like rabbit brain). Later on, kaolin or kaolin-cephalin become available commercially which is used nowadays as tissue factor. But this division is somewhat confusing as intrinsic pathway is relevant *in vitro* (outside body), whereas extrinsic pathway is relevant *in vivo* (within body), after vascular injury/damage.

Q2. Why kaolin and phospholipids are added for doing APTT?

Ans: Kaolin leads to surface contact activation. So, initiating the intrinsic system of coagulation from factor XII onwards. Hence, it is called activated PTT or APTT. On the other hand, exogenous phospholipids (platelet factor 3) act as alternative to blood platelets and are necessary for coagulation. Within body, platelets are source of phospholipids. This exogenous phospholipid also known as partial thromboplastin. Hence, APTT is also known as partial thromboplastin time.

Q3. Why is incision made parallel to antecubital fossa during template method of bleeding time (BT) estimation?

Ans: Incision is made parallel to antecubital fossa so that no wound is formed as there will be absence of retraction of skin. But if incision is made right angle or obliquely to the antecubital fossa, the skin edges will be retracted and a spindle-shaped wound/scar will be formed.

Q4. What are the effects of circulating anticoagulants in blood?

Ans: Circulating anticoagulants are inhibitors of coagulation like heparin and other anticoagulants present in blood. Presence of circulating anticoagulants cause prolonged PT and APTT.

Q5. If a patient has normal PT but prolonged APTT, then what are the possibilities?

Ans:

- Defect in intrinsic pathway or congenital deficiency of factors require for intrinsic pathway. As for example, haemophilia A, haemophilia B, deficiency of factor XI, factor XII, prekallikrein and HMWK (high molecular weight kininogen).
- Deficiency of von Willebrand's factor (vWF) in von Willebrand's disease (also prolonged bleeding time).
- Presence of heparin and other anticoagulants in blood (circulating anticoagulants).

Q6. If a patient has prolonged PT and normal APTT, then what are the possibilities?

Ans:

- Congenital deficiency of factor VII, which is very rare.
- Initiation of oral anticoagulant therapy (warfarin).

Q7. In above scenario (question No. 6), how would you ascertain the exact cause?

Ans: When a patient has prolonged PT and normal APTT, then prothrombin (PT) is repeated with 1:1 mixture of patient's plasma and normal plasma (control) as substrate. If the prolongation is corrected, then the cause is factor VII deficiency. The normal plasma present in 1:1 mixture supplies factor VII required for normal prothrombin time. If it is not corrected, then the cause is presence of an inhibitor in blood.

Q8. If a patient has prolongation of both PT and APTT, then what are the possibilities?

Ans: In this case, both tests are repeated with 1:1 mixture of patient's plasma and normal plasma (control) as substrate. If both PT and APTT now become normal, then the patient has deficiency of one or many of these factors; factors I, II, V, and X (common for PT and APTT). After that, individual specific assay is done to find out deficiency of particular factor.

Q9. What is thrombin time (TT)? What are the causes of prolonged TT?

Ans: This is time required for testing the conversion of fibrinogen into fibrin. It depends on adequate fibrinogen levels.

Prolonged thrombin time (TT) is seen in afibrinogenaemia, dysfibrinogenaemia, DIC (disseminated intravascular coagulation) and heparin like inhibitors.

Q10. What is clot retraction study?

Ans: A clot forms at the end of blood coagulation. In normal circumstances, the clot undergoes contraction. When serum is expressed from the clot, the clot becomes denser. The platelets release one substance (called thromboplastin) which is responsible for clot retraction. Normal clot retraction begins within 30 seconds after the blood has clotted, and at 1 hour it is about 30% normally.

Clot retraction test is done when there is suspicion of haemorrhagic disorders related to platelets. Poor clot retraction is seen if platelet count is low (thrombocytopenia) or in poor platelet function with normal platelet count (thrombasthenia).

Q11. Why 3.2% trisodium citrate is used nowadays instead of 3.8%?

Ans: Trisodium citrate 3.8% was used earlier but is no longer recommended as it causes longer PT and APTT results and discrepant INR values. Moreover, INR values based

on WHO protocols have been derived using 3.2% and are not validated for 3.8% trisodium citrate.

Q12. How corrections are done in abnormal haematocrit value/polycythemia?

Ans: In polycythemia, volume of plasma is low, whereas haematocrit/ RBC count is high. For this reason, excess of anticoagulant (sodium citrate) remains in test tube. This excess anticoagulant subsequently binds to calcium reagent and cause prolon-

gation of PT and APTT. So, for accurate results volume of anticoagulant should be reduced by using one of these two formulas:

i. Volume of citrate (3.2%) needed

$$= \frac{(100 - \text{Haematocrit}) \times \text{Total tube volume}}{595 - \text{Haematocrit}}$$

ii. Volume of anticoagulant = (Volume of blood in ml) $\times$ (100 – Haematocrit) $\times$ 0.00185.

Blood Grouping and Rh Typing

The **term blood group** refers not only to erythrocyte antigen system but also to the immunologic diversity expressed by other blood constituents including leukocytes, platelets and plasma.

International Society of Blood Transfusion (ISBT) Working Party recognizes 35 significant blood group systems though there are about 200 red cell antigens. Landsteiner first discovered **ABO system** in 1901. After that, **MNS system** in 1927, **P system** in 1927, **rhesus or Rh system** in 1939, **Lutheran system** in 1945, **Kell system** in 1946, **Lewis system** in 1946, **Duffy system** in 1950, **Kidd system** in 1951 were discovered.

Other significant systems of those 35 systems (recognized by ISBT) are Diego, Yt, Xg, Scianna, Dombrock, Colton, Landsteiner–Wiener, Chido/Rodgers, Hh, Kx, Gerbich, Cromer, Knobs, Indian, Ok, Raph, John Mitton Hagen, I, Globoside, GIL, RHAG.

Most blood group genes (with a few exceptions) are located on the autosomal chromosomes and are inherited following Mendelian rules of inheritance. These blood group genes are expressed equally when inherited in a co-dominant manner (i.e. two allelic forms are expressed equally when inherited in a heterozygous state). The specific alleles at a particular locus of gene in an individual constitute the genotype. Outward expression of this genotype is known as phenotype.

With regard to blood transfusion practice, most important blood group systems are ABO and Rh systems. Because, A, B and Rh D antigens are most immunogenic (they are capable of eliciting a strong antibody response on stimulation) and their alloantibodies can cause destruction of transfused RBCs or they may induce haemolytic diseases of newborn (HDN). ABO antigens are also important for organ transplantation (graft rejection).

Apart from A, B, Rh D antigens other important antigens in transfusion medicine as per their strong immunogenicity are D, K, C, FY^a, c, E, k, e, JK^a, S and s.

Almost always, an individual has the same blood group for life, but very rarely an individual's blood type changes through addition or suppression of an antigen in infections, autoimmune diseases or malignancies. Another rare cause in blood group change is bone marrow transplant.

ANTIBODIES TO RED CELL ANTIGENS

Mainly there are two main types: Naturally occurring and immune or acquired antibodies.

1. Naturally occurring antibodies: These antibodies are formed without any antigenic stimulus (RBC antigens). These are present in the serum of persons who lack

that particular antigen(s) in the RBC, e.g. isoagglutinins (antibodies) of ABO blood grouping system. These antibodies are IgM in nature and react to corresponding antigen at a temperature below 37°C. These antibodies very rarely may be seen in other blood group systems.

It is presumed that these antibodies develop due to antigenic stimulus from the similar type of antigens present in the intestinal bacteria or foods consumed by newborns. The infant regards these antigens as foreign and develops antibodies to those foreign antigens which are not present in their own cells/RBCs. Hence, blood group A persons develop anti-B antibodies, blood group B persons develop anti-A antibodies, blood group O persons develop both anti-A and anti-B antibodies while blood group AB persons do not have any antibody.

2. **Immune or acquired antibody:** These antibodies develop when different antigens either in RBC or body fluids are introduced in a person who do not have these antigens. The person consider those antigens foreign and immune or acquired antibodies (agglutinins) are formed. Example: Mismatched blood transfusion or after pregnancy. Most of these antibodies are IgG in nature and react best at 37°C.

ABO SYSTEM

There are four main types of blood groups: A, B, AB and O. Blood group A again can be subdivided into A₁ and A₂. The much rarer A subgroups are A₃, A_x and A_m. But the A₁ and A₂ subgroups are important only. So, ABO system increases to six: A₁, A₂, B, A₁B, A₂B and O. About 80% of blood group A and blood group AB belong to A₁ or A₁B respectively. ABO antigens are found predominantly on erythrocyte membrane protein band 3 and 4.5, membrane glycoprotein and structural glycolipids. In addition to red cells, these antigens are also expressed on

platelets, white blood cells and various body tissues. As ABO antigens are found on most tissues of the body, they are often referred to as "histo-blood group" antigens. They may be found in soluble form in various body secretions (in secretors).

Although ABO antigens have been detected on erythrocytes in a six-week old foetus but these antigens are poorly expressed at birth. Antigenicity increases gradually and becomes fully expressed around one year of age (some believe full expression needs three years of age).

The blood groups A, B, AB and O are determined by presence or absence of A and B antigens on the red cell membrane (Fig. 8.1, Tables 8.1 and 8.6). There is another antigen called O antigen which remains silent.

Actually, these A, B or O antigens on red cells are controlled by three allelic genes on the long arm of chromosome 9. The A and B genes are co-dominant but the O antigen is amorph or silent (it has no effect on antigenic structure).

The cellular expression of A and B antigens is determined by another gene, called H gene. The H gene (genotype HH or Hh) produces a transferase enzyme, which changes precursor or substance present on RBCs into **H substance**. The A and B genes produce specific transferase enzymes that convert H substance into A and B antigens respectively. But the O gene produces an inactive transferase so that H antigen persists unchanged on red cells.

In the absence of H genes (designated as hh), the precursor substance remains unconverted and H substance is not synthesized. So, A and B genes, if present cannot be expressed and **Bombay blood group (Oh)** type results. Their red cell type is group O. But unlike group O individuals, Bombay blood group persons (Oh) have no H antigen on RBC. As there is no expression of A, B antigens and absence of H antigen, their plasma contain antibodies against all of them, i.e. anti-A, anti-B and anti-H. These antibodies are active at 37°C. Therefore,

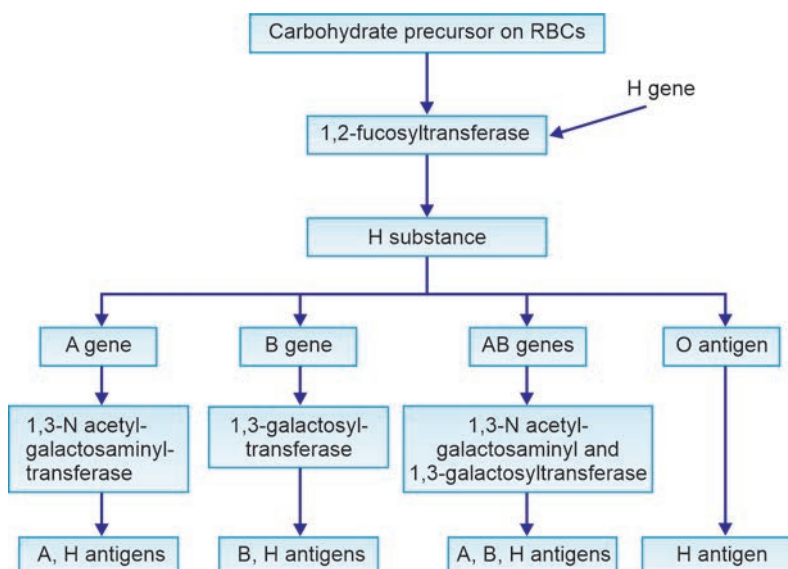


Fig. 8.1: Formation of A, B and H antigens on RBCs

Bombay blood group persons should be transfused only with Oh blood.

Secretors and Nonsecretors

Secretors are persons who secrete A, B, and H antigen (called ABH substances) into body fluids (such as plasma, saliva, sweat, tears, semen, milk, etc.). These ABH substances are secreted in water-soluble form of glycoprotein. The ability of secretion is dependent on presence of a dominant secretor gene (Se). About 80% of Caucasian persons are secretors (genotype Sese or SeSe) and remaining are nonsecretors (sese). Both secretors and

nonsecretors express ABO antigens on red cells.

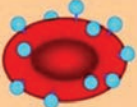
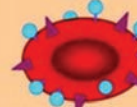
Differences between A₁ and A₂ Subgroups of Blood Group A and AB

Subgroups of A are distinguished by their lack of agglutination with anti-A₁ lectin prepared from *Dolichos biflorus* or with anti-A₁ reagent derived from serum of group O or B persons that has been absorbed with A₂ cells.

In India, frequency of blood group A is 22.88%, blood group B is 32.26%, blood group AB is 7.44% and blood group O is 37.12%.

Features	A ₁	A ₂
Quantitative differences		
• Reaction with diluted anti-A	++++	++
• Antigenic sites:		
a. In adults	1,000,000	250,000
b. In newborns	310,000	140,000
Qualitative differences		
• Reaction with <i>Dolichos biflorus</i> (Anti-A ₁) lectin	++++	0
• Anti-A ₁ in serum	Absent	1–8%
• N-acetylgalactosaminyl-transferase activity	Normal activity (optimal at pH 6)	Decreased activity (optimal at pH 7)

Table 8.1: Antigens, antibodies and other features in ABO blood grouping system

ABO blood groups				
Antigen (on RBC)	Antigen A 	Antigen B 	Antigens A + B 	Neither A nor B 
Antibody (in plasma)	Anti-B antibody 	Anti-A antibody 	Neither antibody	Both antibodies 
Blood type	Type A Cannot have B or AB blood Can have A or O blood	Type B Cannot have A or AB blood Can have B or O blood	Type AB Can have any type of blood Is the universal recipient?	Type O Can only have O blood Is the universal donor?

METHOD OF ABO BLOOD GROUPING

It can be done by slide or tube method or by microplate method. Slide method is satisfactory but not as sensitive as tube method because slide method cannot detect weak anti-A or anti-B reverse serum grouping. Monoclonal anti-A and anti-B reagents are used for blood grouping.

Again, ABO blood grouping can be of two: Forward grouping and reverse grouping.

Forward grouping: Here, one drop of 2–5% red cell suspension is tested with one drop each of commercially prepared anti-A and anti-B.

Reverse grouping: Here, two drops of patient or donor serum are tested against one drop of reagent erythrocytes of known A (usually A₁) and B phenotype. But this is not done in infants under 4 months of age as the corresponding antibodies are normally absent.

Preparing red cell suspension

Depending upon the specific technique employed 2, 5, 10, 20, or 50% red cell suspensions are required. These can be

prepared by suspending in saline the packed red cell obtained from citrate or oxalate blood or from a skin puncture into saline.

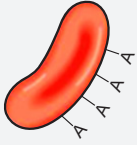
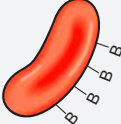
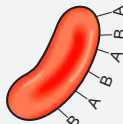
Preparation of 2% red cell suspension

1. Take 5 ml of normal saline in a test tube. To it, add several drops of anticoagulated or fresh blood.
2. Centrifuge in order to get packed red cells (3000 r.p.m. for 10–20 minutes).
3. Withdraw the supernatant fluid as completely as possible.
4. Add 0.1 ml of this packed red cells to a test tube which contains 4.9 ml of normal saline. Mix them well. This represents 2% red cell suspension (as 0.1 ml of packed red cell in total 5 ml of suspension).

For preparation of 5% red cell suspension, add 0.25 ml of packed red cell to 4.75 ml of saline. For preparation of 10% red cell suspension add 0.5 ml of packed red cells to 4.5 ml of saline in the last step (step 4) of the above procedure.

Slide Method (ABO Grouping)

This method is satisfactory but less preferable compared to tube method. However, in small

Blood group	Antigen + antibody(ies) present	As donor	As recipient
A	 Antigen A  Makes anti-B	Compatible with: A and AB Incompatible with: B and O, because both make anti-A antibodies that will react with A antigens	Compatible with: A and O Incompatible with: B and AB, because type A makes anti-B antibodies that will react with B antigens
B	 Antigen B  Makes anti-A	Compatible with: B and AB Incompatible with: A and O, because both make anti-B antibodies that will react with B antigens	Compatible with: B and O Incompatible with: A and AB, because type B makes anti-A antibodies that will react with A antigens
AB	 Antigens A and B Makes neither anti-A nor anti-B	Compatible with: AB only Incompatible with: A, B and O, because All three make antibodies that will react with AB antigens	Compatible with all groups universal recipient AB makes no antibodies and therefore will not react with any type of donated blood
O	 Neither A nor B antigen  Makes both anti-A and anti-B	Compatible with all groups universal donor O red cells have no antigens, and will therefore not stimulate anti-A or anti-B antibodies	Compatible with: O only Incompatible with: A, AB and B, because type O makes anti-A and anti-B antibodies

Red Blood Cell Compatibility Table

Recipient	Donor							
	O–	O+	A–	A+	B–	B+	AB–	AB+
O–	✓	✗	✗	✗	✗	✗	✗	✗
O+	✓	✓	✗	✗	✗	✗	✗	✗
A–	✓	✗	✓	✗	✗	✗	✗	✗
A+	✓	✓	✓	✓	✗	✗	✗	✗
B–	✓	✗	✗	✗	✓	✗	✗	✗
B+	✓	✓	✗	✗	✓	✓	✗	✗
AB–	✓	✗	✓	✗	✓	✗	✓	✗
AB+	✓	✓	✓	✓	✓	✓	✓	✓

laboratories, resource poor set ups, in emergency situations or during mass blood group screening this can be done.

Sample: A 20% red cell suspension is used. Alternatively, fresh blood by finger prick or cells from clotted blood may be used.

Reagent: Monoclonal anti-A and anti-B reagents (commercially available) blue-coloured vial: Anti-A, yellow-coloured vial: Anti-B. Monoclonal anti-AB is also available.

Test procedure (Fig. 8.2A to D and Fig. 8.4)

1. Take a clean glass slide. Mark anti-A on left side corner and anti-B on middle and control on right side corner of that slide.
2. Two drops of blood or 20–40% red cell suspension are placed by a Pasteur pipette at each three demarcated areas.
3. On the left side a drop of anti-serum, on the middle a drop of anti-B serum and on the right a drop of normal saline are added separately.

4. The serum cell suspensions are mixed separately with the help of glass rod or by the corners of another clean slide. Alternatively, wooden swabstick breaking off can also be used.
5. The slide is then tilted with hands gently and carefully for 2–3 minutes.

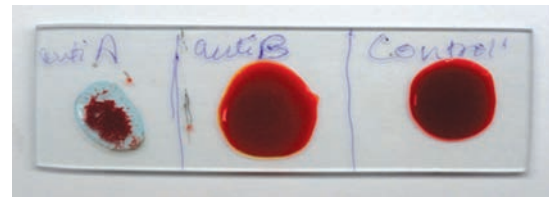
Test result: Results are read within 2–5 minutes depending on the appearance of agglutination of red cells (positive or negative) as follows:

- i. Blood group A: Agglutination with anti-A serum (left corner) but not with anti-B serum (middle).
- ii. Blood group B: Agglutination with anti-B (middle) but not with anti-A (left corner).
- iii. Blood group AB: Agglutination on both left and middle portions.
- iv. Blood group O: No agglutination on left and middle portions.

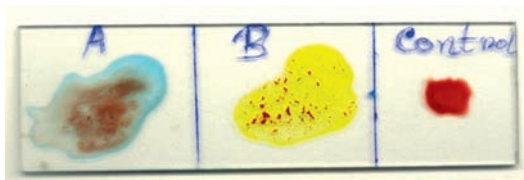
Control should be negative. If there is agglutination in the control that means either the method or technique is faulty or the reagents are of expired/poor quality.



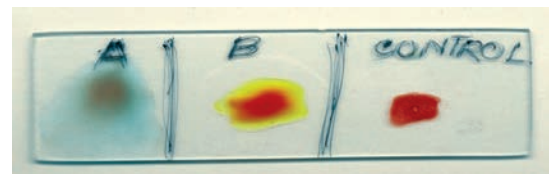
A



C



B



D

Fig. 8.2A to D: ABO blood grouping. (A) Monoclonal anti-A (green coloured), anti-B (yellow coloured) and anti-AB (colourless) serum. Also anti-D serum (colourless) on rightmost side (**upper left**); (B) Blood group A (**upper right**); (C) Blood group B (**lower left**); (D) Blood group O (**lower right**)

Tube Method (ABO Grouping)

(Fig. 8.3A and B, and Table 8.2)

- Take 3 glasses or plastic tubes (75 × 12 mm) and add one drop each grouping reagent to 3 tubes to labelled anti-A, anti-B and anti-AB respectively.
- Put 1 drop of 2–5% of red cell suspension to each tube.
- Mix the suspension by tapping the tubes and leave them undisturbed for 15–30 minutes. Check agglutination.

**A**

Test result: Agglutination is judged by the macroscopic appearance of agglutination in round bottom tubes. If positive it will show “graininess”, whereas in the absence of agglutination the sedimented cells appear as a smooth round bottom. This agglutination is better visualized under microscope. A scoring system depending upon agglutination pattern can be employed.

For determining the particular blood group following the agglutination pattern in the slide method described early.

