Section

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Haematology

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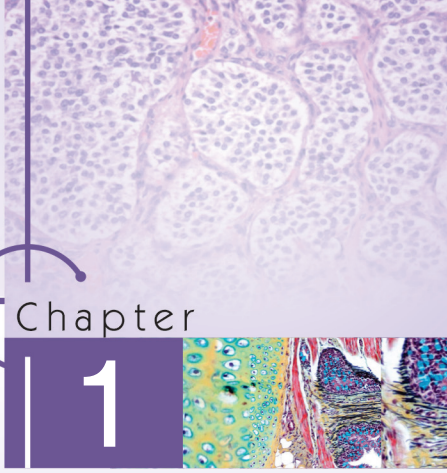


Chapter 1

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 gm%), globulins (2.5 gm%) and fibrinogens (0.3 gm%). 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:

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Venous blood is best withdrawn from an antecubital vein by means of a dry glass syringe or disposable plastic syringe.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

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 vacuotainer 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

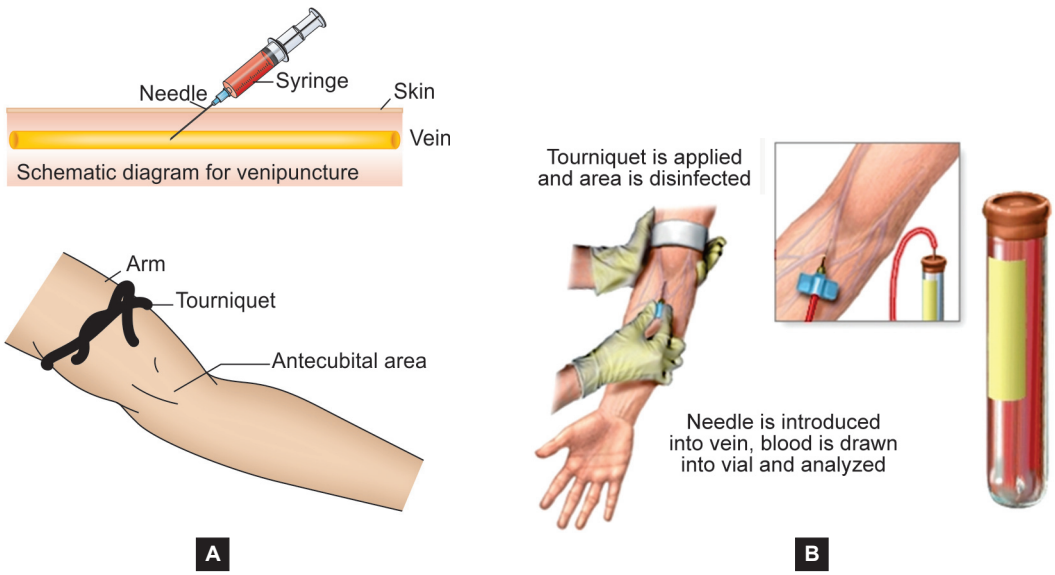


Fig. 1.1A and B: Blood collection procedure

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 pCO_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 pCO_2 and pO_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

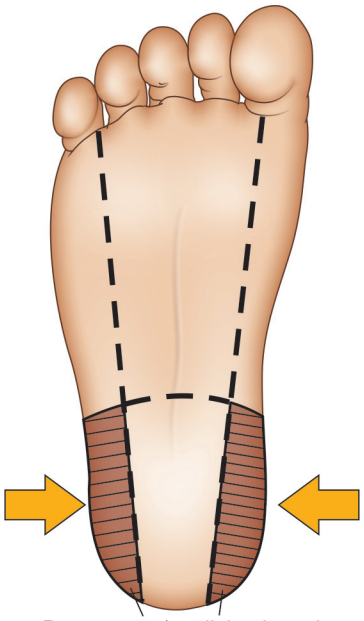


Fig. 1.2: Heel puncture method

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.

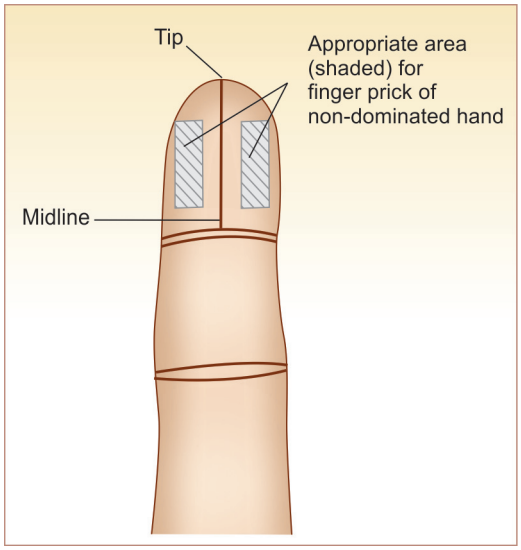


Fig. 1.3: Finger prick (stick) procedure

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 γGT, 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 ($Na_3 C_6H_5O_7, 2H_2O$) 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.

Advantage: 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 gm
- Potassium oxalate: 0.8 gm
- 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