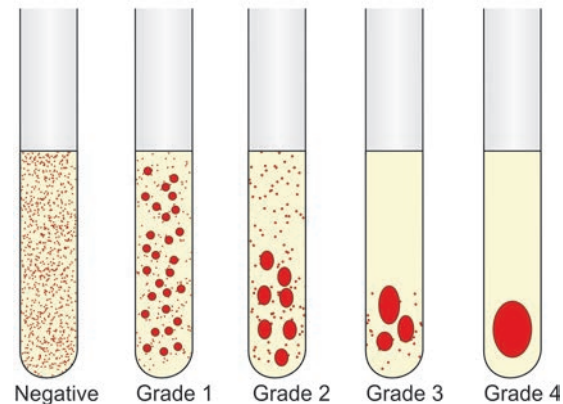
**B**

Fig. 8.3A and B: (A) Test tube methods for blood grouping; (B) Scoring system (grades 1 to 4) in test tube method of blood grouping

Table 8.2: Scoring of results in red agglutination tests

Description	Symbol	Agglutination score*
a. Negative result: All cell-free and evenly distributed	–	0
b. Cell button dislodges into fine granules, only visible microscopically	+ or weak (W)	3
c. Cell button dislodges into finely granular clumps, just visible macroscopically	1 +	5
d. Cell button dislodges into many small clumps, macroscopically visible	2 +	8
e. Cell button dislodges into several large clumps, macroscopically visible	3 +	10
f. Cell button remains in one clump, macroscopically visible	4 + or complete (C)	12

***Agglutination score:** Further classification depending upon the number of red cells present in the clump. Hence, clumping of average 12 cells (score 4+), average 10 cells (score 3+), average 5 cells (score 1+).

Serum Grouping (Reverse Grouping)

In the serum grouping or reverse grouping, patient's serum is tested against known red cell antigens on RBCs (Table 8.3). If the patient's serum contains anti-A, anti-B or both it will agglutinate known antigen A, antigen B or both antigens in red cells. Red cells form blood group A contains antigen A, blood group B contains antigen B, blood group AB contains both antigens and blood group O contains no antigen (serves as control) on their red cells. For A antigen, A₁ subtype is commonly used. A₂ cells should be used if there is suspected presence of anti-A₁.

Microplate Method

ABO grouping may be carried out on one U well plate (96 wells) and monoclonal reagents (anti-A, anti-B, anti-AB) are used. Using Pasteur pipette (or commercial reagent dropper) 1 drop of monoclonal reagent or antiserum is added to each well of the U well plate. Then 1 drop of 2–5% patient's red cell suspension is added to these rows. This is done for forward grouping.

For reverse grouping at the same time 1 drop patient's serum or plasma are added to separate wells of the plate. To this known antigens 1 drop (antigen A, antigen B or antigen AB) are added to the serum/plasma. Control may be used.

Now, mix them on a microplate shaker. Leave the microplate at room temperature (20°C) for 15 minutes. Then centrifuge the plate at 700 r.p.m. for 1 minute.

The plate can be read for agglutination by one of the two methods

1. Streaming (microplate set an angle) or agitation.
2. Automated microplate readers.

False Positive Results (ABO Grouping)

- **Rouleaux formation:** Marked rouleaux formation can simulate true agglutination. In reverse grouping the two can be distinguished by repeating the test using serum diluted 1 in 2 or 1 in 1 saline. The rouleaux will disappear but agglutination will persist. If rouleaux are apparent in forward grouping test, then tests should be repeated after washing the patient's red cell thoroughly with normal saline.
- **Cold agglutinins:** Sometimes true agglutination of red cells develops due to cold agglutinins at or below 20°C. If ABO compatibility is ruled out, then the presence of cold agglutinins like anti-P₁ or anti-I antibodies may be the cause of this false positivity. In that case, patient's red cells are washed several times with warm (37°C) saline and blood grouping (ABO)

Table 8.3: Forward and reverse groupings

ABO blood group	Forward grouping		Reverse grouping	
	Anti-A	Anti-B	A ₁ antigen on RBC	B antigen on RBC
1. Blood group A	+	–	–	+
2. Blood group B	–	+	+	–
3. Blood group AB	+	+	–	–
4. Blood group O	–	–	+	+

is repeated. There will no chance of false positivity.

- **Warm antibodies:** These antibodies are absorbed to red cell of patient. Agglutinin other than anti-A, anti-B or anti-D (Rh typing) which cause agglutination at 37°C are occasionally seen. Presence of antibodies like anti-Lu, anti-M, anti-K or anti-S antibodies are responsible for this.
- **Bacterial contamination:** Infection of RBCs by bacteria both *in vivo* and *in vitro* may cause polyagglutination in normal sera. Bacterial enzyme expose T receptors on red cell surface. As most human sera contain anti-T antibodies, such infected red cells get agglutinated in normal serum. Hence, blood group O may appear as blood group AB.

False Negative Results (ABO Grouping)

1. Failure of agglutination or weak reactions are usually due to improper sera. If the sera are kept at room temperature for longer time the potency is lost.
2. Failure to add grouping reagent or insufficient volume will cause false negative results.
3. In reverse grouping test, failure to recognize lysis as a positive result may end up in giving false negative reports. To avoid lysis, reagents (anti-A and anti-B) should contain EDTA to prevent complement activation in presence of fresh patient's serum as seen in tile method of grouping.
4. Miscellaneous: Wrong technique, poor quality of red cells, etc.

Rh System

The rhesus (Rh) system was so named because Landsteiner and Wiener (1940) published studies of animal experiments involving the immunization of guinea pigs and rabbits with rhesus monkey erythrocytes. The anti-serum produced agglutinated 85% of human erythrocytes, and the antigen defined was called the **Rh factor**.

Using five basic antisera anti-D, anti-C and anti-E, anti-c and anti-e, Wiener identified five different factors or antigens and named them as Rh₀, rh', rh''hr', hr''. While Fisher postulated that Rh antigens (C, c, D, d, E, e) are determined by three pairs of closed linked allelomorphous genes which are located on chromosome 1 (Table 8.4). These three pairs are C or c, D or d and E or e. Every human carries one member of these three pairs from each parent. Each gene can control production of a specific antigen. But the antigen controlled by D locus is the strongest immunogen, called the Rh D antigen. The six Rh genes give 8 allelomorphs and 8 antigenic patterns. The nomenclature suggested by Fisher later on accepted by WHO expert by WHO Expert Committee in 1977 (Table 8.5).

Table 8.4: Comparison of Wiener, Fisher and Rosenfield nomenclature

Wiener	Fisher	Rosenfield
Rh ₀	D	Rh 1
rh'	C	Rh 2
rh''	E	Rh 3
hr'	c	Rh 4
hr''	e	Rh 5

Table 8.5: The Rh genes and antigens as per Fisher's nomenclature, accepted by WHO (1977)

	Genes	Antigens
Rh positive	CDe	CDe
	cDE	cDE
	cDe	cDe
	CDE	CDE
Rh negative	Cde	Cde
	cdE	cdE
	cde	cde
	CdE	CdE

Rh D is the most strong antigen amongst those 8 antigens and other antigens (which do not have D) are much less antigenic than D and do not have clinical relevance. In

clinical practice, therefore, Rh positive or Rh negative depends on the presence of D antigen on surface of red cells which can be detected by adding strong anti-D serum and noting agglutination. Nearly about 95% Indians, 90% Chinese and 85% Caucasians have this Rh D antigen and hence they are Rh positive (Rh+). Only 5–6% Indians do not have Rh D antigens and are Rh negative (Rh–).

During blood transfusion, after ABO compatibility, Rh+ blood can be safely transfused to Rh+ persons. But if the person is Rh– then not only RHD negative blood but also presence of CE antigens should be checked by adding anti-C and anti-E serum. When all these three antigens (CDE) are negative, then only it should be transfused to Rh– persons. This anti-D serum is actually IgG in nature.

There are six antisera corresponding to the Rh antigens. They are anti-C, anti-c, anti-D, anti-d, anti-E and anti-e. But in humans only five of these antisera have been detected and anti-d is absent. This may be probably because d antigen is not immunogenic or amorph to produce antibody against it.

Rh Grouping Method (Fig. 8.4)

Slide Methods

1. Place one drop of anti-D (commercially available) onto a slide.
2. Add two drops of red cell suspension (50%) or whole blood (citrated/oxalated/fresh).
3. Mix well and distribute over a large area of the slide.
4. Tilt it for 2–3 minutes.

Result: Clumping of cells (both macroscopic and microscopic) indicates Rh D positive and no clumps indicate Rh D negative.

Tube Methods

1. Add one drop of anti-D serum in a test tube.
2. Add one drop of 5% red cell suspension and mix well.

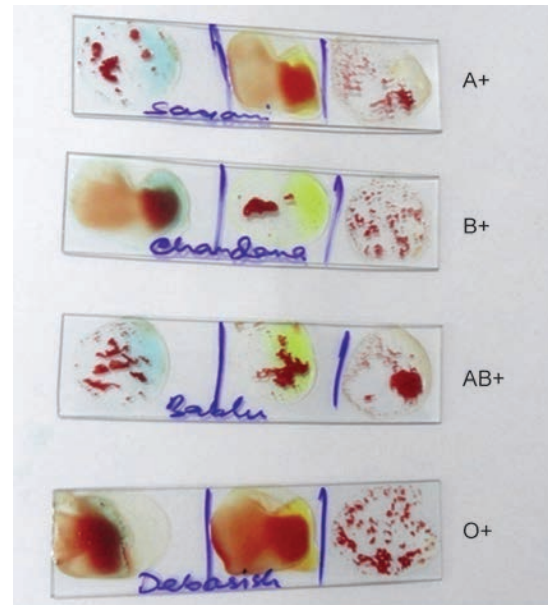


Fig. 8.4: Three compartments. First drop of blood on left side mixed with anti-A serum, middle drop of blood mixed with anti-B serum and last drop of blood on right side mixed with anti-D (Rh) serum. All are Rh group positive with ABO blood grouping of A, B, AB and O respectively

3. Incubate in a water bath at 37°C for 30–60 minutes.
4. Check for agglutination.

Result: Agglutinations or clumps seen under the bottom of the tube indicate Rh D positive and no clumps indicate Rh D negative.

REACTIONS OR COMPLICATIONS FOLLOWING BLOOD TRANSFUSION

A. Immediate complications

1. Rigor followed by pyrexia
2. Allergic urticaria over the body and anaphylaxis
3. Air embolism
4. Haemolytic transfusion reactions leading to haemoglobinuria, haemolytic shock, and renal failure.
5. Cardiac failure due to massive transfusion (volume overload).

6. Systemic infection due to contamination of blood.

B. Delayed complications

1. Infections: Hepatitis, malaria, HIV, syphilis, cytomegalovirus, Epstein-Barr virus (EBV), etc.
2. Past transfusion thrombocytopenic purpura.
3. Delayed haemolytic crisis due to immune body production.
4. Pulmonary microembolism.
5. Thrombophlebitis.

CONCEPT OF UNIVERSAL DONOR OR UNIVERSAL RECIPIENT

Universal donor: Blood group O person is often considered as universal donor because of absence of antigen A or antigen B on red cells. Plasma of blood group O person contains anti-A and anti-B. But these agglutinins

or antibodies get diluted when blood is transfused to the recipient (patient) because of large serum in the recipient. So, these antibodies become inactive and no untoward reactions occur.

Universal recipient: Blood group AB person is often considered as universal recipient because of absence of anti-A or anti-B isoantibody in the serum. Any blood group when transfused to these persons, the red cells of donor's blood do not find any antibody to react with. Hence, there is no transfusion reaction.

The above definition is based on ABO grouping system. But when it is combined with Rh typing then persons with blood group O and Rh D negative (Rh-) are called universal donors. Likewise, persons with blood group AB and Rh D positive (Rh+) are universal recipients (Table 8.6).

Table 8.6: Comparison of ABO and Rh blood grouping

Parameter	ABO blood group	Rh blood group
1. Antigenic locus on gene	Chromosome 9	Chromosome 1
2. Antigens	A, B, AB	D (only clinically significant)
3. Distribution of antigens	RBCs, platelets, body fluids, many tissues (called histo blood group)	RBCs only
4. Nature of antibody	Naturally occurring	Immune or acquired
5. Development of antigens	Weak expression at birth (full expression after 1 year of age)	Fully developed at birth
6. Antibody class	IgM	IgG
7. Optimal reaction temperature of antibody	4°C	37°C
8. Whether antibody can fix complement	Yes	No
9. Optimal reaction medium	Saline	Anti-human globulin
10. Haemolysis following mismatched transfusion	Intravascular haemolysis due to complement mediated haemolysis	Extravascular and predominantly in spleen by mononuclear phagocyte (MP) or macrophage system

GEL CARD METHOD (COLUMN AGGLUTINATION OR MICROTYPING SYSTEM)

Microtyping system or column agglutination method is based on gel technology. In this test, gel (dextran acrylamide gel) is held in microtubes contained in a plastic card (hence the name gel card). This sephadex gel presents in each microtube is prepared in a buffer solution. This gel contains group specific antisera/antibody/antiglobulin (for Coombs' test) and sodium azide as preservative. These are incorporated into the gel during manufacture.

Principle of the Test

Cells are poured over the microtube first. As for example, for blood grouping RBC suspension is poured. Then the microtubes are incubated at 37°C followed by centrifugation which pulls the cells downward and react with the antibody incorporated into gel. If antigen over cells are present against particular antibody, haemagglutination will occur (Figs 8.5 and 8.6). Normal RBCs can pass through the gel but the agglutinated

RBCs cannot because of their adherence and large size and gel matrix acts as a sieve. The agglutinated RBCs get trapped at various sites within the gel and thus formed a red line (positive test).

If the specific antigen is absent on RBCs, agglutination does not occur and RBCs easily pass through the gel and reach the bottom of the tube. No red line is formed due to absence of agglutination (negative test).

Uses

- Blood grouping (ABO and Rh typing) and cross-matching
- Coombs' antiglobulin test
- For diagnosis of sickle cell anaemia, PNH (paroxysmal nocturnal haemoglobinuria)
- For diagnosis of different infective organisms (diphtheria, syphilis, measles, parvovirus, etc.)
- Antibody identification.

Advantages of gel card method

- This method is easy, accurate, standardized.

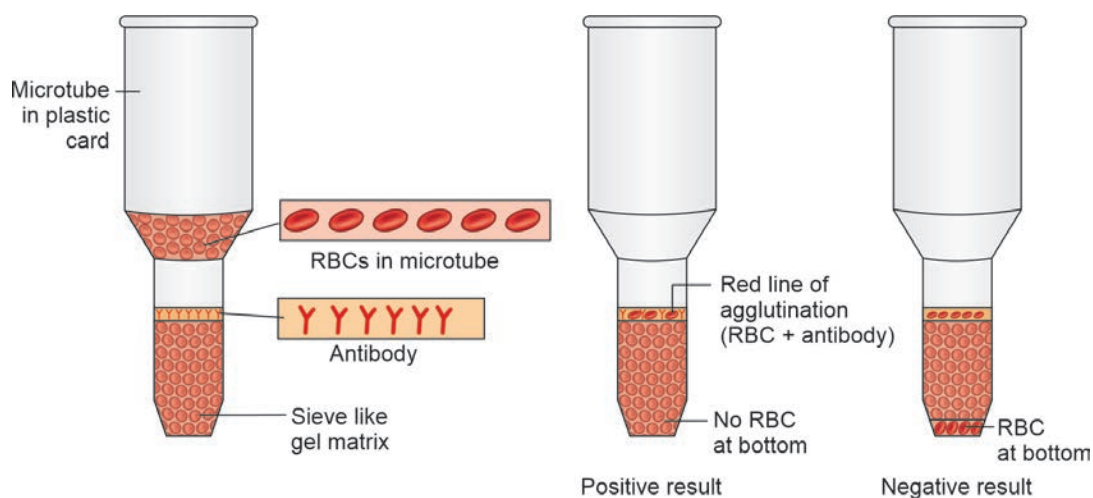


Fig. 8.5: Gel card method of blood grouping (column agglutination or microtyping system)

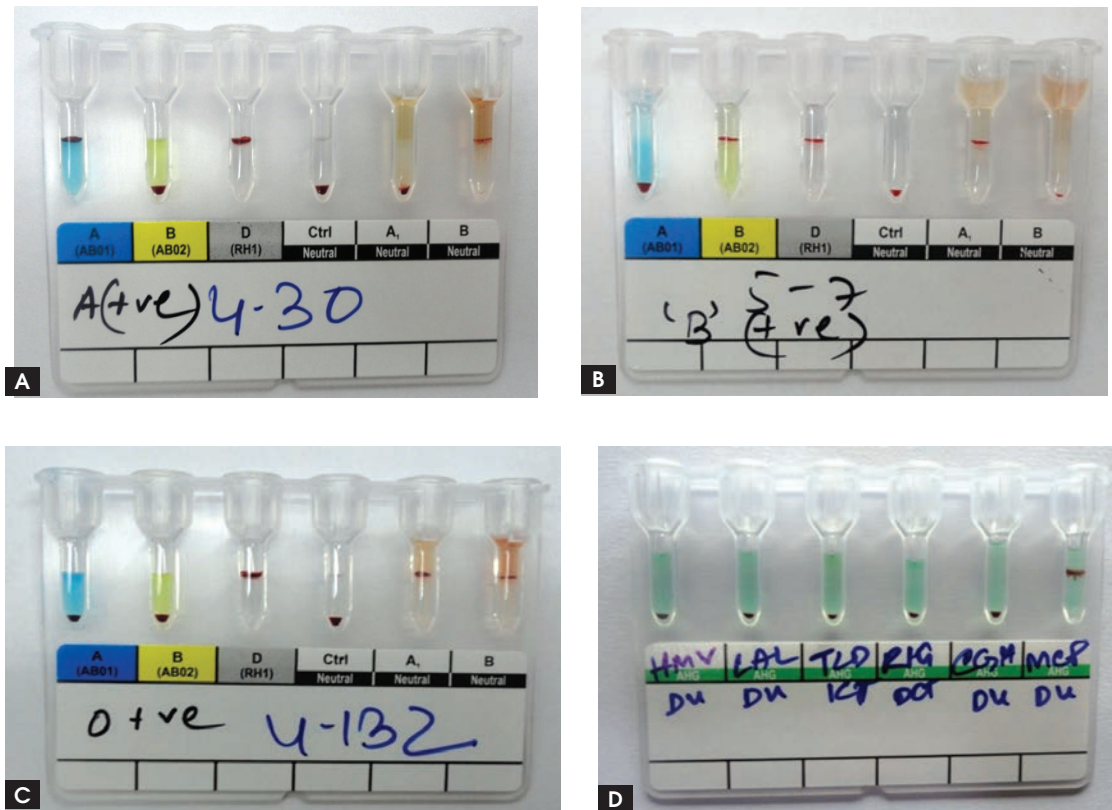


Fig. 8.6A to D: (A to C) showing ABO blood grouping in gel card method. (A) Blood group A+; (B) Blood group B+, (C) Blood group O+. There are six tubes. First one contains anti-A serum, second one contains anti-B serum, third tube contains anti-D serum, fourth tube is control, fifth tube contains antigen A₁, and sixth (last) tube contains antigen B. Particular agglutination is marked by a ring in the middle of that corresponding tube; (D) It shows Du blood grouping system. Among the six cases, only last case is Du positive

- ii. It needs small sample volume and reduces exposure to biohazardous samples.
- iii. This method has greater sensitivity compared to conventional methods.
- iv. Cell washing is not required for Coombs' test.
- v. Chances of error is almost absent as this is not done manually.

Disadvantage: It is expensive compared to conventional glass and tube method. Also it needs a special centrifuge.

SOLID PHASE ADHERENCE TECHNOLOGY

This method of cell grouping uses a microplate in which solid phase walls are coated

with reagent RBCs or red cell stroma. Serum sample is added to it and if there is antibody in the serum it is captured by the antigen(s) over RBC surface/stroma. Then indicator RBCs (coated with monoclonal IgG) are added and the mixture is centrifuged. This indicator RBCs now attach to the antibody which was captured by coated RBCs. Agglutination occurs and is indicated by diffuse adherence of indicator red cells along the microwell (positive test). If there is no antibody in serum, then there is no agglutination (haemagglutination). This is indicated by RBCs forming a button at the bottom of the microwell (negative test) (Tables 8.7 and 8.8).

Table 8.7: Cause of unexpected results (false-positive or false-negative) during blood grouping

Cell grouping (forward grouping)	
Unexpected negative	Unexpected positive
i. ABO subgroup ii. Antisera stored improperly iii. High levels of soluble blood group substances iv. Antigenic suppression seen in leukaemia or cancer	i. Polyagglutinable RBCs ii. Acquired B antigens, seen in gastric or colon cancer, intestinal obstruction iii. Foetomaternal haemorrhage iv. Wrong (out-of-group) transfusion v. RBCs coated by Wharton's jelly of umbilical cord vi. Bone marrow transplantation

Table 8.8: Serum grouping (reverse grouping)

Unexpected negative	Unexpected positive
i. ABO subgroup ii. Newborn and elderly persons iii. Immunosuppression iv. Hypogammaglobulinaemia	i. Monoclonal antibodies (immunoglobulins) ii. Transfusion of plasma components iii. Cold-reacting agglutinins

Q1. What is Bombay blood group?

Ans: Both antigens A and B on RBCs are formed from H substance (H antigen). The dominant H gene is located on chromosome 19. This H gene encodes for an enzyme that converts a carbohydrate precursor substance present in red cell into H substance (H antigen). A and B genes encode for specific transferase enzymes that convert H substance into A and B red cell antigens. O gene encodes for inactive transferase enzyme that cannot convert H substance in blood group O red cells.

Rarely, persons do not inherit H gene (very rare HH genotype) and they are unable to produce H substance (H antigen). So, they cannot produce A and B blood group antigens on RBC membrane. This rare blood group is called Bombay blood group or Oh group. It was first discovered in Bombay, Maharashtra, among Marathi speaking people. Hence, it was named Bombay blood group. As, there is absence of blood group antigen A, B and H on RBC membrane, the plasma of Bombay blood group people will contain anti-A, anti-B and anti-H antibodies.

Q2. What are the subtypes of blood group A?

Ans: There are two subtypes—A₁ and A₂ in blood group A. A₁ subgroup is 80% and A₂ subgroup is 20% based on the presence of A₁ antigen or A₂ antigen on RBCs. A₁ has greater number of antigenic site than A₂ (10⁶ site vs. 250,000 site). A potent anti-A₁ serum (anti-A₁) can agglutinate A₁ red cells but not A₂ red cells. There is no specific anti-A₂ serum. So, A₂ red cell does not agglutinate and pretends to be blood group O.