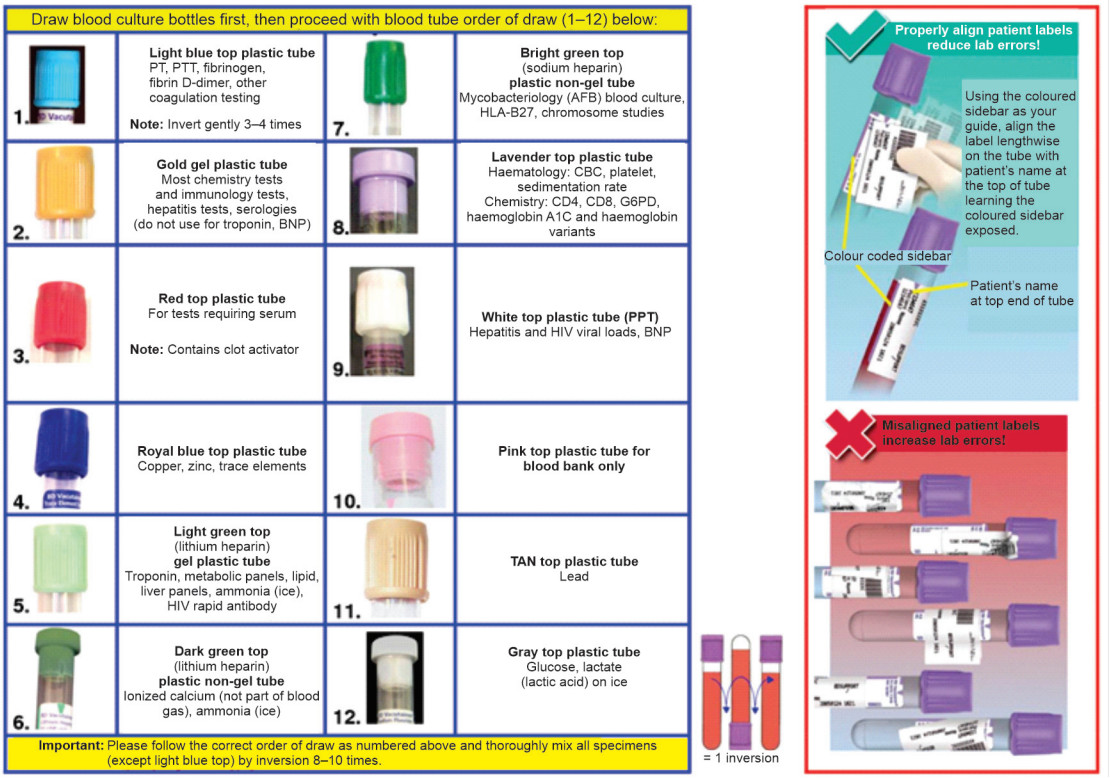


Fig. 1.4: Colour code of top of blood tube/vial and their significance

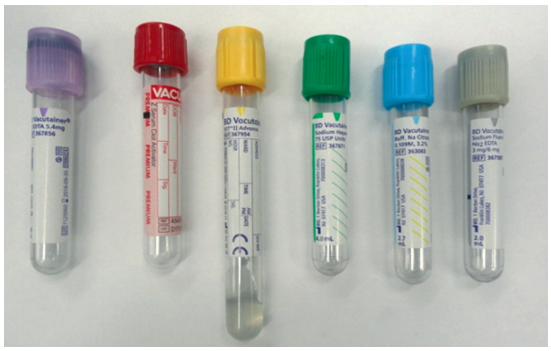


Fig. 1.5: Different blood collection tubes/vials with colour code

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).

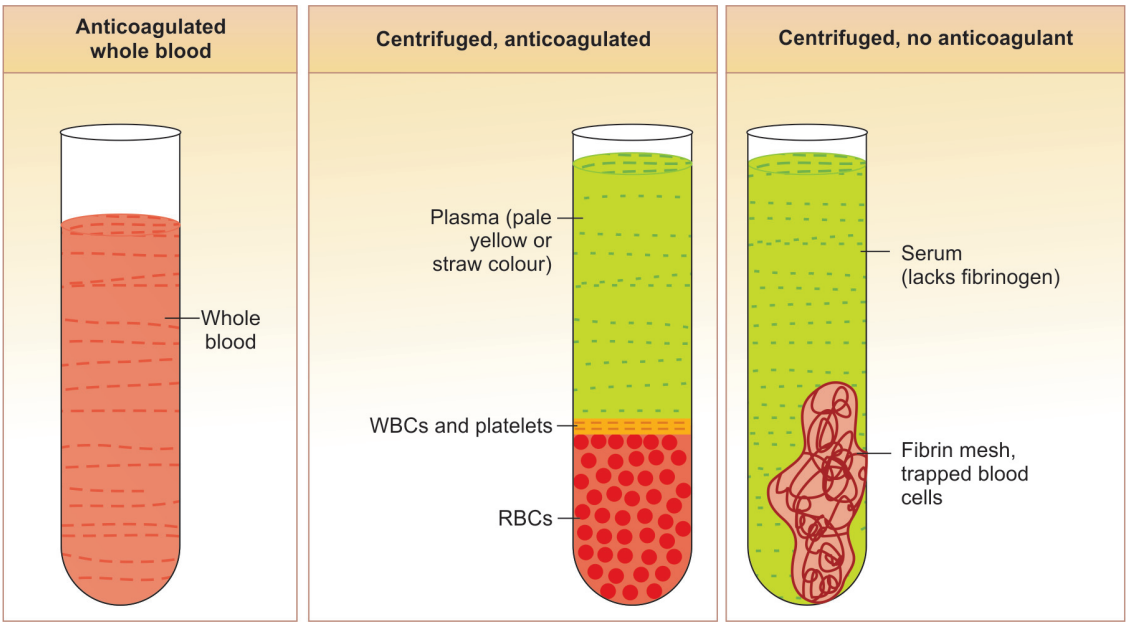


Fig. 1.6: Left tube contains anticoagulated whole blood, middle tube showing plasma and last tube showing serum

VIVA VOCE

Q1. What are differences between serum and plasma?

Ans:

Serum	Plasma
1. Serum is formed as supernatant when blood undergoes clotting	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 centri-fugation.
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.	2. It contains all the clotting factors including fibrinogen. But calcium ion is absent.
3. It is used to estimate different biochemical parameters and serum enzymes like uric acid, electrolytes, urea, creatinine, SGOT, SGPT, alkaline phosphatase, etc.	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. Serum is clear fluid.	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, haemato-logical 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 anticoagulant 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 anticoagulant?

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 vaccum 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

Chapter 2

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 hold 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.

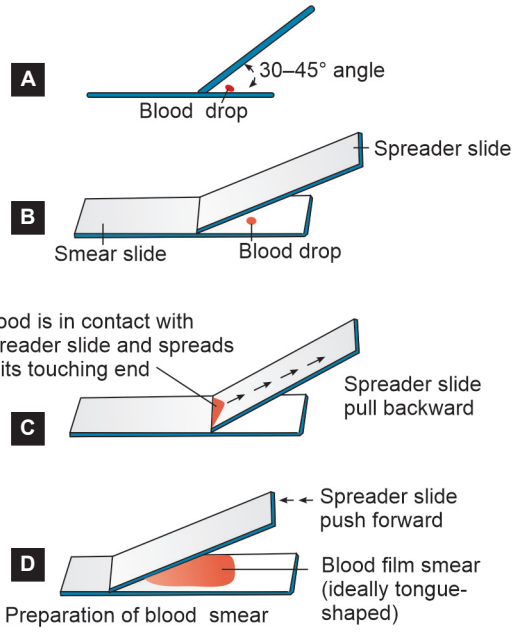


Fig. 2.1A to D: Preparation of blood smears. (A) Small blood drop from 1 to 2 cm from one end of glass slide; (B) Place the spreader slide at an angle of 30° to 45° over the smear slide; (C) Pull back the spreader slide so that it touches the blood drop and spreads throughout the edge of the spreader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

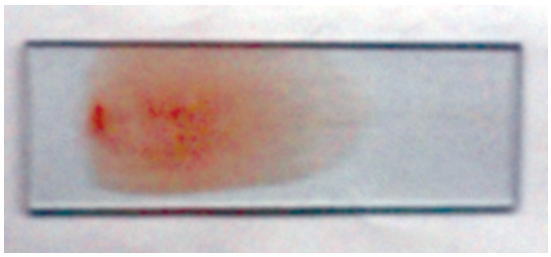


Fig. 2.2: Ideal thin blood smear

Note

a. The blood drop should be such that it can produce the blood film 3–4 cm in length.
b. 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.
c. The film should not cover the entire surface of glass slide.
d. 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).
e. The blood film should have an even, smooth appearance and should be free ridges, waves or holes.
f. The edge of spreader slide must be very smooth. Roughed edges will produce ragged tails containing many leucocytes (WBCs).
g. 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).
h. At a given angle, increasing the speed of spreader slide will cause increase in the thickness of the film.
i. At a given speed, increasing the angle of spreader slide will also cause increase in the thickness of the film.
j. 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.
k. 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.

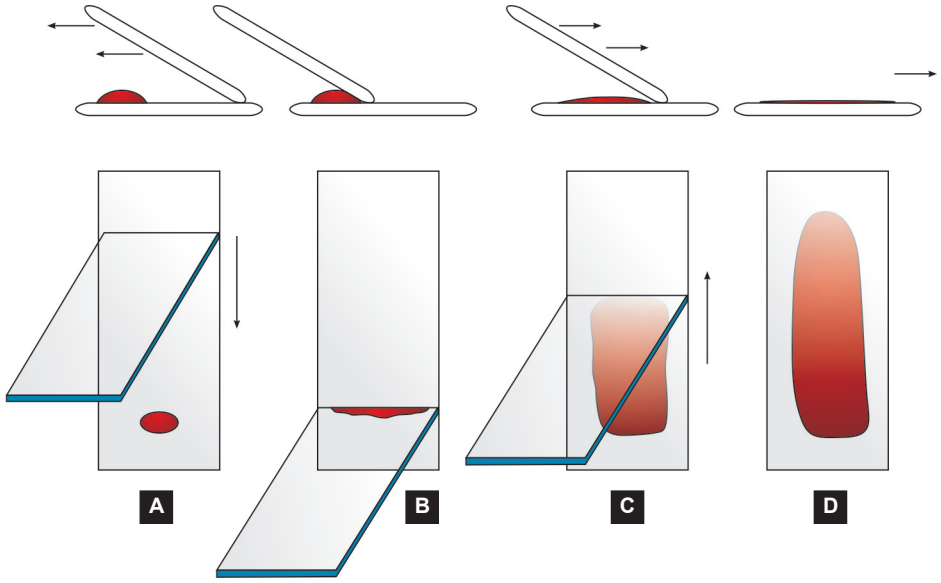


Fig. 2.3A to D: Upper row showing side view of different steps of making a thin blood film. Lower row showing front view of different steps of making a thin blood film

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.

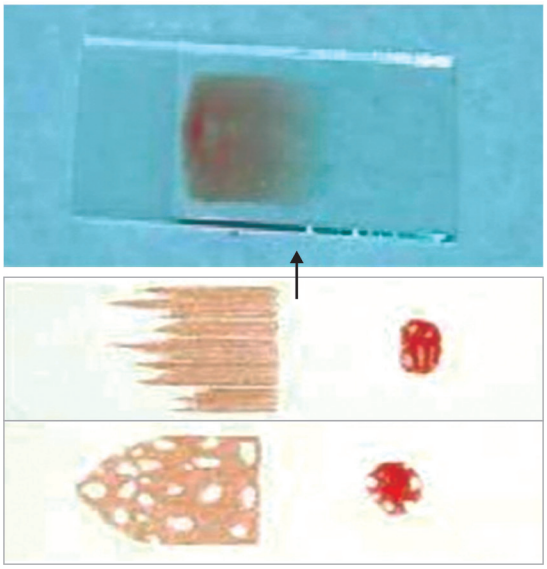


Fig. 2.4: Good and badly prepared thin blood film

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 gm/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 spreaded 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 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.

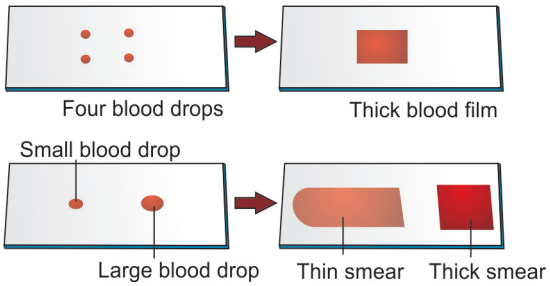


Fig. 2.5: Preparation of thick and thin blood films in the upper panel. Preparation of both thin and thick blood films on the same slide.

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.