However, a saline extract of seeds of *Dolichos biflorus* is now routinely used in subtyping A blood cells (RBCs). It agglutinates A₁ blood cells and A₁B blood cells.

Saline extract of seeds of *Ulex europaeus* is used to agglutinate A₂, A₂B and O cells. It is actually an anti-H lectin.

Q3. Why reverse grouping (serum grouping) is not done before 4 months of age in infants?

Ans: There are no corresponding natural antibodies as regard to antigen(s) present on RBCs in infants below 4 months of age. So, reverse grouping (serum grouping) will give inaccurate result. It will pretend to be blood group AB as serum lacks any natural antibody.

Q4. Which method is better for blood grouping—tube method or slide method and why?

Ans: The tube method is better because the reaction between red cells and anti-serum is enhanced because of centrifugation to make red cell suspension. So, even the weaker antigen like A₂ can be detected apart from other/strong antigen(s). Tube method is recommended for both ABO and Rh blood grouping.

Q5. How would you distinguish agglutination from rouleaux formation?

Ans: When dilution with saline is done, it disperses rouleaux but cannot disperse agglutinated cells. Because agglutinated cells (antigen–antibody bonds) are firmly attached with each other.

Q6. Other than alloagglutination, what are the other causes of agglutination?

Ans: i. Autoagglutination: It is due to presence of cold agglutinin.

- ii. Pseudoagglutination: It is aggregates red cells due to nonimmunological cause like excess rouleaux formation as seen in multiple myeloma, or in macroglobulinaemia (due to high level of paraproteins in blood).
- iii. Polyagglutination or pan agglutination: If RBCs are contaminated by certain bacteria like *Pseudomonas aeruginosa*, then RBCs may be agglutinated by all blood group sera (antibody) or even by normal human serum. This phenomenon is known as **Thomson-Friedenreich phenomenon**. It is due to unmasking of a particular antigen (T or Tk antigen), which is present on human RBCs. Human serum contains anti-T antibody normally. So, when T antigens on RBCs are unmasked, agglutination occurs in blood groups. This is very rarely observed *in vivo*. *Ex vivo (in vitro)*, it can be demonstrated by use of an anti-T lectin prepared from peanut.

Q7. What do you mean by one unit of blood?

Ans: Usually one unit of whole blood means 350 ml of whole blood mixed with 49 ml of anticoagulant CPDA-1 (citrate phosphate dextrose adenine 1).

Q8. How blood and blood products are stored?

- Ans:**
- i. Whole blood and packed red cells: At 2 to 6°C in refrigerator for 35 days.
 - ii. Platelet concentrate: At 20 to 24°C for 3 days with continuous agitation.
 - iii. Fresh frozen plasma: Below -25°C for one year.
 - iv. Cryoprecipitate: Below -25°C for one year.

Q9. What is chimerism that can be found unexpectedly during blood grouping?

Ans: Sometimes a blood sample may contain more than one population of red cells. It may result from (i) transfusion of ABO

compatible but not ABO-identical blood, (ii) foetomaternal haemorrhage and (iii) bone marrow transplantation (when blood group of donor is different from that of recipient).

Q10. What do you mean by major cross-match and minor cross-match?

Ans: The name 'cross-match' came from the past practice of testing, the recipient's serum against donor's RBCs (major cross-match) and donor's serum against recipient's RBCs (minor cross-match). However, minor cross-match is less important as antibodies in donor blood becomes diluted or neutralized in recipient's plasma (volume is more than donor unit). Minor cross-match is also less important for antibody screening and identification.

Q11. What are the advantages of gel card method for blood grouping?

Ans: In gel card method, the monoclonal antibodies are used (unlike polyclonal antibodies in other methods). So, it is very sensitive and weak antigens can also be detected.

Q12. When blood group of a person can be changed to other blood group?

- Ans:**
- i. Almost always, an individual has the same blood group for life, but very rarely an individual's blood type changes through addition or suspension of an antigen in infections, autoimmune diseases or malignancies.
 - ii. Another rare cause in blood group change is bone marrow transplantation.
 - iii. Bacterial contamination: Infection of RBCs by bacteria both *in vivo* and *in vitro* may cause polyagglutination in normal sera. Bacterial enzymes expose T receptors on RBC surface. As most human sera contain anti-T antibodies, such infected red cells get agglutinated in normal serum. Hence, blood group O may appear as blood group AB.

- iv. Scientist has discovered a particular enzyme called 98 glycoside hydrolase, extracted from a strain of *Streptococcus pneumoniae*. The enzyme can cut away A or B antigens in blood group A, B or AB to make them more like blood group O. The scientist published their results in *Journal of the American Chemical Society*.

Q13. How different blood groups are associated with disease?

Ans: Persons with blood group A, B or AB will have more chance of heart attacks and heart diseases (due to coronary artery disease) and diabetes compared to blood group O. These people are more likely to develop cognition and memory loss (may be due to high blood pressure and high cholesterol). People with blood group A have been found to have a higher risk of stomach cancer. In blood group O, malaria is less severe. People who are Duffy antigen negative get protection from *P. vivax* infection.

Blood Group A : Heart disease, Stomach cancer.

Blood Group B: Heart disease, pancreatic cancer

Blood Group AB: Thromboembolism (blood clots), dementia

Blood Group O: Skin cancer, renal cancer

Q14. Why blood grouping of partners is done before marriage?

Ans: It is a good idea to know partner's blood group in the event of an emergency (for blood donation). Also knowing blood group is important for pregnancy and Rh factor should be known. If father is Rh +ve and mother is Rh -ve, it may cause Rh incompatibility (usually second and next pregnancies). This Rh incompatibility may cause hemolytic diseases of newborn (HDNB). So, in these cases pregnancies are monitored closely. Also, if needed Rh immunoglobulin (RhoGAM) is given to mother in the 7th month of pregnancy and again within 72 hours after delivery.

Haemoparasites

HAEMOPARASITES (PARASITES IN PERIPHERAL BLOOD AND BONE MARROW)

Some parasites are found in peripheral blood and bone marrow.

Parasites Found in PBS (Peripheral Blood Smear) and Bone Marrow

- Malaria: Most common parasite found in PBS
- Microfilaria of *Wuchereria bancrofti*
- *Leishmania donovani* or LD bodies of kala-azar: Found free or inside monocytes. Mainly found in the reticuloendothelial cells (macrophages) and megakaryocytes of bone marrow.
- *Trypanosoma cruzi* (causative organism of Chagas' disease)
- Others: Babesia, Brugia, Mansonella, etc. rarely.

Some parasites can be blood-borne. That means

- i. The parasite can be found in the blood stream of infected people; and
- ii. The parasites may spread to other people through exposure to an infected person's blood (for example, by blood transfusion or by sharing needle or syringes contaminated with blood).

Examples of parasitic diseases which can be blood-borne include African trypanosomiasis (caused by *Trypanosoma brucei*), babesiosis (caused by Babesia, a type of Apicomplexa and also known as Nuttallia), Chagas' disease, leishmaniasis, malaria and toxoplasmosis (caused by *Toxoplasma gondii*). In nature, many blood-borne parasites are spread by insects (vectors) like mosquitoes, sandflies, bugs, ticks, lice, mites, etc. So, they are called vector-borne diseases. But *Toxoplasma gondii* is not transmitted by a vector (by eating infected cooked food which contains cysts).

A parasite is an organism that lives in another organism, called the host, and often harms it. They depend on their host, and often harms it. They depend on their host for survival.

Without a host, a parasite cannot live, grow and multiply. Parasites, unlike predators, are usually much smaller than their host and they reproduce at a faster rate. A parasite either lives within the parts of the body of host or it lives on (outside but in contact) host. Parasites themselves are not diseases but they can spread diseases. The parasite uses host's resources to fuel its life cycle. It uses host's resources to maintain itself.

Endoparasites: These live inside the host. Examples are tapeworm, hookworm, round-

worm, etc. Endoparasites rely on a third organism, known as vector, or carrier.

Epiparasites: These feed on other parasites in a relationship known as hyperparasitism. As for example, a flea may live on a dog, but the flea may have a protozoan in its digestive tract. The protozoan is the hyperparasite.

Types of Parasites

- i. **Protozoa:** A protozoa can only multiply or divide within the host. Examples are Plasmodium (malaria), leishmanias, Trypanosoma in blood or bone marrow, Entamoeba histolytica (amoebiasis) or Trichomonas in intestine.
 - ii. **Helminths:** These are worm parasites. Examples are Schistosomiasis, pinworm, tapeworm, roundworm, etc.
 - iii. **Ectoparasites:** These live on their hosts (not inside). Examples are lice and fleas.
- The important haemoparasites are: (1) Malarial parasites, (2) microfilaria, (3) trypanosomes, (4) leishmaniasis and (5) babesiosis.

Infection with haemoparasites (blood parasites, particularly malaria and filaria may cause enormous human sufferings especially in Indian subcontinent. Malaria and microfilaria can be diagnosed by careful examination of PBS but bone marrow examination is necessary for diagnosis of Leishmania.

MALARIA

There are four species of Plasmodium (malarial parasites which infect humans: *Plasmodium vivax*, *Plasmodium falciparum*, *Plasmodium malariae* and *Plasmodium ovale*. Among these four species *P. vivax* is the most widely distributed species in the world. In India, incidence is *P. vivax* (70%), *P. falciparum* (25–30%), mixed infection of *P. vivax* and *P. falciparum* (4–8%) and *P. malariae* (<1%). *P. malariae* has been reported in the Eastern India state of Odisha (Sharna et. al, 2006), while *P. ovale* appears to be extremely rare, if not absent. Odisha has the highest incidence

of malaria (40% of all malarial cases in India as per ICMR, 2019).

P. vivax and *P. ovale* both of which cause benign tertian malaria (febrile episodes typically occurring at 48 hours intervals). *P. falciparum*, which is accountable for most deaths due to malaria (malignant malaria and febrile episodes typically occurring at 72 hours intervals). *P. malariae* and *falciparum* may cause nephrotic syndrome (Table 9.1 and Figs 9.1 and 9.2).

Role of Genetic Factors in Malaria

- **Duffy antigen:** People with **Duffy antigen negative** blood group or the Fy (a⁻ b⁻) phenotype in blacks confers protection against *P. vivax* infection. This is due to the fact that *P. vivax* parasite enters RBC at glycoprotein receptors present on Duffy antigen site. Black people of West Africa have this immunity towards *P. vivax* as they lack Duffy antigens on red cells. But this protection is available to homozygous people (homozygotes) only.
- **G6PD (Glucose-6-phosphate dehydrogenase) deficiency:** G6PD deficient female heterozygotes get protection against falciparum malaria (*P. falciparum* infection).
- **Sickle cell trait:** Heterozygous patients of sickle cell trait will have comparatively milder form of falciparum malaria. But the homozygotes do not have this advantage.
- **β-Thalassaemia trait:** They also get protection against falciparum malaria.
- **HbC and pyruvate kinase deficiency** also give protection against malaria.
- **Newborns:** Newborn infants have high level of foetal haemoglobin or HbF (4–6 months of early life) until replaced by adult haemoglobin. HbF is markedly increased in a disease called hereditary persistence of foetal haemoglobin (HPFH) which may be present both in children and in adults. HbF in red cells suppresses the growth of malarial parasite. So, newborns (4–6 months) and patients of HPFH get protection against malaria.

- **HLA subtype:** Certain HLA antigens like class I antigen HLA-BW53 and class II antigen HLA-DRBI 1302 offers some sort immunity against malaria.
- Renal impairment [serum creatinine (>3.5 mg/dl)]
- Hypoglycaemia (blood glucose <40 mg/dl)
- Unconsciousness or coma
- Prostration (extremely weak or subservient)
- Circulatory collapse or shock
- Multiple convulsions
- Clinical jaundice along with dysfunction of vital organs
- Haemoglobinuria
- Respiratory distress/acidotic breathing
- Metabolic acidosis

WHO Criteria for Severe Malaria

If anyone if these features is present, it is called severe malaria:

- Hyperparasitemia (>5% parasitised RBCs in low endemic area and >10% in hyper-endemic area).

Table 9.1: Different features of *P. vivax* and *P. falciparum*

Features	<i>P. vivax</i>	<i>P. falciparum</i>
Distribution	Tropical countries, subtropics, temperate zones	Tropical countries
Incubation period	8–10 days	8–10 days
RBC infected	Young red cells	All stages of RBCs
Severity of disease	Moderate to severe	Severe
Periodicity of symptoms	48 hours	36–48 hours
Relapse	Yes (due to hepatic hypnozoites)	No
Nephrotic syndrome	Rare	May be
CNS involvement	Rare	Frequent
Microscopic examination		
Form in PBS	Trophozoites (ring form), schizonts and gametocytes	Trophozoites (ring form) and crescents (gametocytes) only. No schizont.
Trophozoite and ring form	Usually single size 2.5 µm; cytoplasm opposite nucleus is thicker. Ring 1/3 diameter of RBC	Single and/ or multiple rings, size of ring smaller 1.25–1.5 µm; cytoplasm regular in outline ring 1/6th diameter of RBC
Nuclei	Single chromatin dots	Single or multiple chromatin dots
Infected RBCs	RBCs are enlarged and pale, Schüffner's dots present	Normal size RBC but crenated, raddish violet colour, Maurer's dot (6–12 in number) in cytoplasm of RBC
Accole' or Applique	Absent	Present. As the parasite attaches itself to the margin or the edge of host cell (RBC), the nucleus and a small part of cytoplasm remains almost outside giving the appearance of 'form applique' or 'accole'.
Schizonts	Size 9–10 µm regular almost completely fill RBC	Size 4–5 µm, fills 2/3 of RBC
Merozoites	12–24 in number, irregularly arranged (grape-like clusters) yellowish brown, fine granules	18–32, arranged like grape-like clusters

Contd.

Table 9.1: Different features of *P. vivax* and *P. falciparum* (Contd.)

Microscopic examination	<i>P. vivax</i>	<i>P. falciparum</i>
Gametocyte	Spherical or globular much larger than RBC	Crescentic or sickle-shaped larger than a RBC but host cell (RBC) hardly recognisable
Haemozoin Schüffner's dots or Maurer's dots in cytoplasm	Yellowish-brown, fine granules Schüffner's dots	Dark brown or blackish Maurer's dots

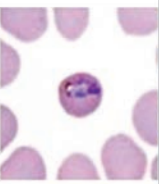
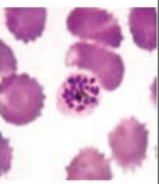
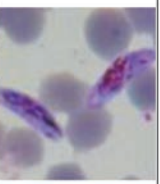
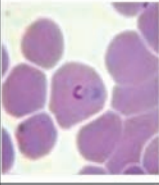
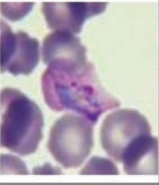
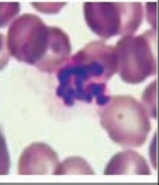
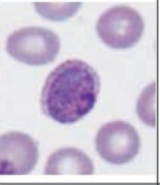
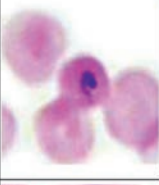
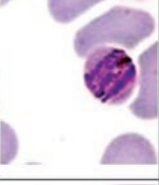
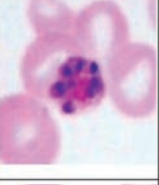
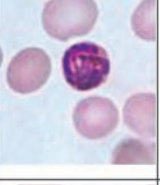
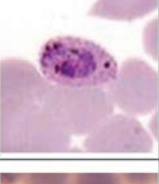
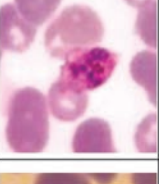
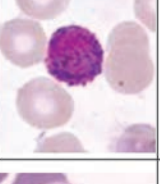
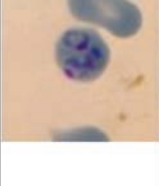
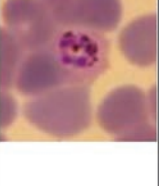
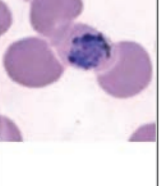
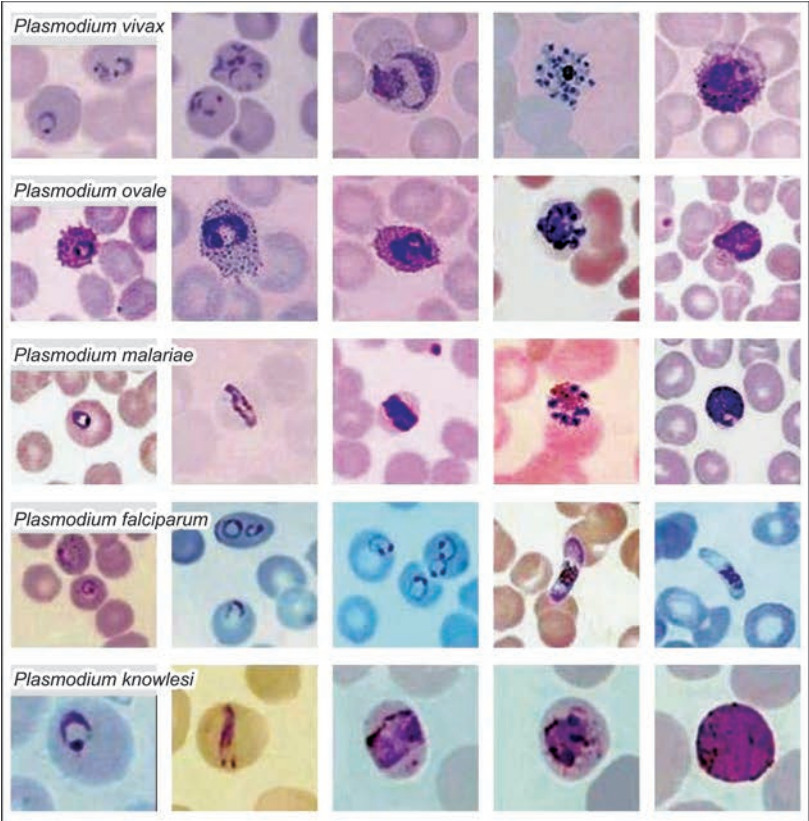
Stages Species	Ring	Trophozoite	Schizont	Gametocyte	
<i>P. falciparum</i>					- Parasitised red cells (pRBCs) not enlarged. - RBCs containing mature trophozoites sequestered in deep vessels. - Total parasite biomass = circulating parasites + sequestered parasites.
<i>P. vivax</i>					- Parasitised prefer young red cells - pRBCs enlarged. - Trophozoites are amoeboid in shape. - All stages present in peripheral blood.
<i>P. malariae</i>					- Parasitised prefer old red cells. - pRBCs not enlarged. - Trophozoites tend to have a band shape. - All stages present in peripheral blood
<i>P. ovale</i>					- pRBCs slightly enlarged and have an oval shape with tufted ends. - All stages present in peripheral blood
<i>P. knowlesi</i>					- pRBCs not enlarged. - Trophozoites, pigment spreads inside cytoplasm, like <i>P. malariae</i>, band form may be seen - Multiple invasion and high parasitaemia can be seen like <i>P. falciparum</i> - All stages present in peripheral blood.

Fig. 9.1: Human malaria



Gametocytes		Schizonts		Trophozoites		
Female	Male	Mature	Immature	Old	Young	
						<i>P. falciparum</i>
						<i>P. vivax</i>
						<i>P. malariae</i>
						<i>P. ovale</i>

Fig. 9.2: Different species of human malaria

MICROSCOPIC EXAMINATION FOR MALARIA

- **Collection of blood:** Blood smear should be obtained before or at the onset of fever and chills. Some advocate that blood should be collected 2–6 hours after the peak of the febrile paroxysms, as number of parasites is more during this period, collection of blood immediately following a paroxysm of fever usually do not show intraerythrocytic parasites because of lysis of parasitized red cells. Blood sample should be taken before giving antimalarial drugs (if given, parasites to number become less in bloody).
- **Smear preparation:** Both thin, and thick smears made from a finger-prick (skin puncture). Then smears are made immediately. Anticoagulated blood is not preferred. If necessary, blood is collected in EDTA and smears are prepared as early as possible, otherwise, morphologic changes of parasites will occur and diagnosis may be difficult.
- **Staining of smears:** One thick smear and one thin smear are stained with Giemsa or Leishman stain. Thick smear also can be stained by Field's stain. For preparation of thin and thick smear refer to Chapter 2. Before staining, thin smear is fixed with methanol for 1–2 minutes.
- **Microscopic fields to be examined:** At least 100 oil immersion fields (requires 5 minutes) of thick smears and 200 oil immersion fields (requires 15 minutes) of thin smears should be examined 'before issuing a negative report (Figs 9.3 and 9.4).