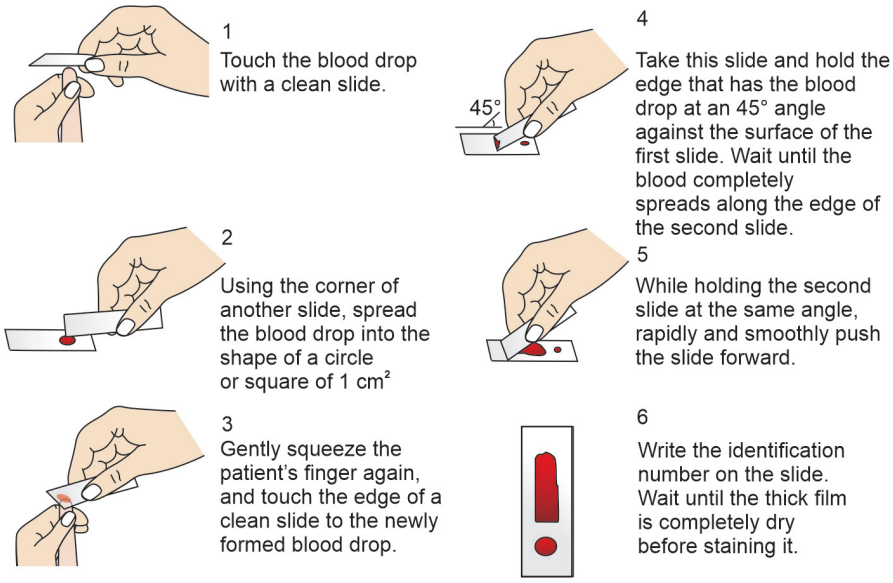


Fig. 2.6: Preparation of a thin and a thick blood film on the same slide

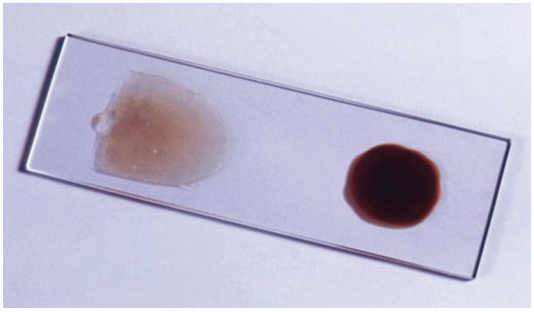


Fig. 2.7: Thin and thick blood films on the same slide. Thin blood film is typically tongue-shaped while thick blood film is circular or square-shaped

This alcohol (methyl alcohol) denatures the proteins present in the blood and hardens 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 to 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 gm
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 gm
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 gm
- Glycerol: 50 ml
- Acetone-free methyl alcohol: 50 ml

Giemsa powder (0.6 gm) 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 gm
2. Acetone-free methyl alcohol: 100 ml

Dissolve the 0.3 gm 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)

a. Methylene blue: 0.26 gm
b. Azure B (optional): 0.1 gm
c. Disodium hydrogen phosphate: 2.5 gm
d. Potassium dihydrogen phosphate: 1.25 gm
e. 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)

a. Eosin Y (yellow eosin, water soluble): 0.26 gm
b. Disodium hydrogen phosphate: 2.5 gm
c. Potassium dihydrogen phosphate: 1.25 gm
d. 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

1. Fix the film for 10–15 seconds in methanol.
2. Pour off the methanol and put 12 drops of diluted stain B (1:4 dilution in water)
3. Immediately add 12 drops of stain A.
4. Agitate the slides to mix the stains.
5. After 1 minute, rinse the slide in water.
6. Differentiate the slide in phosphate buffer for 5–10 seconds at pH 6.6.
7. Wash the slide in water.
8. Place it on end to drain and then dry.

Note

i. A pH to the alkaline side of neutrality accentuates the azure component of Romanowsky stain at the expenses of the eosin and vice versa.
ii. A pH of 6.8 is usually recommended for general or routine use.
iii. 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*).

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

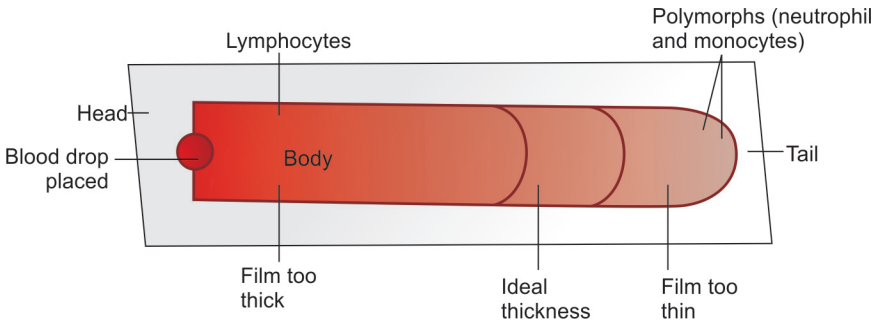


Fig. 2.8: Three zones in stained smears. The head, body and tail

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.

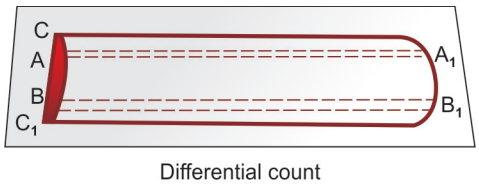


Fig. 2.9: Linear or straight line method of differential count of WBC

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).

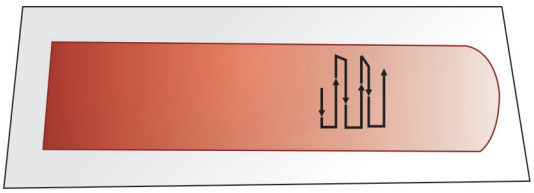


Fig. 2.10: Serpentine counting pattern (area is in between body and tail end of smear)

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 mm in diameter, average 7–7.5 mm)
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/mm^3. Leukocytosis refers to TLC more than 11000/mm^3. Leucopenia less than 4000/mm^3.

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").

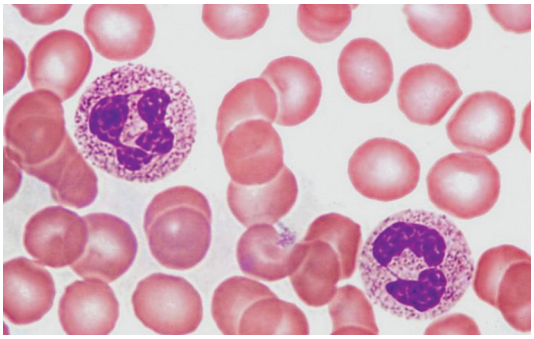


Fig. 2.12: Neutrophils

- 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

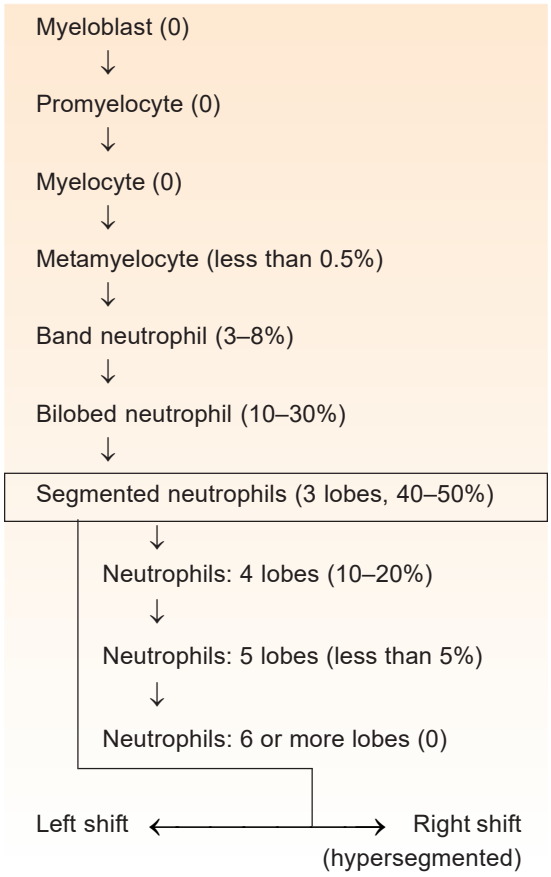
and is about 1.5 mm in diameter. It indicates the inactive X chromosome and corresponds to **Barr body**.

Hypersegmented neutrophils (Fig. 2.13): If the peripheral smear shows ≥5% of neutrophils having 5 lobes or ≥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.

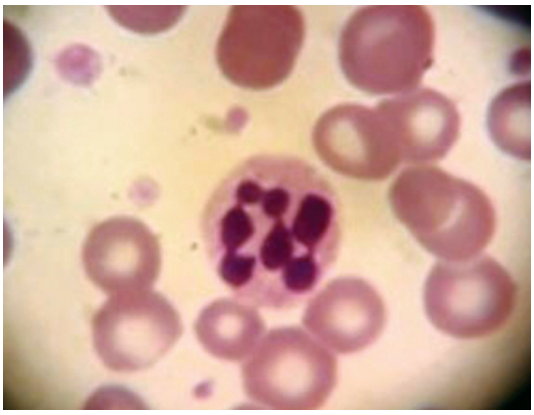


Fig. 2.13: Hypersegmented neutrophils (7 lobes) in megaloblastic anaemia

With the three-lobed neutrophil as a marker, shift to the left (less nature) or to the right (hypermature) can be understood. A left 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 ½ 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 $7500/mm^3$ 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 $<1500/mm^3$.

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/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/mm^3 is high risk factor for sepsis, whereas a count <200/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 ≤10% are large.

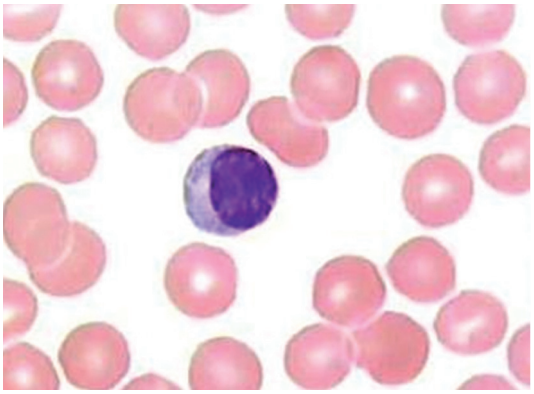


Fig. 2.14: Lymphocyte

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 azurophil 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 immunoblastosis (10 μm in diameter) with a round nucleus and abundant deeply basophilic cytoplasm. Ultimately these cells will transform into

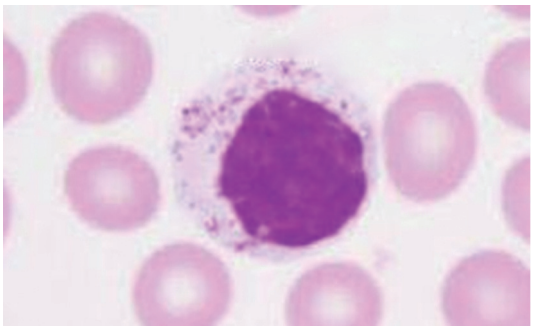


Fig. 2.15: Large granular lymphocyte

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

Activated lymphocytes: These cells are seen in PBS in virals 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/mm^3 (or >43%) in adults, >7200/mm^3 in adolescents and >9000/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, pertusis (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/mm^3 (<18%) in adults, and <3000/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; 15–18 μm in diameter and constitute 2–10% of leucocytes in PBS.

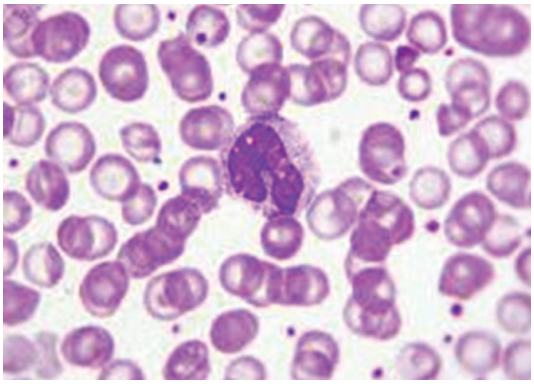


Fig. 2.16: Monocyte

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/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 mm). They usually have two lobes in the nuclei bit 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.

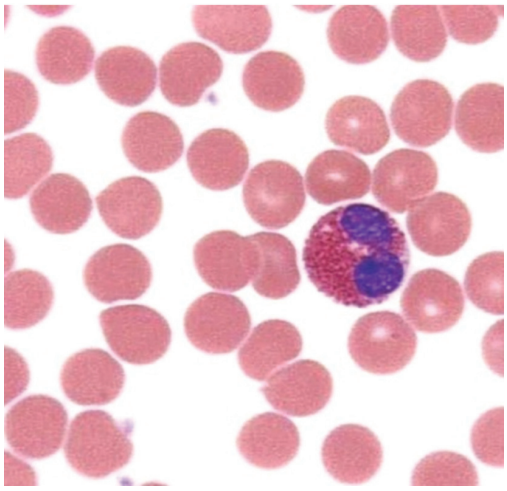


Fig. 2.17: Eosinophil

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/mm^3 or >8% of DLC (some use a cut-off value of >>450/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 to 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.