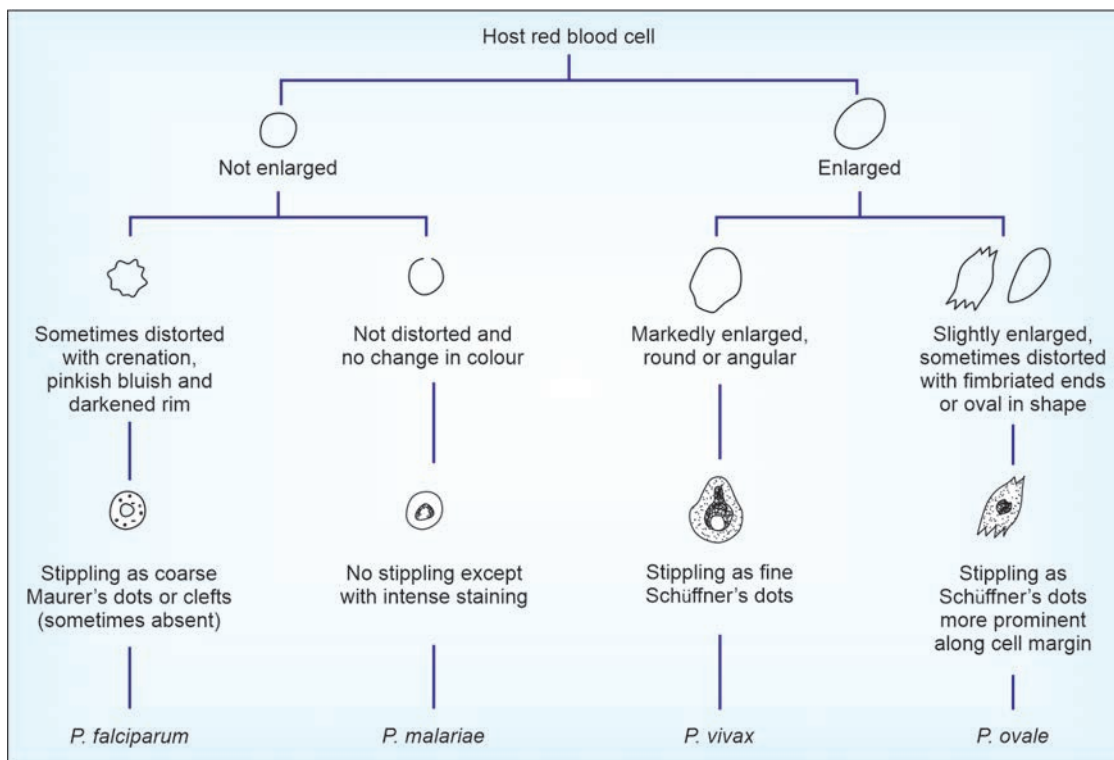


Fig. 9.3: Schematic diagram to diagnose four different species of malaria

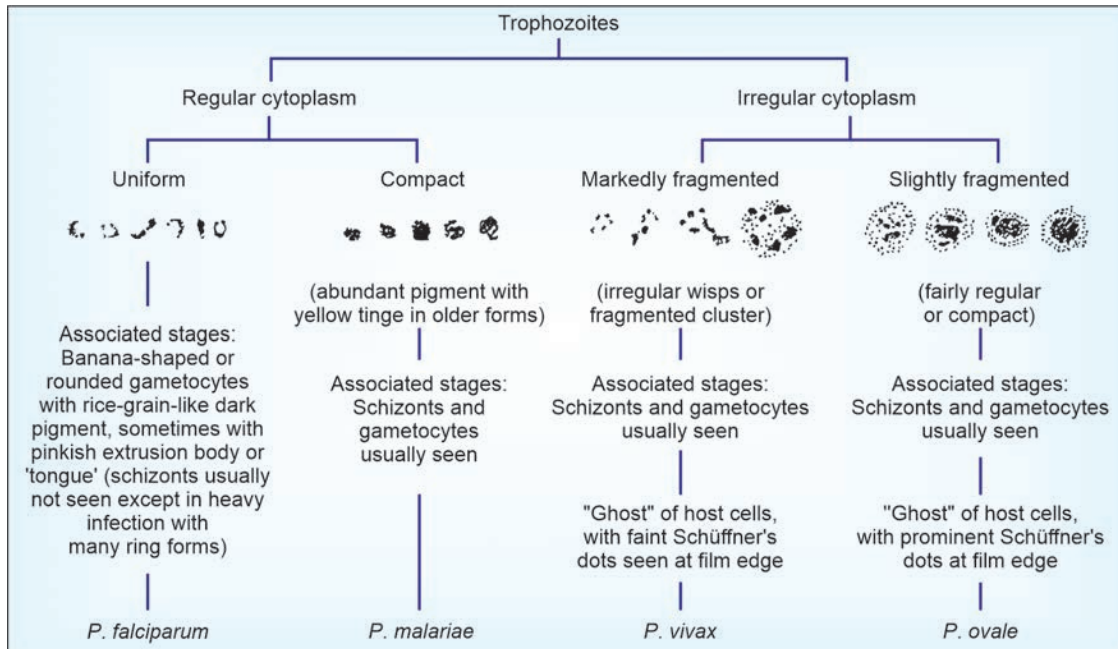


Fig. 9.4: Species differentiation of malarial parasites by cytoplasmic pattern of trophozoites in Giemsa-stained thick blood films

PLASMODIUM FALCIPARUM (*P. FALCIPARUM*)

In the peripheral blood trophozoites, and gametocytes are present. Schizonts are rarely found in PBS.

- **Early trophozoites:** A delicate small uniformly fine cytoplasmic ring with 1–2 small chromatin dots. Ring may be attached (protruding) to the red cell margin (accolé form).
- **Late trophozoites:** Compact blue ring with 1–2 red chromatin dots.
- **Schizont:** Very rarely seen except in cerebral malaria. It contains 18–32 merozoites which fill 2/3rds of the RBC.
- **Gametocyte:** Crescentic or sickle or sausage or banana-shaped and larger than RBC (Figs 9.5 and 9.6).

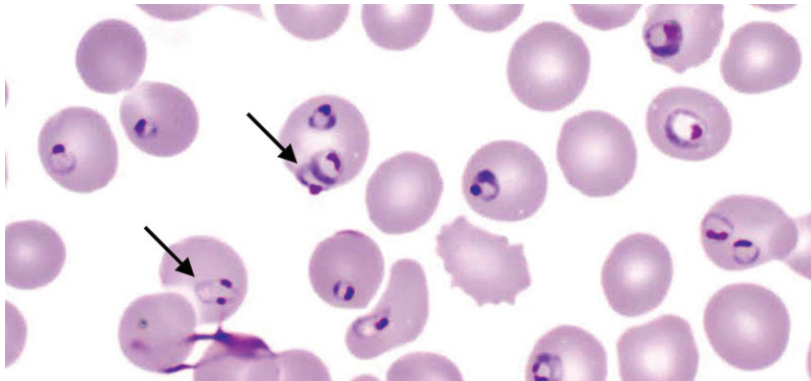


Fig. 9.5: Trophozoites of *P. falciparum*

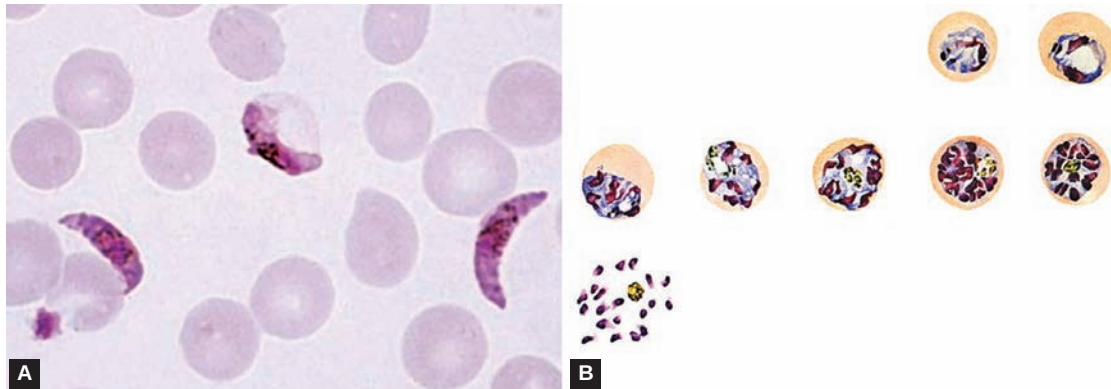


Fig. 9.6: (A) Gametocytes of *P. falciparum*; (B) Schizonts of *P. falciparum*

Note

- **Signet ring form** and **marginal form** usually seen with young or early trophozoites.
- **Maurer's dots** are seen in the cytoplasm of RBC with early trophozoites. Maurer's dots (also called Maurer's clefts) are red colored granules present in the cytoplasm. They are usually larger and more coarse than Schüffner's dots (found in *P. vivax* and *P. ovale*). Like Schüffner's dots, Maurer's dots appear to play a role in the metabolic pathways of the infected RBCs.
- In late or mature trophozoites showing haze of pigments may be seen throughout the cytoplasm. Maurer's dots may be found in the cell.
- Gametocytes may be microgametocyte or macrogametocyte or it may be immature or mature. Remember only mature macrogametocytes are crescentic or banana-shaped. Gametocytes may be male gametocyte (cytoplasm pale blue, nucleus diffuse and larger) or female gametocyte (cytoplasm blue, nucleus small and compact).

Plasmodium vivax (*P. vivax*) (Fig. 9.7)

Trophozoites, schizonts and gametocytes, i.e. all stages may be found in the peripheral blood (PBS).

- Early trophozoites: Blue cytoplasmic ring, 1/3rd the diameter of RBC. One side of the ring thicker; red chromatin at thinner part of ring.
- Late trophozoites: Irregularly thick cytoplasmic ring (amoeboid form); large red chromatin dot.

- Schizonts: 12–24 merozoites arranged like a rosette with granular yellow-brown pigment in center.
- Gametocytes: Large and spherical. Much larger than RBC.

Note

- Pseudopodia may be found in polychromatophilic erythrocyte
- Microgametocytes (male): Size: 9–10 μm ; cytoplasm: stains light blue; Nucleus: Diffuse large, lies laterally
- Macrogametocytes (female): Size: 10–12 μm , cytoplasm: stains deep blue; Nucleus: Small compact, lies peripherally.
- Early trophozoite has a cytoplasmic ring (blue), a red nuclear mass (chromatin dot), and an unstained area called **nutrient vacuole**.
- The trophozoites possess a very active amoeboid movement and constantly thrusts out pseudopodia inside the RBC, giving rise to diverse forms.
- After a period of about 10 hours, yellowish brown pigment (hemozoin) granules appear in the cytoplasm.
- Schizont appears after a period of growth of about 36–40 hours and represent full grown (mature) trophozoites. At this stage the parasite becomes rounded in shape and has lost all amoeboid activities. According to the stage, the schizont may be immature, schizont (nucleus not divided) and mature schizont (nucleus divided) (Fig. 9.5).

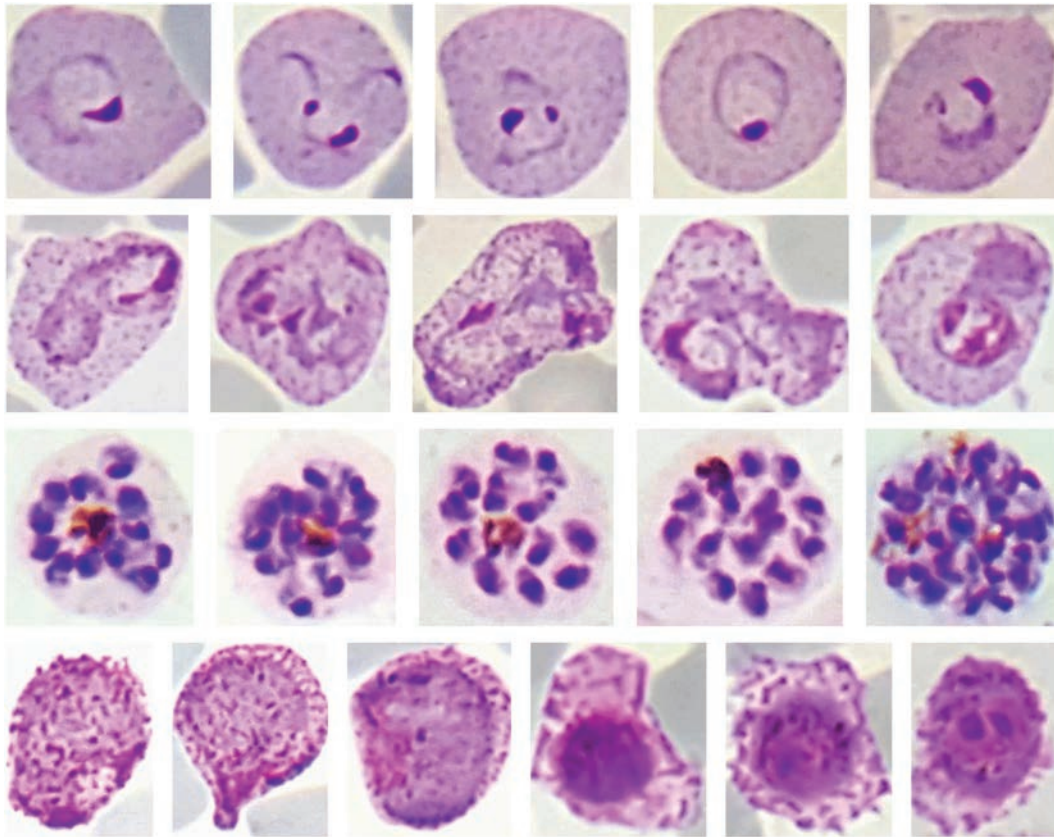


Fig. 9.7: *Plasmodium vivax*. First row: Early trophozoites, Second row: Late trophozoites, Third row: Schizonts, Fourth row: Gametocytes

THICK SMEAR FOR MALARIAL DETECTION

Preparation and straining: Thick smear is prepared by spreading a large drop of blood from center of a glass slide. It is spreaded with the corner of a spreader slide or a stick in such a manner that an evenly spread circular or rectangular smear of size 15×15 mm is obtained.

After that smears are air dried. For better results, thick smears can be dried in an incubator at 37°C for 15 minutes. Thick smears are not fixed in methanol (unlike thin smears) as they will be dehaemoglobinised.

The smears are then given a few dips in tap water until red coloured solution comes out (**dehaemoglobinisation of the smear**) Now, the smear is fixed in methanol (after

dip in tap water) and stained with Leishman's or Giemsa or Field's stain.

Thick smear is a concentration method for malarial parasites. One microscopic field (oil immersion) of thick smear is equivalent to 40–50 microscopic fields of a thin smear. However, thin smear is essential for morphology and to characterize the type of malarial parasites. Thick smear is of use for quick detection or for mass surveys (Fig. 9.8).

Malarial Pigments

Due to parasitemia of RBCs, physiology of red cell is disturbed, globin part of the haemoglobin is broken down and resynthesized into parasite protein. The unutilized

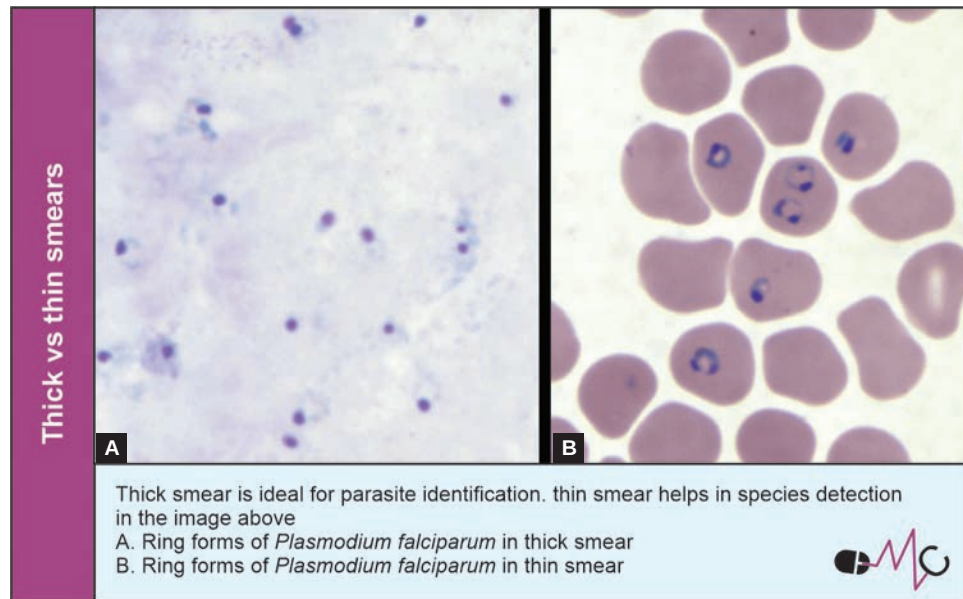


Fig. 9.8: *Plasmodium falciparum*; (A) Thick smear; (B) Thin smear

part of globin is called **haemozoin**—a brown coloured pigment presents in the parasite and known as **malarial pigment**. Haemozoin is responsible for the pigmentation of various organs like liver, spleen, brain, etc. (Fig. 9.9).

Parasite Density (PD)

High density of parasite is associated with a severe disease. The prognosis becomes worse if mature form of parasites (with

malarial pigment) predominates in blood. This means prognosis will be better if PBS shows only ring forms (early trophozoites) and >50% of all forms. Prognosis will be bad if PBS shows many mature trophozoites and schizonts (>20% in number).

To calculate parasite density, 200 WBCs are counted on a **thick smear**. Number of parasites present per μl of blood is calculated from this formula:

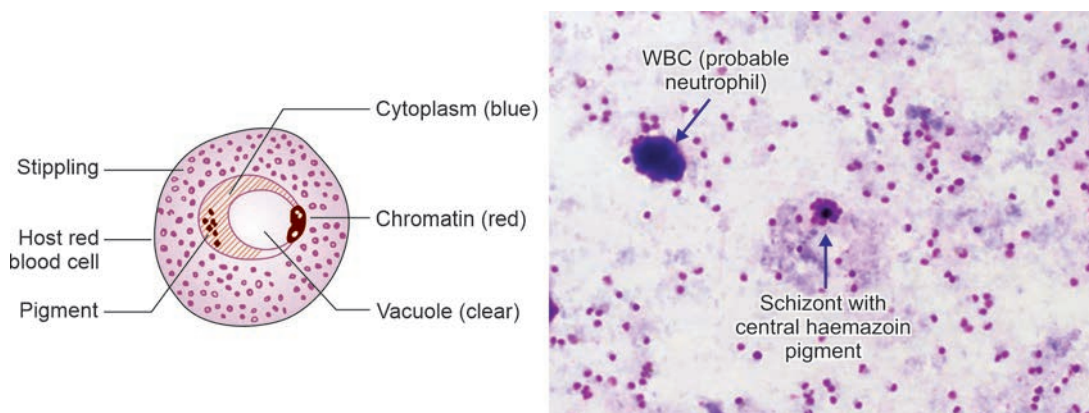


Fig. 9.9: Malarial pigments

Parasite density (PD) on thick smear
= Total WBC count/ μl

$$\times \frac{\text{Number of parasites}}{200}$$

that is (total WBC count/ μl $\times$ Number of parasites) $\div$ 200

Assuming a WBC count of 8000/ μl , the number of parasites is multiplied by 40 to get PD. However, if accurate WBC count is known, then a better estimate of PD is obtained.

In a **thin smear**, number of parasites amongst 1000 RBCs is counted and reported as a percentage. Number of parasites in 1 μl of blood can be calculated if RBC count in millions/ μl is known.

PD on thin smear = Red cell count in millions/ μl $\times$ Parasite percentage, i.e. number of parasites in 1 μl of blood or PD.

If the RBC count is not known, then it can be arbitrarily taken as 5 millions/ μl .

Significance of parasite density (PD)

- i. Calculation of PD is of significance in *P. falciparum* infection. PD >10% is an indication for exchange transfusion.
- ii. For effective antimalarial treatment (treatment response), percent parasitemia should be calculated (PD) daily till no more parasites (excluding gametocytes) are found.

Parasite Index (PI)

This is calculated by estimating the number of parasitised RBCs among 1000 RBCs. Only asexual forms (ring, trophozoite and schizont) are included for calculating the PI (parasite index). The parasite load is graded on a scale of I–IV corresponding to 0–5%, 6–10%, 11–20% and > 20%. This is calculated on thin smears So,

PI (Parasite index) =

$$\frac{\text{Number of parasitised RBCs}}{1000 \text{ RBCs}} \times 100\%$$

Blood Alterations in Malaria

PBS also shows monocytosis with moderate leukopenia. Two-thirds of patients infected with *P. falciparum* infection also show anaemia with to increased reticulocyte count (due to haemolysis) and thrombocytopenia.

Anaemia progressively becomes progressively severe due to hypersplenism.

Reporting of Smear (Result)

In the presence of malarial parasites, blood smear should be reported as follows:

- Smear is positive or negative for malarial parasite
- Name of species (*P. vivax* or *P. falciparum* or mixed, etc.)
- Red cell stages (early trophozoite, ring form, late trophozoite, schizont, gametocyte, accolé form, amoeboid form, etc.)
- Parasite density (especially in *P. falciparum*).

RAPID TEST FOR MALARIA: MICROTYPING TEST (GEL CARD)

Recently, simple nonmicroscopic, rapid diagnostic kits are available in the market. These tests detect malarial antigen by immuno-chromatographic method. The antigens against which commercial test kits presently available are:

- **Parasite, lactate dehydrogenase (pLDH):** This is an enzyme of glycolytic pathway which is present in all four human malarial species. But there are forms of LDH for each species. Level of pLDH correlates with parasite density.
- **Parasite aldolase:** Aldolase based tests are less sensitive for non-falciparum species.
- **Histidine rich protein 2 (HRP-2):** It is synthesized by asexual stages and young gametocytes of *P. falciparum* and expressed on red cell surface.

Of the different antigenic tests, **gel card**, or **microtyping test** for malarial pLDH antigen

is very popular. For this test, 10 µl of fresh/frozen/dried whole blood is taken. RBCs are lysed and pLDH from parasitized RBCs is released. Presence of pLDH is detected using monoclonal antibodies against specific epitomes of pLDH (immunochromatographic test: Optimal).

Test based on HRP-2 is useful to detect *P. falciparum* infection. But HRP-2 antigens remain longer in circulation than pLDH. So, more false + ve cases may be reported even after malarial treatment: (immunochromatographic test: Optimal).

Positive test indicates active Plasmodium infection and it can differentiate various species of malarial species. It can also determine strains of Plasmodium resistant to therapy by repeating the test 48 hours after treatment (Fig. 9.10).

Other Methods of Malarial Detection

- i. **Fluorescent microscopy:** Nucleic acids of the parasite are stained, with fluorescent dyes and visualized by fluorescent microscopy.
- ii. **PCR based test:** These tests are based on the detection of nucleic acid sequence specific to *Plasmodium* species. Primers targeting the *Plasmodium* species 18S rRNA genes are used.

Artifacts Mimicking Malaria on Smears

- The most common artifact in thin smear/films are blood **platelets superimposed on red blood cells**. These platelets should be readily identified because they do not have a true ring form. Platelets do not show differentiation of the chromatin and cytoplasm and do not contain pigments.
- **Clumps bacteria or platelets** may be confused with schizonts.
- At times, masses of fused platelets may resemble gametocytes of *P. falciparum* but do not show the differential staining or the pigment.
- Precipitated stain and contaminating bacteria, fungi, or spores may also be confused with these parasites.

Filaria (*Wuchereria bancrofti*)

Wuchereria bancrofti is confined to tropical and subtropical regions. In India, the disease is confined to the regions along the banks of big rivers, and sea coast. Adult worms localize in the lymphatic vessels and lymph nodes causing lymphatic obstruction to lymph flow resulting in lymphedema and elephantiasis. Embryos of *W. bancrofti* (microfilaria) pass through lymph nodes, lymphatics and enters circulating blood.

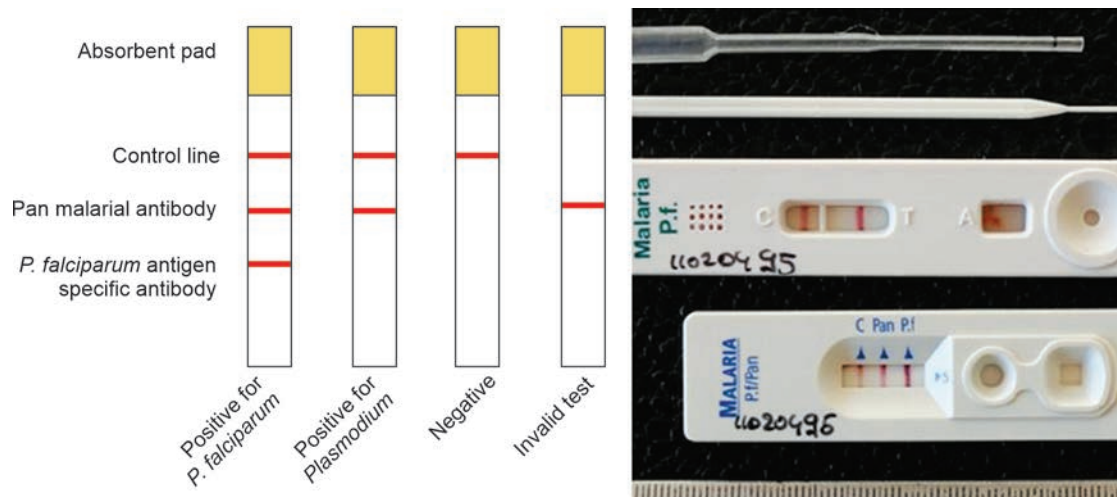


Fig. 9.10: Rapid diagnostic kits for malaria

Demonstration of this microfilaria in PBS is diagnostic clue of filariasis.

Collection of blood: The blood sample should be collected at night between 10 pm and 2 am as microfilariae exhibit nocturnal periodicity. Midnight smears can also be examined.

From blood sample, microfilariae can be demonstrated in

- i. Thin blood smear
- ii. Thick blood smear
- iii. Unstained wet preparation: For this, 2–3 drops of blood are taken in a slide and a coverslip is placed. Microfilariae are recognised by their movements causing agitation of the wet smear and can be confirmed by examining under a low power objective.
If the smear is to be examined next morning then the coverslip is sealed with Vaseline to prevent drying of blood drop.
- iv. Concentration method: Take 5 ml of blood in EDTA vial. Centrifuge at 2500 r.p.m for 5 minutes. Discard the plasma and take buffy coat and sediments of cells. Make 4–6 smears out of it. Air dry, fix in methanol and stain in Romanowsky stain.

Alternatively, 5 ml blood + 10 ml distilled water is taken in a tube and the mixture is shaken vigorously till blood is completely haemolysed. Centrifuge the tube at 2500 r.p.m. for 15 minutes. Deposit after centrifugation is examined under for microfilaria.

Morphology of filaria: Microfilaria measures about 290 μm , in length and 6 μ in breadth. A hyaline sheath engulfs the larval body but hyaline sheath is longer than body. Somatic cells appear as granules and extend from head to terminal tail sheath, which is a distinguishing feature of *W. bancrofti* microfilaria (tail end free).

Bone marrow examination: Sometimes, bone marrow examination also reveals microfilariae of *W. bancrofti* (Fig. 9.11).

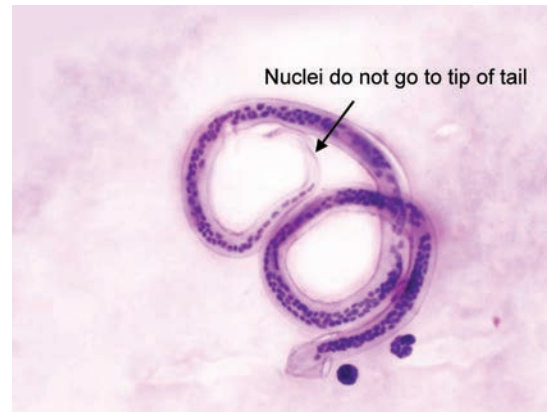


Fig. 9.11: Filaria (*Wuchereria bancrofti*) in blood

Rapid immunochromatographic test: This is a card test, highly sensitive and specific test for diagnosis of active infection caused by *Wuchereria bancrofti*. This card test is available in the market (ICT diagnostics).

This test detects circulating antigens of filaria in a finger prick blood sample. In contrast to microscopic examination, blood sample can be collected at any time of the day. There is no cross-reactivity with other filarial organism like *Brugia malayi*.

Positive result is obtained even microfilariae are not present in blood and adult worms live in lymphatics/lymph nodes. But the test may give positive result for up to 18 months following successful treatment of filariasis.

Leishmaniasis (*Leishmania donovani*)

The parasite *Leishmania donovani* (LD) causes kala-azar or visceral leishmaniasis. In India, it is endemic in the states of Bihar, Odisha, Chennai and Eastern parts of Uttar Pradesh. **Amastigote forms, known as LD bodies** can be found in the reticulum cells of bone marrow and spleen. So, **bone marrow examination and splenic puncture** is done to identify LD bodies (**amastigote form**).

LD bodies are small, round, 2-4 μm in diameter with a nucleus and a pod-shaped kinetoplast.

Rarely LD bodies are seen free on in monocyte in peripheral blood when parasitemia is very high. So, it may be found from buffy coat preparation of peripheral blood.

PCR (molecular diagnosis): Target regions for PCR analysis are genes encoding r-RNA, repetitive nuclear DNA sequences and kinetoplast DNA. PCR which amplify a 120-bp fragment of kinetoplast DNA has the highest sensitivity (Fig. 9.12).

Trypanosomes/Trypanosoma

Trypanosoma is a unicellular parasitic protozoon. The name is derived from the Greek

trypano (borer) and *soma* (body) because of their corkscrew-like motion. Most trypanosomes are heterogenous (requiring more than one obligatory host to complete cycle) and most are transmitted via a vector. Trypanosoma is a motile flagellate protozoon. The kinetoplast contains the mitochondrial DNA and the nucleus contains the genomic DNA.

Trypanosomes infect variety of hosts and cause various diseases, including the fatal human diseases like sleeping sickness caused by *Trypanosoma brucei* and Chagas disease caused by *Trypanosoma cruzi* (Fig. 9.13).

The diagnosis can be made by demonstrating **trypomastigote forms (amastigotes of trypanosoma)** in PBS or bone marrow.

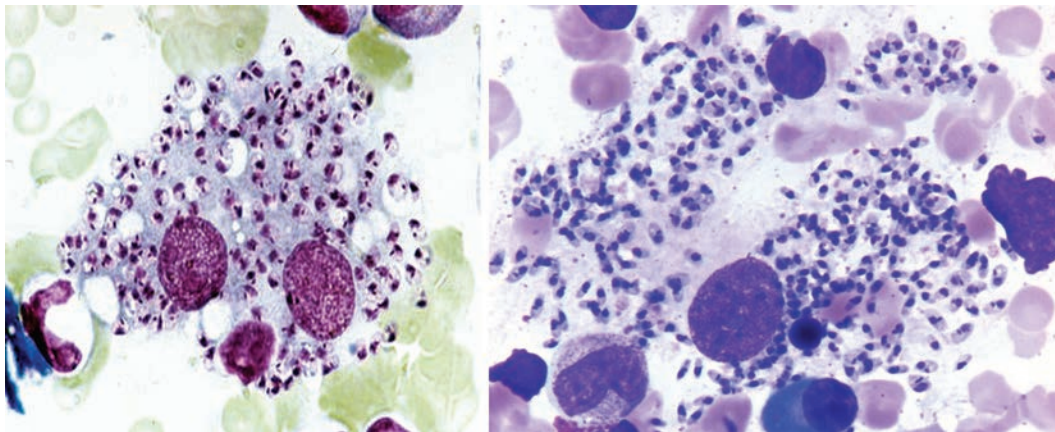


Fig. 9.12: LD bodies in bone marrow

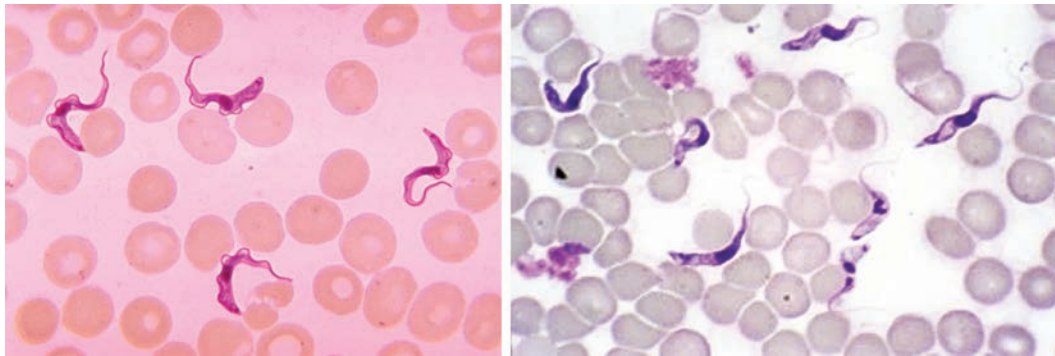


Fig.9.13: *Trypanosoma cruzi* in peripheral blood smear

Babesiosis (Fig. 9.14)

Babesiosis is a malarial-like parasitic disease caused by infection with *Babesia*. Human babesiosis is transmitted by tick bite.

Diagnosis can be done by demonstrating *Babesia* parasites in RBCs on PBS. Morphologically it looks like ring form of malarial

parasite and can be confused with *Plasmodium* infection (Table 9.2 and Fig. 9.16). Diagnosis can also be done by antibody testing (indirect fluorescent antibody test, IFA). The antibody level (titer) rises in about 2–4 weeks after infection and declines/wanes at 6–12 months (Fig. 9.15).

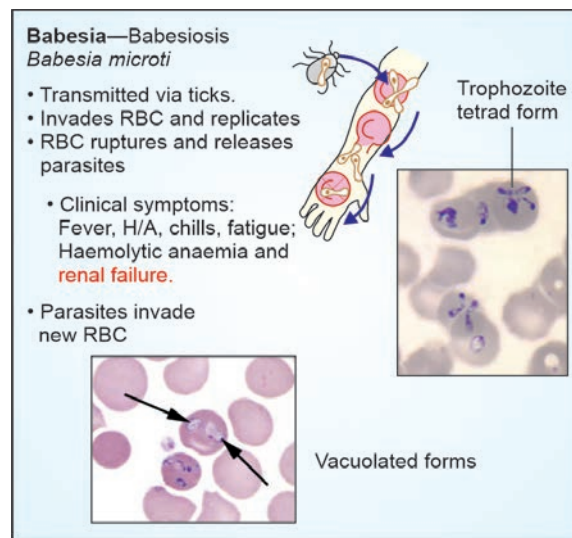


Fig. 9.14: Schematic diagram of babesiosis

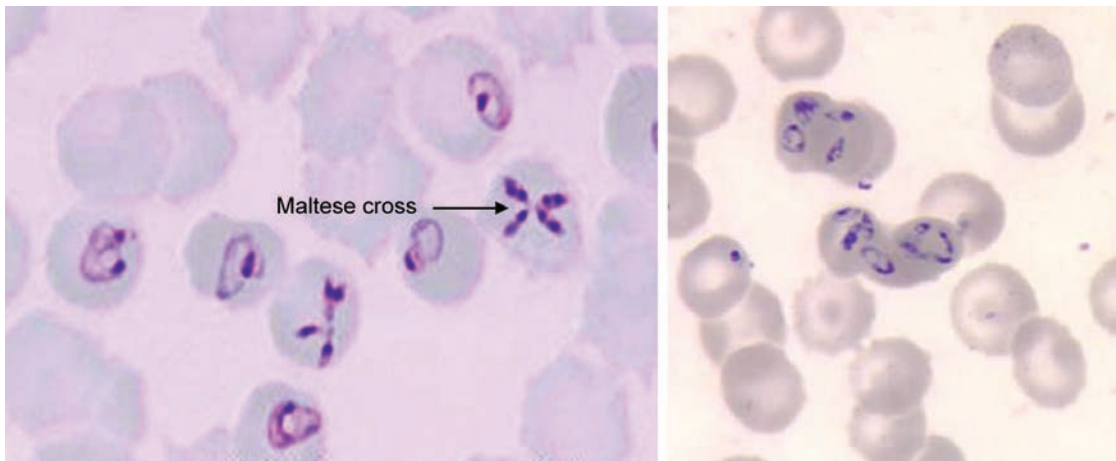


Fig.9.15: Babesiosis on peripheral blood

Table 9.2: Morphological differentiation of malaria parasites (Fig. 9.16)

	<i>P. vivax</i>	<i>P. falciparum</i>	<i>P. ovale</i>	<i>P. malariae</i>
Infected RBC	Enlarged, Schüffner dots	Normal size, Maurer clefts	Enlarged, oval and fimbriated, Schüffner dots	Normal or microcytic, stippling not usually seen
Ring form (early trophozoites)	Large, thick, usually single (occasionally 2) in cell, large chromatin dot	Delicate, frequently 2 or more accolé, small chromatin dot	Thick, compact rings	Very small, compact rings
Late trophozoites	Amoeboid, central vacuole, light blue cytoplasm	Compact, vacuolated, sometimes 2 chromatin dots	Smaller than <i>P. vivax</i> , slightly amoeboid	Band across cell, deep blue cytoplasm
Gametocytes	Spherical, compact, almost fills cell, single nucleus	Crescent or sausage shaped, diffuse chromatin, single nucleus	Oval, fills three-fourths of cell, smaller than <i>P. vivax</i> but similar	Round, fills one-half to two-thirds of cell, similar to <i>P. vivax</i> but smaller
Schizonts	12–24 merozoites, irregularly arranged	18–24 merozoites, filling two-thirds of cell	8–12 merozoites filling three-fourths of cell	6–12 merozoites in daisy head around central mass of pigment
Pigments	Fine granular, yellow-brown	Dark to black clumped mass	Coarse light brown	Dark, prominent at all stages

In *P. falciparum* infection, percentage of RBCs infected should be reported. Schüffner dots: Fine stippling; Maurer dots or clefts: Large, irregularly shaped, red-staining dots; Accolé forms: Parasite that is marginalized to edge of cell

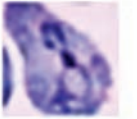
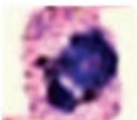
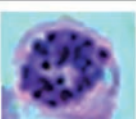
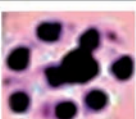
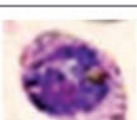
Species Stages	<i>P. falciparum</i>	<i>P. vivax</i>	<i>P. malariae</i>	<i>P. ovale</i>
Ring stages				
Trophozoite				
Schizont				
Gametocyte				

Fig. 9.16: Differentiation of malaria parasite

Blood Transfusion/ Transfusion Medicine

Transfusion medicine is a multidisciplinary speciality concerned with the proper selection and utilization of blood components as well as the removal of blood or blood components in the treatment or prevention of disease.

The term blood banking is being replaced by the term **transfusion medicine** in order to stress the increasing role of patient care and evaluation of clinical results in this speciality. The blood components—red cells, platelets, granulocytes, fresh frozen plasma and cryoprecipitate—are made directly from a unit of whole blood, using different methods of physical separation (i.e. centrifugation and freezing).

Blood derivatives like albumin, plasma protein fraction, immune serum globulin, coagulation factor concentrates are produced by pharmaceutical companies and are usually made from plasma pools of thousands of donor units, using modifications of the Cohn ethanol fractionation technique. The basic principle of this method is that different proteins can be precipitated from plasma, without denaturation, by adjusting the amount of ethanol added.

Whole blood: One unit of donor blood collected in a suitable anticoagulant—preservative solution and which contains blood cells and plasma.

Blood components: A constituent separated from whole blood, by differential centrifugation of one donor unit of plasma by fractionation.

Blood derivatives: A product obtained from multiple donor units of plasma by fractionation (Fig. 10.1).

BLOOD TRANSFUSION

Blood transfusion is the process of transferring blood/blood products from donor into the blood circulation of recipient within stipulated time.

Types of Blood Transfusions

- **Whole blood donation:** In this one unit (350 ml) of whole blood is collected with a suitable anticoagulant.
- **Autologous donation:** In this type, blood is collected for an individual for subsequent transfusion to the same individual from whom the blood is collected.
- **Apheresis donation:** In this method, whole blood is removed from donor and is separated. Only desired portion is retained and the remaining portion is returned to the donor.
- **Massive blood transfusion:** It is defined in adults as replacement of >1 blood volume, e.g. 100% blood volume in

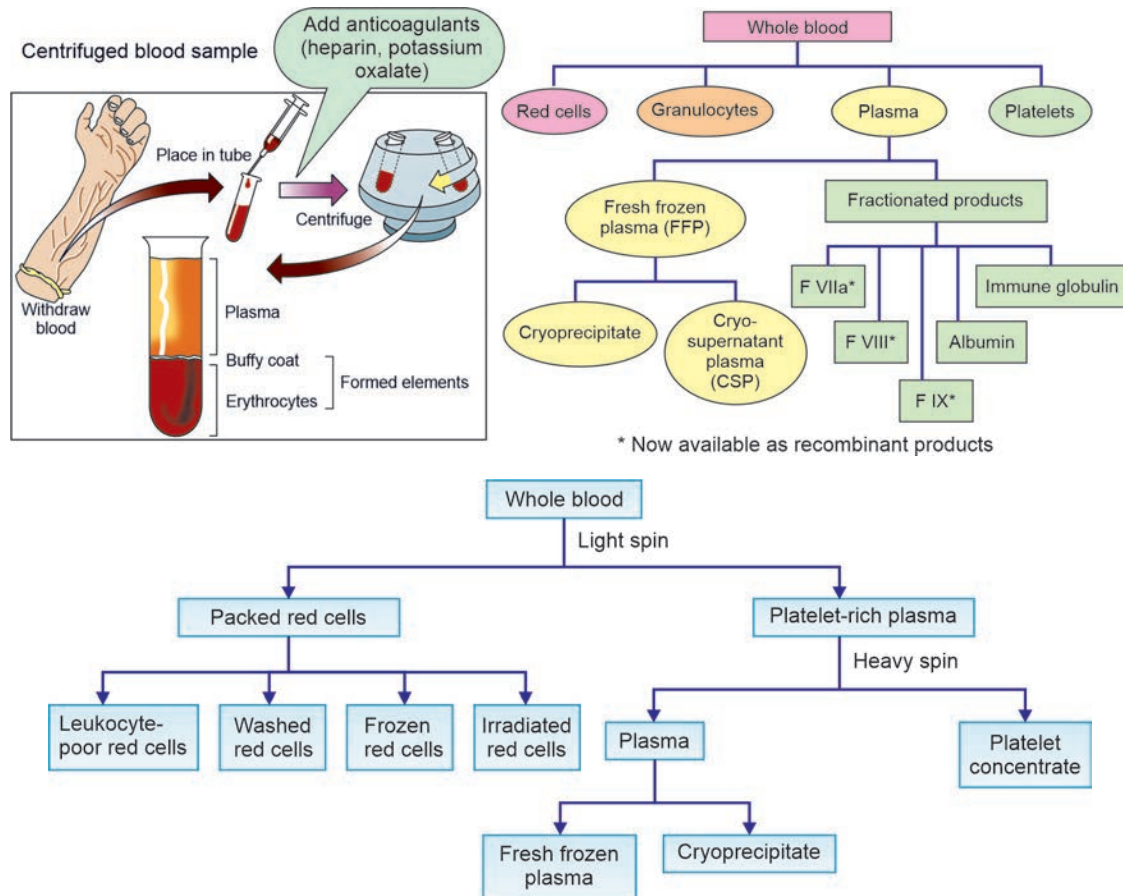


Fig. 10.1: Blood component preparation from whole blood (within 6 hours of collection)

24 hours or >50% of blood volume in 4 hours (adult blood volume is approximately 70 ml/kg). In the past, it was defined as the replacement by transfusion of 10 units of red cells in 24 hours. For children, 100% blood volume in 24 hours or >50% blood volume in 3 hours.

- **Emergency blood transfusion:** If the patient's blood group cannot be determined, then the patient should be transfused with O blood group red cells which are Rh negative with colloids/crystalloids.

Donor Selection for Blood Transfusion

- The donor should be healthy and free from diseases of heart, lung, liver and kidney.

- Donor should not suffer from cancer, diabetes, epilepsy, tuberculosis, bleeding, allergy, malaria, sexually transmitted diseases.
- The donor should be between 18–65 years of age.
- Weight should be >45 kg.
- Time interval between two blood transfusions: 3 months.
- Donor should have eaten something in the last 3 hours.
- Whole blood donation to be deferred for 3 days after platelet/plasmapheresis.
- Blood pressure should be controlled.
- No skin disease at the phlebotomy site.