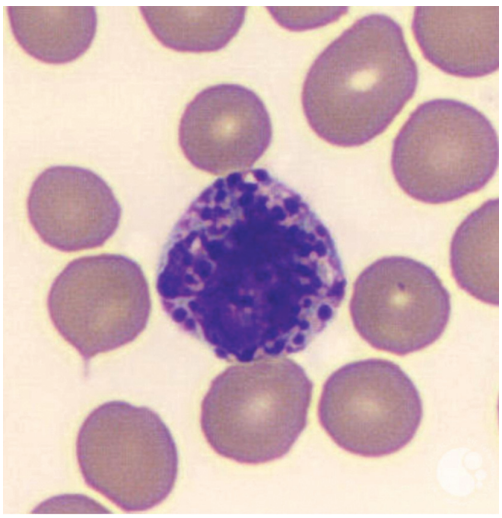


Fig. 2.18: Basophil

Basophilia

Definition: Basophil count is >200/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/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-

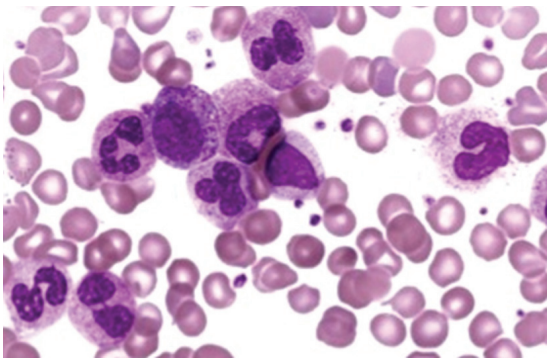


Fig. 2.19: Leukaemoid reactions (neutrophilic)

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 lmphocytes may occur in measles, chickenpox, CMV, pertusis 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 $50 \times 10^3/mm^3$. 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 ≥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 mm 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 mm 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.

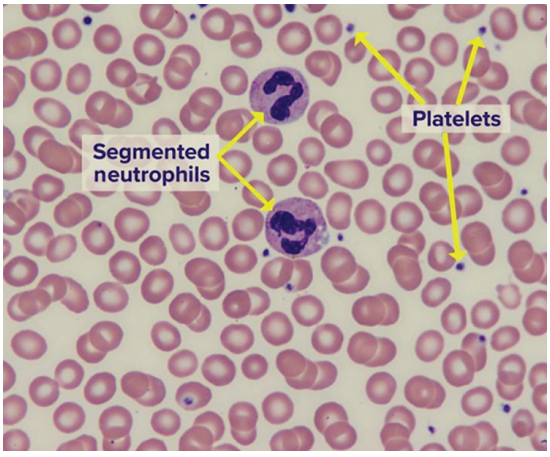


Fig. 2.20: Platelets

Platelet Functions

i. They from 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 routing 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	• Decreased in number
• Several platelets with occasional clumps	• Adequate in number
• Over 25 platelets	• 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 protozoans, such as *Acanthamoeba* and *Trichomonas* species.

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

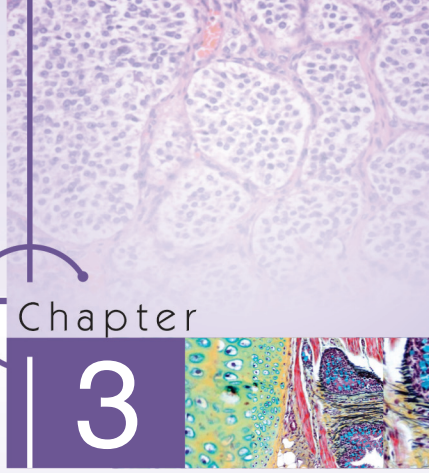
Ans: Ideally, the smear should be tongue shaped and after staining, a well stained blood smear will have pinkish tint at the stained portion and no staining on other portion of the slide.

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 buish 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 trophozoites and 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. 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, trophozoites and schizonts of *P. falciparum* do not appear in PBS.



Chapter 3

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 gm.

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

Most favoured site nowadays

- Spinous processes of lumbar vertebra
- Sternum: It is no longer favoured though was popular in the past.

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:

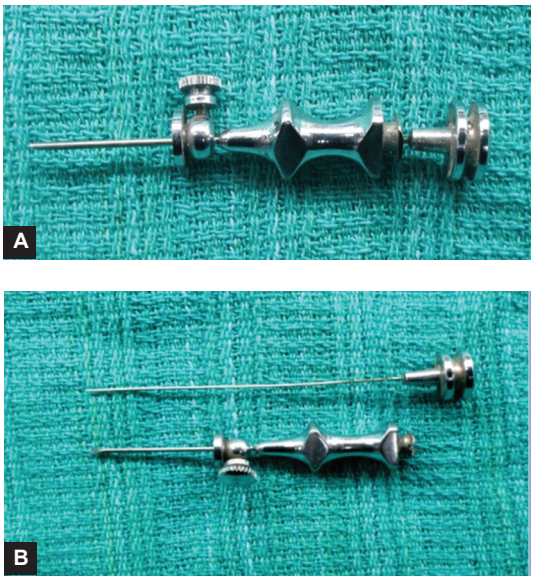


Fig. 3.1A and B: Salah bone marrow needle

   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.

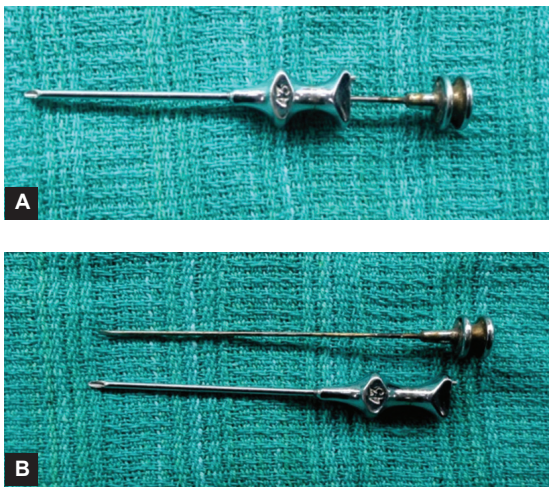


Fig. 3.2A and B: Klima bone marrow needle

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 stares (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, malaria (*Leishmania donovani*)
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

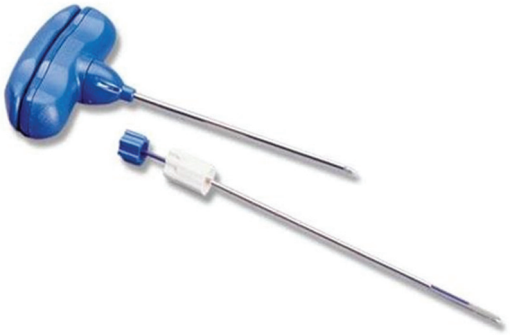
Bone Marrow Aspiration Technique

(Fig. 3.4)