Preference of Blood Group for Routine Blood Transfusion

- The first choice is the donor blood of the same ABO group as that of the recipient.
- If same ABO blood group is not available, then blood of an alternate but compatible group may be transfused as per mentioned below.

Recipient blood group	Donor blood group	
	first choice	Alternative or second choice
A	A	O
B	B	O
AB	AB	A → B → O (in this order)
O	O	Nil

To reduce the risk of haemolysis in a case of non-identical but compatible blood transfusion of ABO group, packed red cells instead of whole blood should be transfused (i.e. most of the plasma which contains anti-A and/or anti-B should be removed).

Salient Features of Blood Transfusion

- Blood should be transfused through a sterile, disposable administration set incorporating a **standard filter (170 µm pore size)**. This filter retains clot or cellular aggregates but permits passage of single cells and microaggregates.
- The usual needle size is 18 or 19 gauge.
- The solution is the blood bag usually contains citrate phosphate dextrose adenine (CPDA)-1. Amount of CPDA-1 is 49 ml for 350 ml of blood or 63 ml for 450 ml of blood.

Function of each component of CPDA is mentioned below

- Citrate:** Anticoagulation by binding of calcium in plasma.
- Phosphate:** Acts as a buffer to minimize the effects of decreasing pH in blood.

- Dextrose:** Maintenance of red cell membrane and metabolism. Also needed for ATP generation for viability of red cells.
- Adenine:** Generation of ATP (energy source).

Collection of Blood for Transfusion

- Blood is collected by phlebotomy under aseptic conditions using sterile, plastic bag with anticoagulant. Nowadays, CPDA1 is used as an anticoagulant.
- Mix the blood with anticoagulant gently and periodically during its collection.

Anticoagulants used as preservatives:

- CPD (citrate phosphate dextrose)
- CPDA-1 (citrate phosphate dextrose adenine-1)
- ACD (acid citrate dextrose): Not used nowadays.

Composition of Preservatives

CPD

- Trisodium citrate: 26.30 g
- Sodium dihydrogen phosphate: 2.28 g
- Dextrose: 25.50 g
- Citric acid: 3.27 g
- Distilled water: To make 1 litre.

CPDA-1

- Trisodium citrate: 26.30 g
- Sodium dihydrogen phosphate: 2.22 g
- Dextrose: 31.8 g
- Citric acid: 3.27 g
- Adenine: 0.275 g
- Distilled water to make 1 litre.

Acid Citrate Dextrose (ACD)

- Trisodium citrate: 22.0 g
- Citric acid: 8.0 g
- Dextrose: 24.6 g
- Distilled water to make a final volume of 1 litre.

Storage of blood (see Fig. 10.14)

- i. Whole blood and packed red cells are stored in a refrigerator at 2–6°C for 35 days.
- ii. Platelets are stored at 20–24°C for 3 days.
- iii. Fresh frozen plasma and cryoprecipitate are stored at ≤–18°C for one year.

Pre-donation check-up

Donor blood: The following tests are routinely performed

- ABO and Rh blood grouping.
- Screening tests for HBsAg, anti-HCV, anti-HIV-1 and HIV-2 and serum alanine aminotransferase (ALT).
- Screening tests for malaria and syphilis.

Recipient blood: The recipient's ABO and Rh blood grouping.

Compatibility Testing (Pre-transfusion)

Before blood or its components are transfused one must know whether donor blood or components are compatible with recipient's blood.

To check compatibility, following tests are done:

- ABO and Rh typing of the donor and recipient
- Cross-matching: But for transfusion of platelets or fresh frozen plasma, cross-matching is not needed.
- Antibody screening test of donor's and recipient's serum.
- Review of patient's past blood bank history and records if done earlier.

CROSS-MATCHING

Cross-matching is very important before any blood transfusion. It detects any antibody present in the patient's serum which will react with the donor's cells during transfusion.

Importance of Cross-matching

- It detects the presence of any clinically significant, unexpected antibodies in the recipient's serum which may react with donor's red cells. So, cross-match prevents transfusion reaction.
- It is the final check of ABO compatibility between donor and recipient.

Types of Cross-match

It can be divided into two major groups, namely major and minor cross-matching. Major cross-matching consists of mixing donor's red cells with recipient's or patient's serum. On the other hand, minor cross-matching consists of mixing patient's (recipient's) red cells with donor's serum (Tables 10.1 and 10.2).

Table 10.1: Types of cross-match

Cross-match type	Donor's component	Recipient's component
Major cross-match	Red cells	Serum
Minor cross-match	Serum	Red cells

Table 10.2: Different techniques of major cross-match

Types of cross-match	Antibody detection in recipient's serum
• Saline cross-match • Antiglobulin test or Coombs test	IgM IgG
• Albumin technique • Enzyme technique	IgG IgG mainly and some IgM

Procedure of Major Cross-match

The simplest method is:

- Place one drop of patient's serum in a glass test tube (Fig. 10.2).
- Add one drop of 5% saline suspension of donor's red cells to the above test tube.

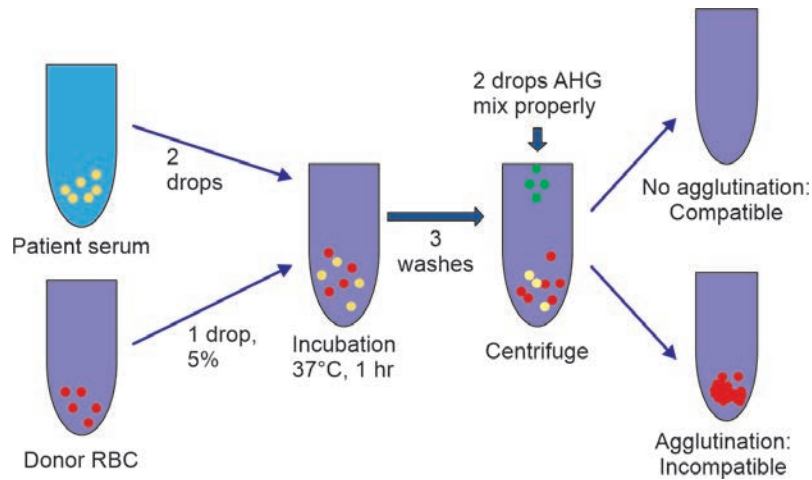


Fig. 10.2: Major cross-match

- Mix them and incubate at 37°C for 30 minutes. Centrifuge at 1000 rpm for 1–2 minutes.
- Examine the tube for agglutination or haemolysis. Also check under microscope.

Interpretation of Result

- If there is no agglutination or haemolysis, that means the donor's blood is compatible with that of patient's blood and can be safely transfused.
- Agglutination or haemolysis indicates mismatching. In this scenario, recipient

should not be transfused with the donor's blood or component.

Procedure of Minor Cross-match

- Place one drop of donor's serum in a glass test tube.
- Add one drop of 5% saline suspension of recipient's red cells to the above test tube.
- Mix them and incubate at 39°C for 30 minutes.
- Centrifuge at 3000 rpm for 1 minute.
- Observe for agglutination or haemolysis (Fig. 10.3).

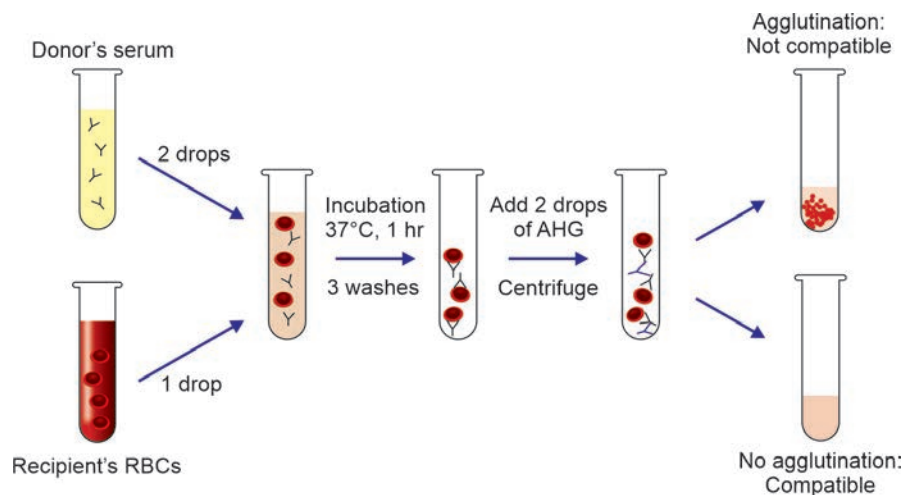


Fig. 10.3: Minor cross-match

INDIRECT COOMBS' TEST (ANTIGLOBULIN TEST)

Saline suspension red cells of donor are incubated with patient's serum. Washed in saline and add antiglobulin agent. Re-centrifuge and examine for agglutination or haemolysis. It will detect any IgG antibodies in patient's (recipient's) serum.

Interpretation of result

- If there is no agglutination or haemolysis, it indicates compatibility of donor unit with patient's (recipient's) serum.
- Agglutination or haemolysis indicates incompatibility and should not be transfused (Fig. 10.4).

Antibody Screening and Identification

Patient (recipient's) serum should be tested for the presence of clinically significant (unexpected or irregular) antibodies. In this procedure, serum of the recipient is tested against a set of blood group O screening cells

of known antigenic type. If there is any clinically significant antibody in patient's serum it will be identified. Then the blood unit without the corresponding antigens is selected for compatibility test.

APHERESIS

Definition: Apheresis is an automated blood collection procedure wherein whole blood is removed from a donor or a patient. Then desired component is separated and the remainder of the blood is returned to the donor or patient.

Types of Apheresis

- Donor apheresis
- Therapeutic apheresis: Component of blood contributing to the disease is removed and the remainder is returned back.

Advantage of donor apheresis: Large amounts of a specific component can be collected; minimizes number of donors to which recipient is exposed.

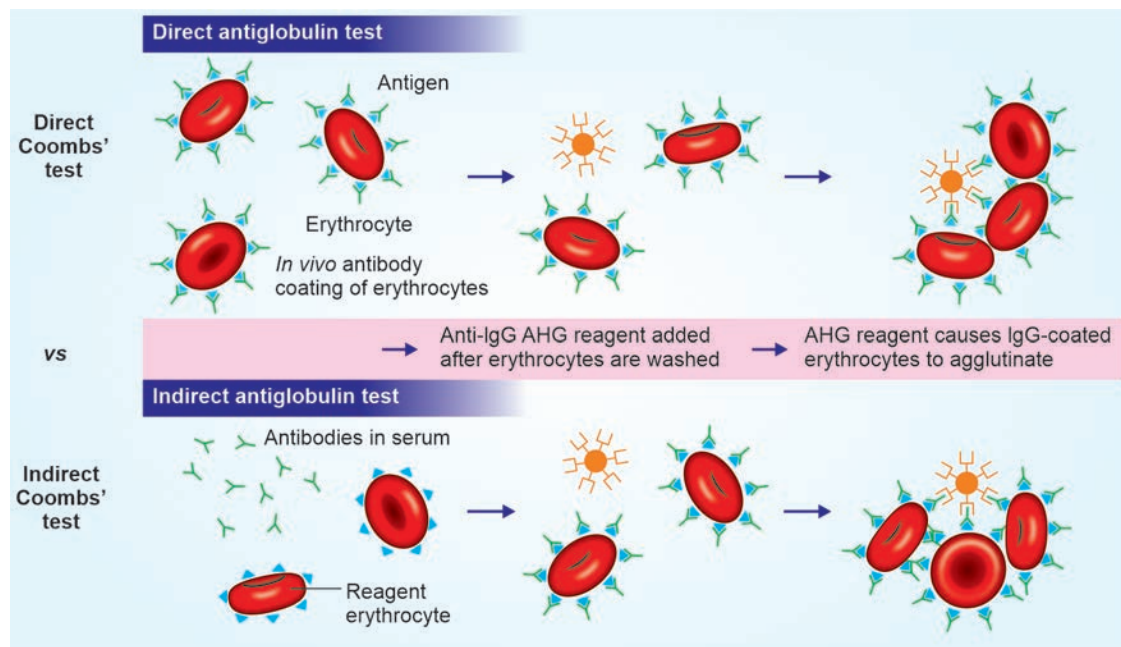


Fig. 10.4: Direct Coombs' test vs indirect Coombs' test

Components Collected in Donor Apheresis

- **Red cell apheresis:** 2 units of red cells can be removed if the donor is larger and has higher haematocrit than for single red cell donations. At least 16 weeks interval needed (deferral) if two units of red cells are collected.
- **Plateletpheresis:** Platelet count of the donor should be $>1.5 \text{ lacs/mm}^3$ (especially for frequent donor). Donation interval should be at least 2 days. Donor should not have taken aspirin for at least 36 hours before donation. It contains $\geq 3 \times 10^{11}$ platelets.
- **Leukapheresis:** Not commonly used. For collection of large number of leukocytes; drugs or sedimenting materials need to be administered to the donor and donor consent for that purpose is needed.
- **Plasmapheresis:** The donor should be larger (≥ 110 pounds or 50 kg) for infrequent plasmapheresis. Donation interval should be at least 4 weeks.

Therapeutic Apheresis

- **Cytapheresis:** For treatment of sickle cell disease (to reduce HbS and replace with HbA), hyperleukocytosis (to remove excess leukocytes in leukaemia), malaria (to reduce parasite load)
- **Plasmapheresis:** Removal of abnormal plasma proteins (e.g. myasthenia gravis, GB syndrome, TTP, monoclonal gammopathies) (Fig. 10.5).

WHOLE BLOOD (Fig. 10.6)

- One unit of blood is collected in a suitable anticoagulant preservative solution (CPD-1). Its total volume is about 400 ml (350 ml of blood + 49 ml of anticoagulant). Shelf life in CPD-1 is 35 days.
- It consists of cellular elements (red cells, white cells and platelets) and plasma. But it contains no liable coagulation factors (factor V, factor VIII) and no functionally effective platelets.

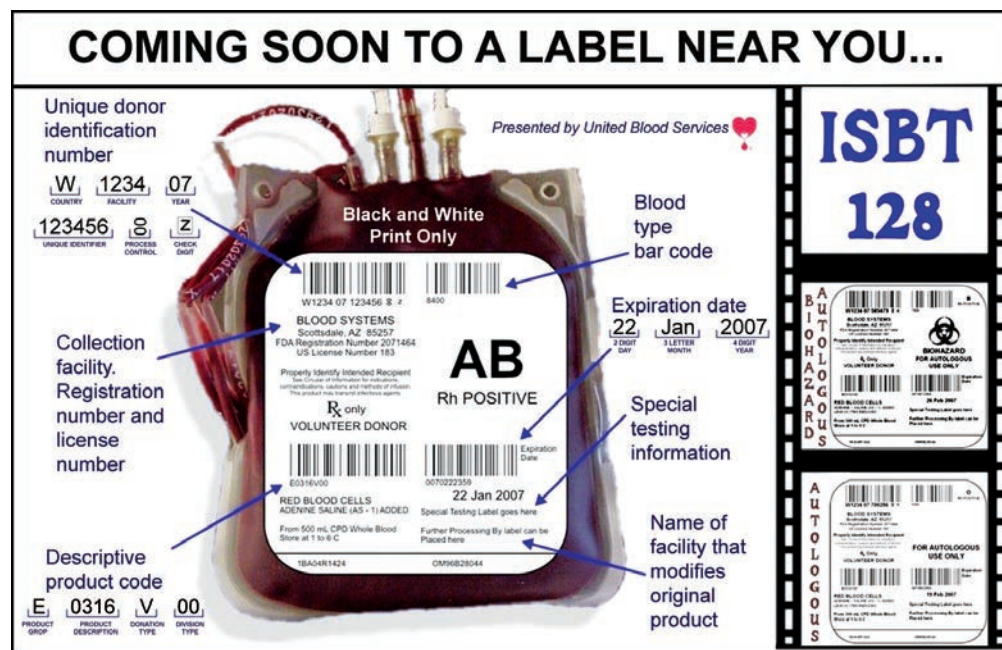


Fig. 10.5: Identification of different points of one unit of blood/blood product

- It is stored at 2–6°C in appropriate blood bank refrigerator.
- Transfusion of whole blood commence within 30 minutes of removal from the refrigerator, and should be complete within 4 hours of starting.
- Transfusion of one unit raises haemoglobin by 1 g/dl or haematocrit by 3%.
- Haematocrit of whole blood 35–45% and haemoglobin 12 g/dl
- Indication: Acute massive blood loss, exchange transfusion, non-availability of packed red cells.
- Risk of volume overload in patients with chronic anaemia and compromised cardiovascular function.
- 450 ml of blood
- 63 ml of anticoagulant solution.
- Hct: 36–44%
- No components have been removed.
- Store at 1–6°C
- Shelf life-
 - Citrate-phosphate-dextrose (CPD)
 - CPDA-1 (adenine): 35 days
 - AS-1, AS-3, AS-5–42 days
- Administer through standard blood filter (150–280 microns)
- Infuse within 4 hours of issue.

RED CELL COMPONENTS

1. Packed red cells (red cell concentrate)

- **Preparation:** Whole blood is either allowed to sediment overnight in a refrigerator 2–6°C or it is spun in a refrigerated centrifuge. Supernatant plasma is separated from red cells in a closed system by transferring the plasma to the attached empty bag. Red cells with small amount of plasma are left behind in the primary blood bag.
- Haematocrit 55–75% or haemoglobin 20 g/dl.
- Raises haemoglobin by 1 g% or haematocrit by 3%
- Stored at 2–6°C and shelf life 35 days in CPDA.
- Ideal haematocrit should be 70–75% after preparation from one unit of whole blood. But haematocrit should not cross 80%, otherwise preservative will not be effective for support of red cells.
- Volume: 250 ml.
- Indications: Replacement of red cells in anaemia and in acute/massive blood loss (along with crystalloid or colloid) (Fig. 10.7).



Fig. 10.6: Whole blood

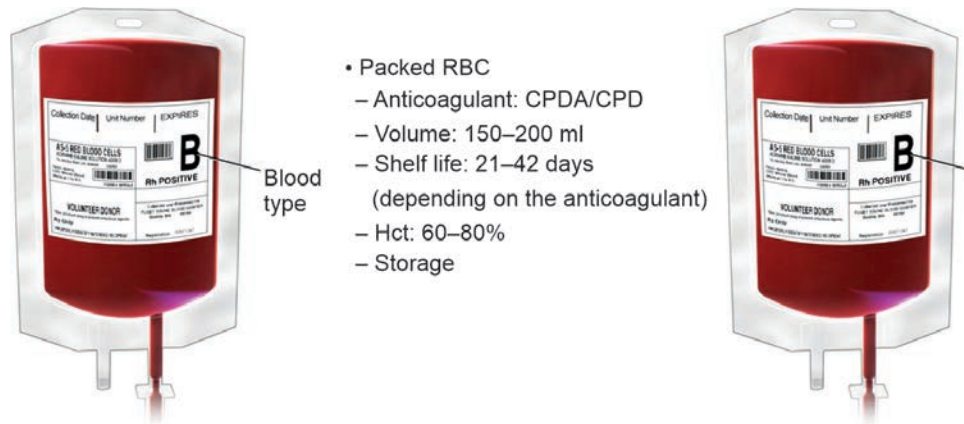


Fig. 10.7: Packed red blood cells

2. Red cells in additive solution (red cell suspension)

These are red cells with minimal residual plasma with an added additive solution. This increases shelf life of red cells (in CPDA) from 35 to 42 days.

Additive solutions like AS -1 AS -3, AS-5 and SAGM (which contains saline, adenine, glucose and mannitol).

Preparation: After collection of whole blood in the primary collection bag (containing CPDA-1), maximum amount of plasma is removed after centrifugation and transferred to a satellite bag (**satellite bag 1**). The additive solution from another satellite bag (**satellite bag 2**) is transferred into the primary collection bag (containing packed red cells) in a closed system within 72 hours of collection.

Advantages of this method are

- i. Red cells with improved viability are obtained (shelf-life increases from 35 to 42 days).
- ii. Maximum amount of plasma can be removed for preparation of plasma components.
- iii. Flow of infusion is improved due to reduction in viscosity.

Indications: Same as for packed red cells.

Contraindication: Red cells in SAGM are contraindicated for exchange transfusion in neonates.

3. Leukocyte-poor red cells

- These are red cells from which most of the white cells have been removed. They are obtained by passing blood through a special leukocyte-depletion filter at the time of transfusion. They can also be prepared in the blood bank.
- By definition, leukocyte-depleted red cells should contain $\leq 5 \times 10^6$ white cells per bag.

Indications

- i. To avoid febrile transfusion reactions in persons who require repeated transfusions or who have earlier been sensitised to white cell antigens.
- ii. To avoid sensitisation to HLA antigens. As for example, in patients with severe aplastic anaemia, who are likely to receive allogenic bone marrow transplant. But it cannot prevent graft-versus-host disease.
- iii. To reduce the risk of transmission of cytomegalovirus (CMV) in some patients.

4. Frozen red cells

- Red cells can be stored frozen for up to 10 years when stored at or below -65°C . But for this, a cryoprotective agent such as glycerol is added. Glycerol prevents red cell dehydration and formation of ice crystals that causes red cell lysis.
- After deglycerolization (washing with decreasing concentration of saline), red cells can be stored at $4-6^{\circ}\text{C}$ for 24 hours, if closed system is not used.
- With this method, virtually all plasma, anticoagulant, leukocytes and platelets are removed.
- Indications: i) It is used for storage of red cells with rare blood groups, for further autologous transfusions. ii) It is used for individuals who have repeated febrile nonhaemolytic transfusion reactions. iii) it can be given to IgA deficient patient as it is safe for them.

5. Irradiated red cell

- Viable T lymphocytes in donor blood can induce life-threatening transfusion asso-

ciated graft-versus-host disease (GVH) in at risk patients.