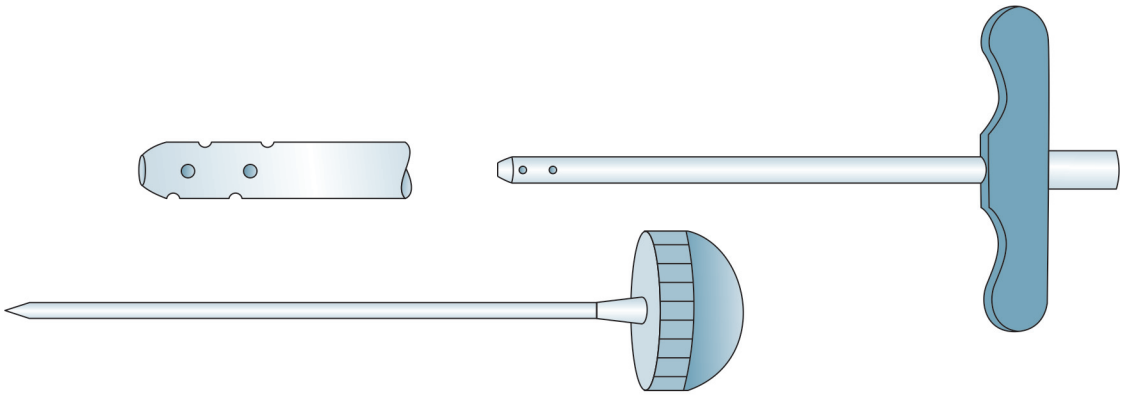
Puncture of Ilium (Superior Iliac Spine and Iliac Crest)

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



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.

B

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.

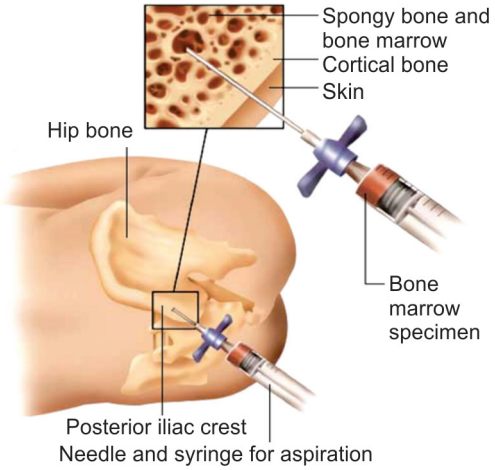


Fig. 3.4: Bone marrow aspiration

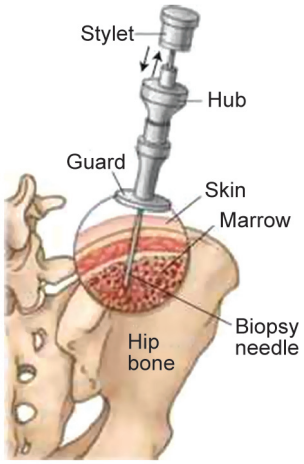


Fig. 3.5: Bone marrow aspiration by Klima's needle

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:

i. **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 → cellularity% = (100 – age)%. So, physiologically children have highest cellularity and elderly lowest cellularity.

ii. **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.

iii. **Type of erythropoiesis:** Whether normoblastic, megaloblastic or dyserythropoietic.

iv. **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.

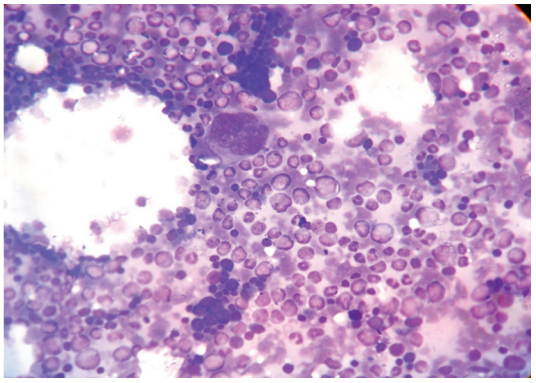


Fig. 3.6: Normal bone marrow shows trilineage haemopoietic cells (oil immersion, Leishman's stain, 1000X)

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 bonds	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

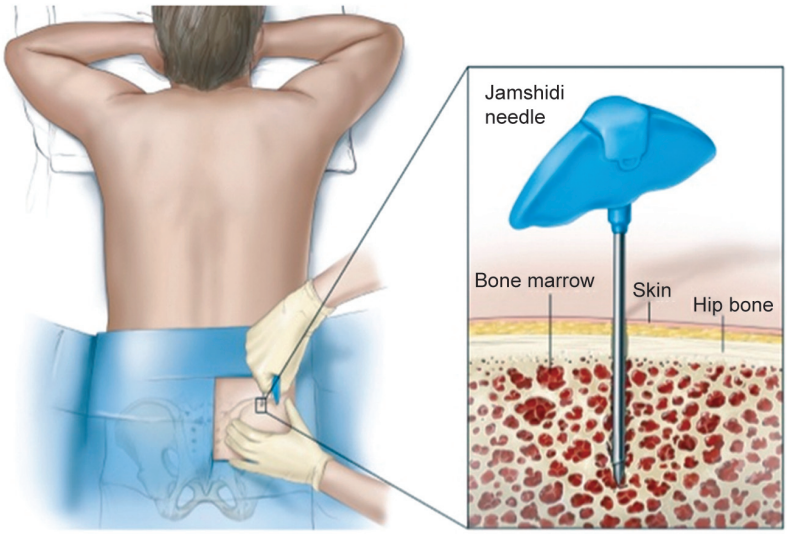


Fig. 3.7: Trephine biopsy by Jamshidi needle

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.

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.
- 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).

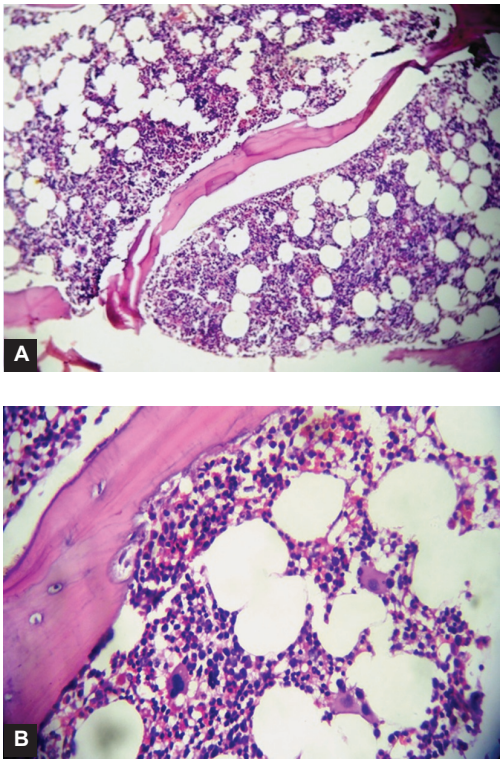


Fig. 3.8A and B: (A) Low power view of trephine biopsy shows marrow tissue, fat spaces and bony trabeculae; (B) High power view shows trilineage (erythroid, myeloid and megakaryocytic/thrombocytic cells) haematopoiesis including megakaryocytes