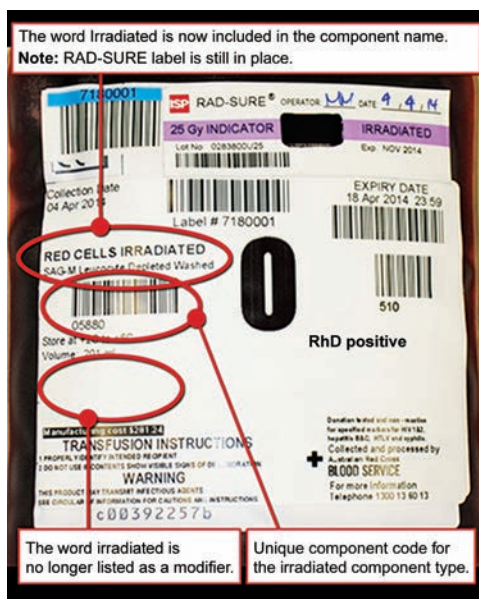
- Gamma-irradiation (250 cGy) of red cells inactivates T lymphocytes and prevents graft versus host disease. This component can be stored at $4-6^{\circ}\text{C}$ up to original expiry date or 28 days from irradiation.

Indications

- It is indicated for prevention of GVHD in susceptible individuals like immunodeficient individuals and patients receiving blood from first degree relatives.
- It is used for intrauterine or premature neonate transfusions (Fig. 10.8).

6. Washed red cells

- Red cells can be washed with normal saline to remove plasma proteins, white cells and platelets.
- Shelf-life of red cells after washing is 24 hours at $4-6^{\circ}\text{C}$. About 20% of red cells are lost in the process.



Indications for irradiated blood

- Pre-/post-haematopoietic stem cell transplant
- Hodgkin's disease
- Low birth weight neonate ($<1,200$ g)
- Neonatal exchange transfusion
- Intrauterine fetal transfusion
- Related donor
- HLA-matched donor or cross-match compatible platelet donor
- Treatment with either fludarabine or 2-CDA

Fig. 10.8: Identification of irradiated blood

- **Indications**

- Use of such cells is restricted for IgA-deficient individuals who have developed anti-IgA antibodies as exposure will lead to anaphylaxis.

PLATELET COMPONENTS

1. Platelet Concentrate (Random Donor Platelets)

- Obtained from whole blood and platelets are separated from whole blood within 6 hours of collection. Remember whole blood should be kept at room temperature.
- One unit of whole blood is centrifuged (light spin) to obtain **platelet-rich plasma (PRP)**. PRP is then transferred to the attached satellite bag and spun at high speed to get platelet aggregates (at the bottom) and **plate-poor plasma or PPP** (at the top). Most of the PPP is return back to the primary collection bag or to another satellite bag, leaving behind 50–60 ml of PPP with the platelets.
- Platelets are stored at 20–24°C with continuous agitation (in a storage device called platelet agitator). Maximum storage period 3–5 days.
- One unit of platelet concentrate contains $\geq 45 \times 10^9$ platelets. Transfusions of one unit of platelet concentrate will rise the platelet count in the recipient by about 5000/ μl .

Indications

- Bleeding due to thrombocytopenia (immune mediated or secondary), where platelet count is $< 20,000/\text{mm}^3$
 - In case of abnormal platelet function or platelet function defects
 - In DIC (disseminated intravascular coagulation).
- The usual adult dose is 4–6 units of platelet concentrate (or 1 unit/10 kg of body weight). These units (which are from different donors) are pooled into one bag before transfusion. This dose will raise the platelet count by 20,000–40,000/ μl .

Note

- Platelets have ABO antigens on their surface but do not express Rh antigen.
- It is advisable to transfuse platelets from Rh negative persons to Rh negative patients/persons.

2. Plateletpheresis (Single Donor Platelets)

- In this method, a donor is connected to blood cell separator machine in which whole blood is collected in an anti-coagulant solution. Then platelets are separated and retained. Remaining components are returned back to the donor.
- With this method, a large number of platelets can be obtained from a single donor (equivalent to 6 units of platelet concentrate or $\geq 3 \times 10^{11}$) and recipient is not exposed to many donors.
- **Indications**
 - It is especially suitable if HLA-matched platelets are required (that means if the patient has developed refractoriness to platelet transfusion due to the formation of allo-antibodies against HLA antigens.)

Contraindications

- TTP (thrombotic thrombocytopenic purpura)
- Haemolytic uraemic syndrome.

Corrected count increment (CCI): After transfusion, platelets will survive for only 3–4 days in circulation. Therefore, patients requiring platelet need frequent platelet transfusions. These patients may develop antibodies against platelets and HLA antigens which will reduce the survival of transfused platelets. It is important to know whether the patient has become refractory or unresponsive to such transfusions. This is done by determining whether there will be anticipated increase in platelet counts by calculating corrected count increment or CCI.

$$\text{CCI} = \text{Post-transfusion platelet count} = \frac{\text{Pre-transport platelet count} \times \text{BSA}}{\text{Number of platelets transfused (in multiples of } 10^{11})}$$

BSA = Body Surface Area (calculated from a nomogram using height and weight)

CCI > 7500/ μl at 10–60 minutes following transfusion indicates adequate platelet count increment.

GRANULOCYTE CONCENTRATE

- **Preparation:** Granulocytes for transfusion can be obtained either from a single donor unit by differential centrifugation or by leukapheresis. Leukapheresis is preferred because of better granulocyte yield, which can further be enhanced by administration of corticosteroids to the donor.
- But granulocyte concentrates are seldomly used. The reasons are:
 - i. When collected from single donor, it contains a smaller number of granulocytes and is heavily contaminated with red cells.
 - ii. Transfusion of granulocyte concentrate often results in complications (e.g. lung infiltration, non-haemolytic transfusion reactions, etc.).
- **Indications**
 - i. Patients with severe neutropenia (when granulocyte count < 500/ mm^3 (agranulocytosis).
 - ii. To combat infections, like in neonatal sepsis and granulomatous disease.
 - iii. Bone marrow depression.

But rather than transfusing granulocyte concentrates, many clinicians prefer to administer growth factors for myelopoiesis like G-CSF/GM-CSF.

PLASMA COMPONENTS

Plasma can be obtained either by centrifugation of a unit of whole blood or by plasmapheresis. Various components can be prepared from plasma. The main plasma

components are: Fresh frozen plasma, plasma frozen within 24 hours of phlebotomy and cryoprecipitate.

1. Fresh Frozen Plasma (FFP)

- **Preparation:** To prepare the FFP, plasma is separated from whole blood by centrifugation, transferred into the attached satellite bag, and then rapidly frozen at -18°C or at lower temperature. This procedure is carried out within 6 hours of collection because after 6 hours, labile factors (factor V and factor VIII) are lost. FFP contains all coagulation factors.
- **Volume:** 200–250 ml
- **Storage:** It can be stored at below -18°C for 1 year. When required for transmission, FFP is thawed between $30-37^{\circ}\text{C}$ for about 30–45 minutes. Then it is temporarily stored at $2-6^{\circ}\text{C}$ and should be used within 24 hours.
- **Indications**
 - i. Patients on anticoagulant drug therapy
 - ii. Antithrombin deficiency
 - iii. Liver disease
 - iv. Vitamin K deficiency
 - v. TTP
 - vi. DIC
 - vii. Inherited deficiency of a coagulation factor for which no specific concentrate is available.
- **Dosage:** 10–20 ml/kg (3–6 units in adults) over a period of 1–2 hours. ABO compatible FFP is preferred to avoid the risk of haemolysis of patient's RBCs by antibodies in donor plasma.

Fresh frozen plasma contains coagulation factors and other plasma protein (per unit or bag)

Volume – 200–250 ml

Factor VIII – 0.6 IU/ml

Fibrinogen – 250–300 mg/bag

Proteins – Albumin, globulin, etc.

1 IU/kg of factor VIII or factor IX (one IU/kg) raises the factor VIII levels in plasma by 2% and factor IX levels by 1% respectively.

Shelf life: One year

Storage tem: -20°C or below

Q.C. requirements: The entire process of preparation and freezing should be completed with 8 hours after collection.

Volume: 50 to 200 ml

Definite indications for the use of FFP

1. Replacement of single factor deficiencies
2. Immediate reversal of warfarin effect
3. Haemorrhagic disease of newborn
4. DIC with evidence of bleeding
5. Thrombotic thrombocytopenic purpura (TTP)

Conditional uses for the use of FFP

1. Massive transfusion
2. Liver disease
3. Cardiopulmonary bypass surgery
4. Newborn with septicaemia

No justification for the use of FFP

1. Hypovolaemia
2. Plasma exchange procedures except TTP
3. Reversal of prolonged INR in the absence of bleeding.

2. Plasma Frozen within 24 Hours after Phlebotomy

Plasma is separated and is frozen at -18°C between 8 and 24 hours of collection. This contains all coagulation factors, but may

have reduced level of factor V and factor VIII in comparison to FFP.

Indications: Similar to FFP.

3. Cryoprecipitate (CRYO)

Cryoprecipitate is prepared from plasma, which has been separated freshly (within 6 hours of collection) by rapidly freezing at a temperature $\leq 18^{\circ}\text{C}$. Then thawed it slowly at $4-6^{\circ}\text{C}$. Plasma and a white flocculent precipitate are obtained. This mixture is centrifuged and supernatant plasma is removed leaving behind the whitish cryoprecipitate in 10–20 ml of plasma.

This prepared cryoprecipitate (one unit) is now refrozen at a temperature $\leq -18^{\circ}\text{C}$ and can be stored for 1 year. When required for transfusion, cryoprecipitate was thawed at $30-37^{\circ}\text{C}$ and can be stored at $2-6^{\circ}\text{C}$ temporarily for 6 hours. This unit can be transferred to patient. Each unit of cryoprecipitate must contain ≥ 80 IU of factor VIII and ≥ 150 mg of fibrinogen. It also contains von Willebrand factor, factor XIII and fibronectin.

Indications: It is mainly used for treatment of fibrinogen deficiency and factor XIII deficiency. Though it can be used for treatment of haemophilia A (factor VIII deficiency) and von Willebrand disease, but nowadays it is not preferred as virus-inactivated factor VIII concentrates and recombinant factor preparations are available (Fig. 10.9).

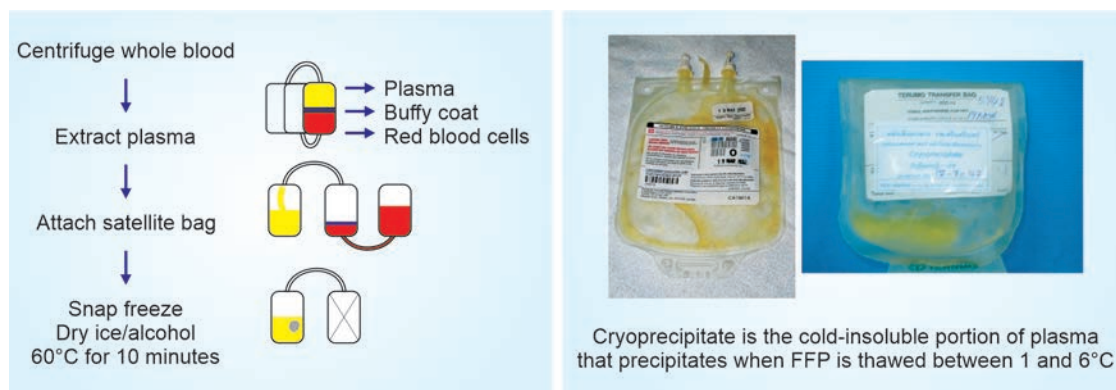


Fig. 10.9: Cryoprecipitate (CRYO)

PLASMA DERIVATIVES

Plasma derivatives are prepared by fractionation of large volumes of pooled human plasma. After fractionation, these derivatives undergo virus-inactivated procedures. Some important plasma derivatives and their uses are listed in Table 10.3.

Recombinant Factor Concentrate

With the advent of recombinant DNA technology, factor VIII, IX and VIIa can be produced by this method. This technique has eliminated the risk of viral transmission. But products produced by this technique are costly. These products are sterile, lyophilized and stable making the administration easier and more scientific compared to blood components.

Indications

- i. Factor VIII concentrate for treatment of haemophilia A, von Willebrand disease
- ii. Factor IX concentrate for treatment of haemophilia B.
- iii. Factor VIIa (rFVII a) for treatment of haemophilia A or B with inhibitors and factor VII deficiency (Table 10.4).

ADVERSE EFFECTS OF BLOOD TRANSFUSION

Blood transfusion is generally safe and effective. But sometimes adverse effects may be seen. These are called blood transfusion reactions. Occasionally, these adverse effects may even cause death to the recipient. Main causes of transfusion-related deaths are:

- Immediate acute haemolytic transfusion reaction due to ABO incompatibility.
- Pulmonary edema and congestive heart failure due to circulatory overload.
- Complications related to infections (mainly bacterial).
- Transfusion associated graft-versus-host disease.

Table 10.3: Important plasma derivatives and their uses

Plasma derivative	Use
• Human albumin solutions	i. It is used as a replacement fluid in therapeutic plasma exchange ii. For treatment of diuretic resistant oedema due to hypo-proteinaemia.
• Factor VIII concentrate	i. Treatment of haemophilia A ii. Severe von Willebrand disease.
• Prothrombin complex	i. Treatment of deficiency of factor IX, factor VIII with inhibitors against factor VIII. ii. Inherited deficiency of factors II, VII and X
• Immunoglobulins	
a. Nonspecific immunoglobulins	i. Treatment of hypogammaglobulinaemia, ii. Autoimmune thrombocytopenic purpura iii. Neonatal sepsis iv. Passive prophylaxis of viral infections like hepatitis, rubella and measles
b. Specific immunoglobulin	i. Anti-RhD immunoglobulin is used for prevention of sensitisation to RhD antigen in Rh negative women giving birth to a Rh positive baby. ii. Tetanus immunoglobulin, hepatitis B immunoglobulin, varicella-zoster immunoglobulin are used for passive prophylaxis of that particular infection

- Transfusion of physically damaged red cells (e.g. heat, cold).

These adverse reactions may be immediate or delayed (Fig. 10.10 and Table 10.5).

Table 10.4: Storage of different components

Cells	Method of preparation	Storage temperature	Lifespan
Packed red cell	5000 g for 5 minutes	2–6°C	35–42 days
Fresh frozen plasma (FFP)	5000 g for 5 minutes at 22°C	–20°C	1 year if frozen, 24 hours if thawed
Platelet-rich plasma (PRP)	2000 g for 3 minutes at 22°C	20–24°C with constant agitation	5 days
Cryoprecipitate	Derived from FFP after thawing at 4°C	Frozen –18°C	1 year if frozen, 16 hours if thawed
Granulocyte concentrate	5000 g for 5 minutes	24°C	Transfuse within 24 hours of collection
Clotting factor concentrate	Lyophilised preparation	+4°C	Half life is 12 hours
Albumin	Plasma concentrate	Room temperature	Half life is 14 days
Protein C and protein S	Fractionation of plasma	+4°C	Several years
Immunoglobulins	Fractionation of plasma	+4°C	21 days

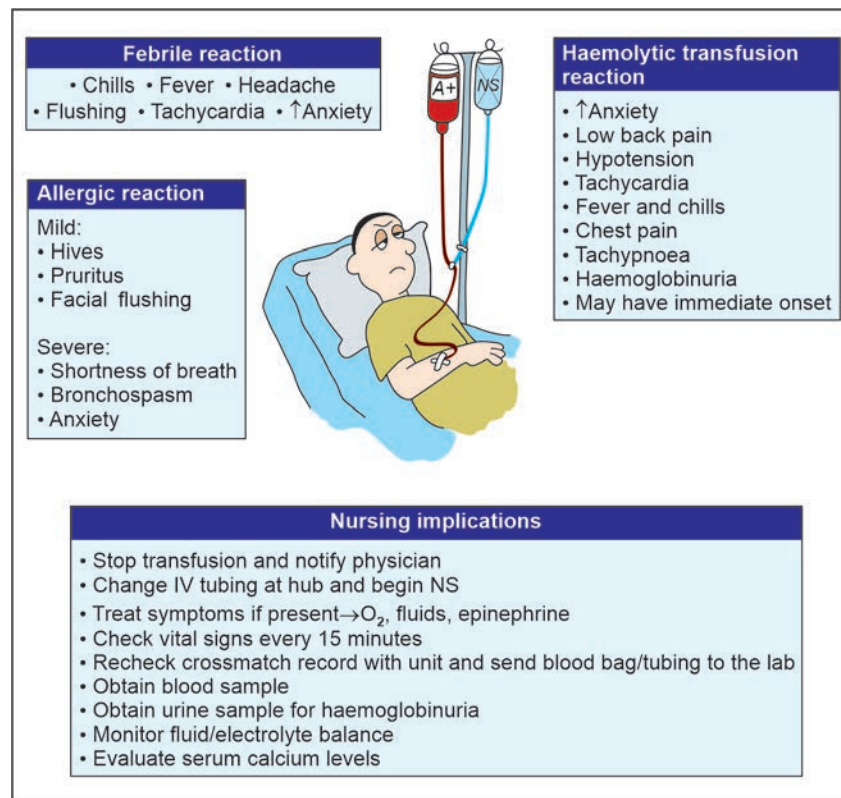
**Fig. 10.10:** Blood transfusion reaction

Table 10.5: Adverse effects of blood transfusion

Immediate (<24 hours)	Delayed (≥24 hours)
<i>Non-immunological causes</i> • Fever and shock (due to presence of antibodies against WBC or platelet antigens). • Haemolysis (due to physical destruction of blood caused by overheating or freezing) • Congestive heart failure (due to circulatory overload following large volume of blood transfusion) <i>Immunological causes</i> • Haemolysis (due to ABO incompatibility) • Anaphylaxis (due to IgE antibody) • Urticaria (due to presence of antibody against plasma proteins of donor) • Febrile reactions (due to antibody against leukocyte antigen of donor) • Non cardiac pulmonary oedema (due to antibody against leukocytes of donor or complement activation)	<i>Non-immunological causes</i> • Hepatitis (mostly hepatitis B and C) • AIDS (if donor is HIV positive) • Iron overload (due to multiple transfusion) • Malaria • Syphilis • Viral infections (e.g. CMV) <i>Immunological causes</i> • Haemolysis (due to secondary response of antibody to RBC antigens) • Graft-versus-host disease (due to donor's leukocyte HLA antigens) • Post-transfusion purpura (due to development of antiplatelet antibody)

Some of the common adverse effects will be discussed here.

Fever (Febrile Non-haemolytic Transfusion Reaction)

- Fever is the most common reaction and constitutes >90% of transfusion reactions and 1% of all transfusions.
- It is defined as an unexplained rise of temperature of at least 1°C during or shortly after transfusion (<6 hours).
- This is due to the presence of WBC and platelet antibodies in the recipient's blood as a result of previous transfusion or pregnancies. It is caused by the release of pyrogenic cytokines such as interleukin - 1(IL-1), IL-6, IL-10 and tumor necrosis factor (TNF) from leukocytes.
- Commonly seen in patients having multiple transfusions in the past.
- Signs and symptoms: Fever, chills and tachycardia.
- Prevention: It can be prevented by transfusing leukocyte poor blood, i.e. blood from which buffy coat has been removed.
- Treatment: Antipyretics like paracetamol.

Haemolysis and Acute Haemolytic Transfusion Reaction (AHTR)

- Haemolysis due to blood transfusion can be due to both non-immunologic and immunologic causes.
- **Acute haemolytic transfusion reaction (AHTR)** is a medical emergency which results from intravascular destruction of donor red cells by antibodies in the recipient. It is usually caused by preformed IgM antibodies against donor red cells.
- AHTR occurs during, immediately after or within 24 hours following blood transfusion.
- AHTR usually results from ABO-mismatched blood transfusion to the recipient due to most commonly a clerical error. Most severe reaction occurs if blood group A is transfused to a recipient of blood group O. Severe reaction may occur with as little as 10 ml of blood transfusion.
- Signs and symptoms of AHTR: Fever, pain at the infusion site, loin or back pain, tachycardia, oozing from venipuncture site, hypotension and haemoglobinuria.

- Another cause of haemolytic reaction may be incompatibility of Rh, Kell or Duffy system. Patient gets iso-immunised and antibodies are formed after many weeks or months of transfusion. These are also called **delayed haemolytic transfusion reaction (DHTR)**.
- Most of these haemolytic transfusion reactions are due to incorrect labelling, wrong identification of patient, error in blood grouping and failure to detect weak antibodies.
- Laboratory findings:
 - i. Haemoglobinaemia (pink coloration of plasma after centrifugation of post-transfusion of blood).
 - ii. Haemoglobinuria
 - iii. Positive direct Coombs' test
 - iv. Raised indirect serum bilirubin
 - v. Schistocytes and spherocytes on PBS.
- excessive administration of blood, plasma or other intravenous blood components.
- It may cause congestive heart failure and pulmonary oedema. This is particularly so in an anaemic patient. Elderly patients, infants and pregnant women in third trimester are more prone to congestive heart failure.
- Signs and symptoms of cardiac failure: Dyspnoea, peripheral oedema, cough, distension of jugular veins and breathlessness.

Graft-versus-host Disease (GVHD)

ANAPHYLAXIS

- GVHD is more common in bone marrow transplant and is rare in blood transfusion. It may occur in patients with immunodeficiency like AIDS, lymphoma, chemotherapy and radiotherapy.
- Though it is rare, but is a lethal complication of transfusion with 90% mortality rate.
- GVHD is mediated by immunocompetent lymphocytes present in blood products which cause immunologic response against the recipient.
- Onset is 3–30 days after transfusion.
- Signs and symptoms: Fever, skin rashes, diarrhoea, infection.
- Patients having risk of GVHD should receive irradiated blood products to prevent immunologic response.
- This rare adverse effect occurs in IgA – deficient recipient who has anti-IgA antibodies (due to previous transfusion) and is now getting blood containing IgA antibodies. This leads to activation of complement and formation of anaphylatoxins (C3a and C5a).
- Signs and symptoms: Nausea, vomiting, flushing, respiratory distress, urticaria, hypotension and shock.

Urticaria

- It occurs due to presence of sensitising antibodies in the recipient which reacts with exogenous antigen, such as milk or egg protein present in the plasma of the donor.
- So, urticaria is common in the patients who suffers from atopic diseases, e.g. asthma, hay fever, etc.

Circulatory Overload

- Transfusion associated circulatory overload (TACO) occurs due to very rapid and

Bacterial Contamination

- The risk is low with plastic closed multi-pack system compared to glass bottles.
- Transfusion of blood product with platelet concentrates carries more risk as platelets are stored at higher temperature (20–24°C).
- The organisms are commonly cold growing gram-negative bacilli such as pseudomonas and also some coliform organisms.
- Platelets are usually contaminated by gram-positive cocci like Staphylococcus, Bacillus, etc. Red cells (stored at 4–6°C)

are contaminated by *Yersinia enterocolitica*, *Serratia liquefaciens* and *Pseudomonas fluorescens*.

- Signs and symptoms: High grade fever, rigors, hypotension and shock.
- Laboratory findings: Inspection of blood bag for discoloration, clots, cloudiness or haemolysis. Gram staining and culture of blood from blood bag and from recipient to detect causative bacteria. Direct Coombs' test is negative.
- Prevention: Blood should be given within 4 hours and prolonged storage of blood at room temperature should be avoided as it causes growth of contaminated bacteria.

Other Infections

- **Hepatitis B virus:** It is a DNA virus and can be transmitted by both cellular and plasma components. Blood and blood components are tested for HBs Ag before transfusion. HBsAg positive donors are excluded from blood donations. Donor screening for HBsAg and anti-HBc is mandatory in some countries. But in India, test for HBsAg is only mandatory.
- **Hepatitis A virus:** It is an RNA virus and is rarely transmitted by transfusion. Donors with hepatitis A or who are in close contact with this virus are deferred for 1 year. Test for hepatitis A antigen or antibody is routinely not done.
- **Hepatitis C virus:** This RNA virus is the **most common cause of transfusion transmitted hepatitis**. This infection is transmitted by both cellular and plasma components. Chronic hepatitis, cirrhosis and hepatocellular carcinoma may occur. Anti-HCV antibody test is done for donor screening.
- **HIV:** This is RNA retrovirus and has two subtypes HIV-1 and HIV-2. HIV can be transmitted by both cellular and plasma components. The test used to detect HIV is anti-HIV-1 and 2 antibodies by **ELISA (screening test)**. **Western blot test is more specific**. To reduce the window period (from 22 days to 10 days), **nucleic acid testing (NAT)** for HIV RNA is recommended.
- **Malaria:** It can be transmitted by transfusion readily. For prevention antimalarial drugs are given in endemic areas. Before transfusion, blood should be checked for malaria (Fig. 10.11).
- **Syphilis (*Treponema pallidum*):** Transmission through transfusion is rare because *T. pallidum* does not survive in refrigerator for storage of blood. It is inactivated at 4°C after 4 days.

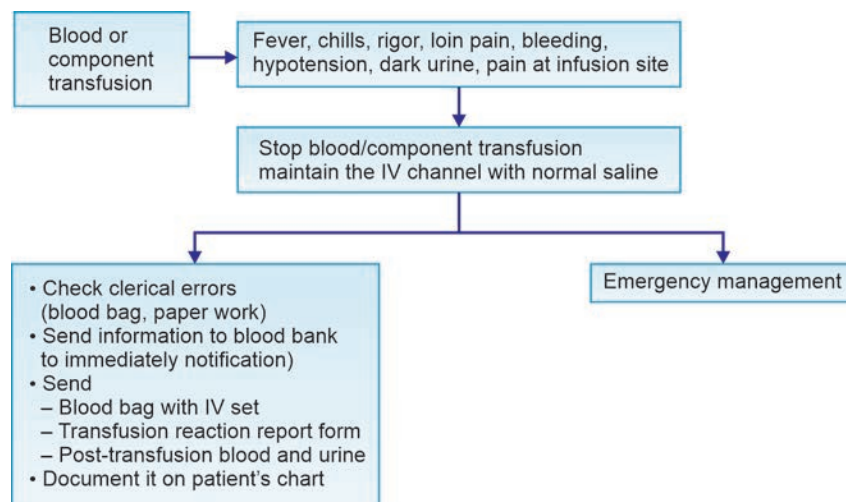


Fig. 10.11: Management protocol after a suspected transfusion reaction

CENTRIFUGATION

Outcome of centrifugation depends on two factors: The relative centrifugal force (RCF) and the duration of centrifugation. The RCF, of g force is the product of $1.118 \times 10^{-5} \times r \times N$ (where r is radius, and N is number of revolutions per minute or r.p.m.). The following speed is useful for preparation of different blood components:

- i. 5000 g for 5 minutes for preparation of packed RBC or platelet concentrate,
- ii. 5000 g for 7 minutes for preparation of cryoprecipitate or cell free plasma. These combinations are called “**heavy spin**” as opposed to 2000 g for 3 minutes, known as a “**light spin**”, which is used to produce platelet-rich plasma.

For preparation of a platelet concentrate, centrifugation is performed at room temperature (20°C to 24°C). For all other blood components, centrifugation is carried out between 1 and 6°C. Balancing the material in opposite sides of the centrifuge head is important and can be easily done with rubber disks different weights.

Platelets are in high demand. The whole blood unit, with its two attached satellite bags is first centrifuged using a **light spin to get PRP (platelet-rich plasma)** in the upper portion and red blood cells in lower portion. The PRP about 250 ml is expressed into an attached satellite bag, leaving the RBC in the primary bag (Fig. 10.12). The three attached bags are recentrifuged using a **heavy spin** to

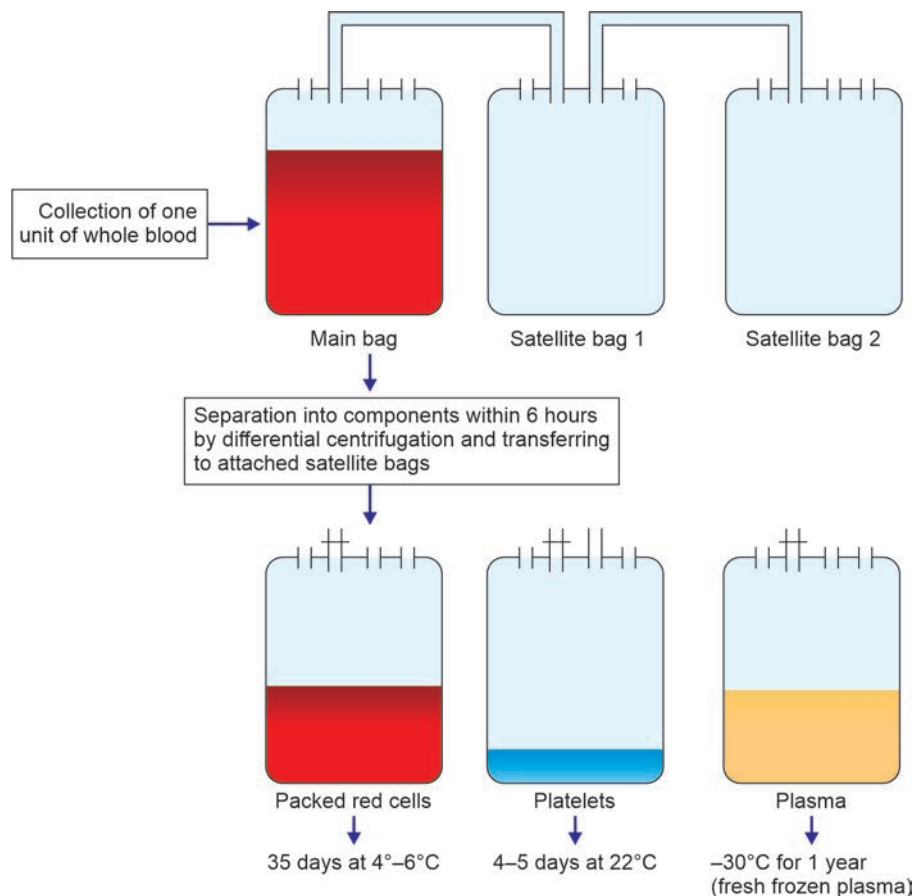


Fig. 10.12: Separation of blood components from whole blood

Table 10.6: Common blood components and derivatives

Blood components and derivatives	Characteristics	Approximate volume	Shelf life	Indications and components
Whole blood	Blood 350 ml, CPD or CPD-1 49 ml	400 ml	ACD or CPD: 21 days. CPD-1:35 days at 1–6°C	Acute massive blood loss, exchange transfusion, non-availability of packed red cells. The flow characteristics are rapid
Packed red cells	Packed RBC with reduced plasma volume. Hct 69%. WBC, platelets and coagulation factors as for whole blood	250 ml	As for whole blood	Replacement of red cells in anaemia. May be used in acute/massive blood loss. Flow characteristic is slow as Hct is high. Can be used with colloids and crystalloid to increase rate of flow.
Random donor platelet concentrate	Platelets (5.5×10^{10}), some WBC (i.e. lymphocyte), 50 ml plasma, few RBC (less than 0.5% haematocrit)	5–60 ml, 4–6 units for adult (1 unit/10 kg)	5 days at room temperature (20–24°C)	Used for quantitative and qualitative platelet disorders. May be used when bleeding (slow ooze) due to thrombocytopenia or for prophylaxis. 6–10 units raise platelet count about $50,000/\text{mm}^3$
Granulocyte concentrate	Granulocyte (1.0×10^{10}) and other WBCs, 250 ml plasma, minimal platelets, RBC about 10% Hct	300 ml	12–24 hours at room temperature	Patients with sepsis, severe neutropenia (neutrophil count $<500/\text{mm}^3$ and bone marrow depression)
Fresh frozen plasma (FFP)	Plasma proteins, all coagulation factors, complement	200–260 ml	1 year at -18°C or colder	Bleeding patient with multiple coagulation deficiency, DIC, liver disease, TTP
Cryoprecipitate	80 units of factor VIII, other plasma proteins, von Willebrand factor, factor XIII, fibrinogen (200 mg), fibronectin	10–15 ml	1 year at $\leq -18^\circ\text{C}$	Most useful for von Willebrand disease, factor XIII deficiency or hypofibrinogenaemia
Factor VIII concentrate	Quantity of factor VIII units are marked on lyophilized bottle	25 ml, as per manufacturer's instructions for reconstitution with diluents	2 years at $2-8^\circ\text{C}$	Used for haemophilia A (factor VIII deficiency). Lacks high molecular weight von Willebrand factor
Albumin	5 g/100 ml; 25 g/100 ml	250–500 ml; 50–100 ml	3 years at $<30^\circ\text{C}$.	Most useful for hypovolaemic shock or hypoproteinaemia

Contd.

Table 10.6: Common blood components and derivatives (Contd.)

Blood components and derivatives	Characteristics	Approximate volume	Shelf life	Indications and components
Immune serum globulin Rh immuno-globulin	Mostly IgG and some IgA and IgM-IgG anti-D (RhO)	10 ml 1 ml	3 years at <30°C 1½ years at 2–8°C	Treatment or prophylaxis of hypogammaglobulinaemia Prevents haemolytic disease of the newborn in Rh negative mother exposed to Rh positive red cells

produce an aggregated platelet button from PRP. About 200 ml plasma (platelet-poor) removed, leaving 50 ml of platelet-poor plasma with platelet button. From this 200 ml **platelet-poor plasma FFP** is prepared (Table 10.6).

Transfusion-related Acute Lung Injury (TRALI)

- Pathogenesis: Presence of antibodies in the donor's plasma against recipient's HLA II antigens located on the neutrophils which leads to neutrophilic aggregation in the pulmonary vasculature and endothelial damage there. This leads to clinical symptom there.
- TRALI develops within 6 hours of transfusion.
- It is a cause of non-cardiogenic pulmonary oedema.
- Fresh frozen plasma (FFP) is the commonest blood product to cause TRALI.

Mostly seen after sepsis and cardiac surgeries.

- Clinically, it is characterised by acute onset of respiratory disorders associated with oxygen desaturation (hypoxaemia) and bilateral lung infiltrates.
- Infections complications: These are common with platelet preparations. These can be prevented by donor screening (Table 10.7).

TRANSFUSION REACTION INVESTIGATION

Most adverse reaction investigations are conducted to make sure that this is not due to acute intravascular immune haemolysis from an ABO-incompatibility or mismatched transfusion. In the suspected case the intravenous line (IV line) must be kept open with crystalloids if immediate treatment is necessary to treat hypotension. Notify the blood bank immediately.

Table 10.7: ABO and Rh compatibility in blood transfusion

Blood type of recipient	Blood type of donor					
	A	B	AB	O	Rh ⁺	Rh ⁻
A	Yes	No	No	S		
B	No	Yes	No	S		
AB	S	S	Yes	S		
O	No	No	No	Yes		
Rh ⁺					Yes	Yes
Rh ⁻					E	Yes

S: Substitute as packed red blood cells or wash to eliminate antibodies. E: Only under extreme conditions, especially if the recipient is a young female

Furthermore, the following investigations should be done in case of suspected mismatched blood transfusion.

- Clerical check of the tag (written information) on the blood bag, the blood bag label and the patient identification for discrepancies.
- Investigation of the pre-transfusion clotted blood specimen an EDTA anticoagulated post-transfusion blood specimen and the blood bag.
- Perform a Gram's stain on the blood in the bag and a culture to identify the causative organism/bacteria.
- Repeat the ABO/Rh typing, antibody screen and the cross match to observe if a patient antibody is directed against donor cells. If an antibody is suspected, perform an RBC panel to identify the antibody.
- Examination of post-transfusion urine.
 - i. Look for dark color of urine, if present, test for haemoglobinuria.
 - ii. Test for haemolysis (extravascular and intravascular).
- If DIC is suspected, then perform PT, APTT, platelet count, fibrinogen and fibrin split products on a post-transfusion anticoagulated blood (Table 10.8).

INTERNAL QUALITY CONTROL IN BLOOD BANK

Internal quality control (IQC) is the backbone of any quality assurance programme. IQC is the set of procedures undertaken by the staff of a laboratory for continuously and concurrently assessing laboratory work and emergent results, to decide whether they are reliable enough to be released.

Drifted trend: Seen when the control value moves progressively in one direction from the mean for a minimum of three days.

Table 10.8: Investigation of haemolysis due to transfusion reaction

1. Check for incompatibility

- Clerical causes: An identification error will show the type of incompatibility
- Serological causes:
 - i. Repeat ABO and Rh grouping of the patient (pre-transfusion and post transfusion) as well as donor's blood
 - ii. Repeat cross-match test with pre- and post-transfusion serum
 - iii. Direct antiglobulin test (DAT) or direct Coombs' test (pre- and post-transfusion samples.
 - iv. Screen for red cell antibodies.

2. Check for haemolysis

- Perform tests for patient's plasma and urine for haemoglobin
- Schistocytes and spherocytes may be present on blood smear

3. Check for bacterial contamination/infection

- Gram stain and culture of donor's blood

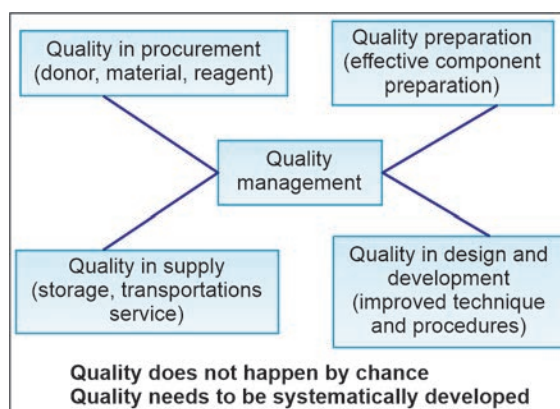
4. Check for disseminated intravascular coagulation (DIC)

- Coagulation screen
- Measure fibrinogen level and fibrin degradation product (FDP)
- Platelet count
- Blood film (red cell fragmentation)

Suggests that a problem is gradually developing, such as deterioration of a reagent or control.

Dispersion: Increase in random errors and lack of precision. Suggests inconsistency of technique or fluctuations in instrumentation function.

Shift: Abrupt changes observed when a problem develops suddenly. May be due to instrument malfunction or an error in technique (Fig. 10.13 and Table 10.9).



The quality requirements involve

- Quality control and proficiency testing
- Internal and external audits
- Personnel and organization
- Premises, equipment and materials
- Blood processing
- Complaints and component recall
- Investigation of errors and accidents

Fig. 10.13: Quality management in blood transfusion

Equipment QC

Equipment	Method of control	Frequency of control	Control executed
Laboratory refrigerator, freezers, water bath	Thermometer, precision thermometer	Daily	Technologist
Blood bag refrigerator, freezer containing transfusates	Graphic recorder plus independent audible and visual alarm for appropriate high and low temperature parameter	Daily	Technologist
Laboratory refrigerator, freezer, water bath	Precision thermometer # For calibration #	Every 6 month	Technologist
Equipment Cell counter	Method of control Calibration, reference samples,	Frequency of control Daily	Control executed by technologist
pH meter	Control solution pH 4–7, 7–10	Each time of use	Technologist
Platelet agitator	Frequency of agitation	Monthly	Technologist
Laminar flow blood	Air pressure	Daily	Microbiologist
Laminar flow blood and sterile area filter	Particle counter	3 times/month	Microbiologist
Cryofuge	Precision RPM meter plus stopwatch to control speed, acceleration and retardation	Twice/month	Technologist

Contd.

Contd.

Equipment	Method of control	Frequency of control	Control executed
Cryofuge	Temperature	Daily	Technologist
Table centrifuge	RPM meter plus stopwatch to control speed, acceleration and retardation	Daily	Technologist
Haemoglobin Spectrophotometer	Calibrate with standard	Daily	Technologist
Haemoglobin Spectrophotometer	Hb-QC sample	Monthly	Technologist
Equipment	Method of control	Frequency of control	Control executed by
Blood mixer	Control weighing and mixing	Twice/month	BM engineer
Blood bag tube sealer	Pressure on bag and tube	Every bag and weld	Technologist
Blood transport container	Temperature control device	Every time on use (on receipt)	Technologist

Table 10.9: List of qualitative (QL) and quantitative (QN) tests done in blood bank

Tests for donor selection	Tests for collected blood product	Tests for final pre-transfusion	Serological test done
• Haemoglobin (QN) • Platelet count (QN) • Serum albumin of donors (QN)	• HIV (QN)/(QL: Card test) • HBsAg (QN)/(QL: Card test) • HCV (QN)/(QL: Card test) • VDRL/RPR (QL) • MP (QL) • Factor VIII (QN) • Fibrinogen (QN) • PTTK (QN), • Pyogenic culture (QN)	• Haemoglobin (QN), • PCV (QN), • Platelet count (QN), • pH (QN) • TRBC (QC) • TLC (QN)	• Blood grouping (QL) • Crossmatching (QL) • Antibody screening/plasma identification (QL) • Direct Coombs' test (QL) • Indirect Coombs' test (QL)

Equipment/Reagents which are Needed for in-house Checks (IQC)

- ELISA reader/kits
- Blood Grouping equipment/reagents
- Crossmatching cards/antibody screening
- Haematology analyzer/reagents
- pH meter
- Rapid cards/RPR (rapid plasma reagin) or VDRL/MP
- Coagulation analyzer

Selecting controls/calibrators: A calibrator has a known concentration of substance (analyte) being measured. Calibrator is used to adjust instrument, kit, test system in order to standardize the assay. Calibrator is not a control. A control has a known range of analyte. Usually 2 or 3 levels (L1, L2, and L3) of controls are used. Control should be run along with patient sample and is used to validate day-to-day reliability of the test systems (Fig. 10.14).

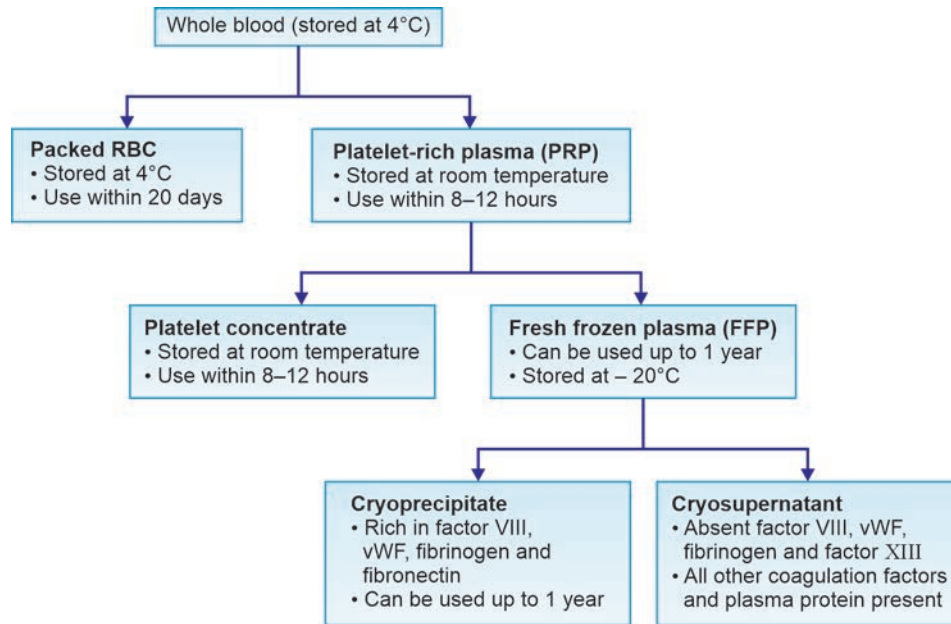


Fig. 10.14: Blood and different components and their use and storage