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

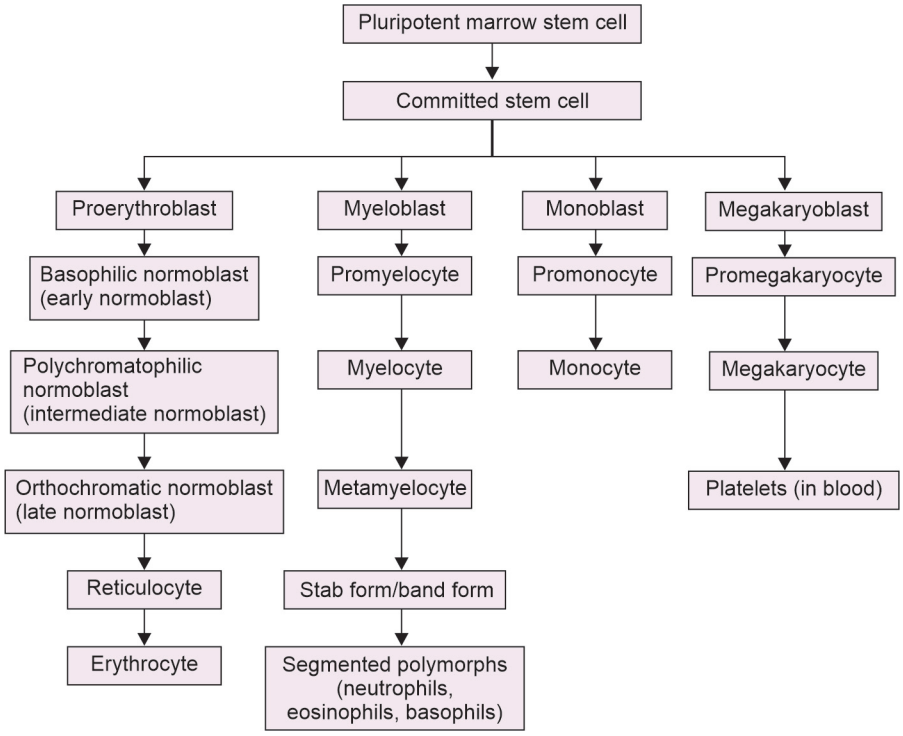


Table 3.2: Comparison of bone marrow aspiration and bone marrow biopsy

Parameter	Bone marrow aspiration	Bone marrow biopsy
1. Site	Illiac 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

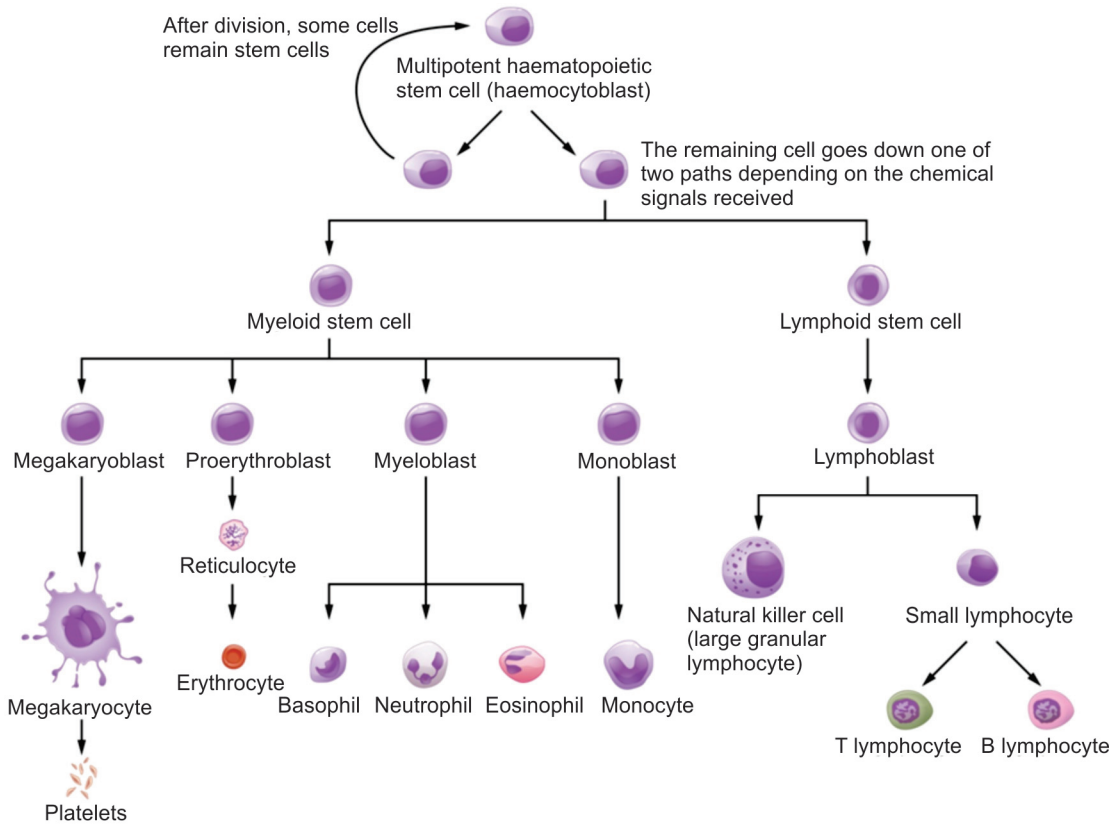


Fig. 3.9: Haematopoiesis of different blood cells in the bone marrow

- 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 (particulary lymphoproliferative disorders and proliferation of megakaryocytes).

VIVA VOCE

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 has a guard which is fixed to the needle with a side screw. Klima's needle has also a guard but unlike Salah's needle it does not has side screw and guard is fixed to the body of needle it does not has side screw and guard is fixed to the body of needle itself by a spiral thread. Klima's needle can be easily readjusted during bone marrow aspiration procedure. Klima's needle is preferable among these two as the guard is on a spiral thread and there is less chance of slipping.

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: i. Macroscopically, by looking at the granularily. When bone marrow smear is touched the surface is irregular and sand-like feeling due to presence of marrow particles. Marrow particles look glistening.

ii. 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).

Chapter 4

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.
- Burker 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

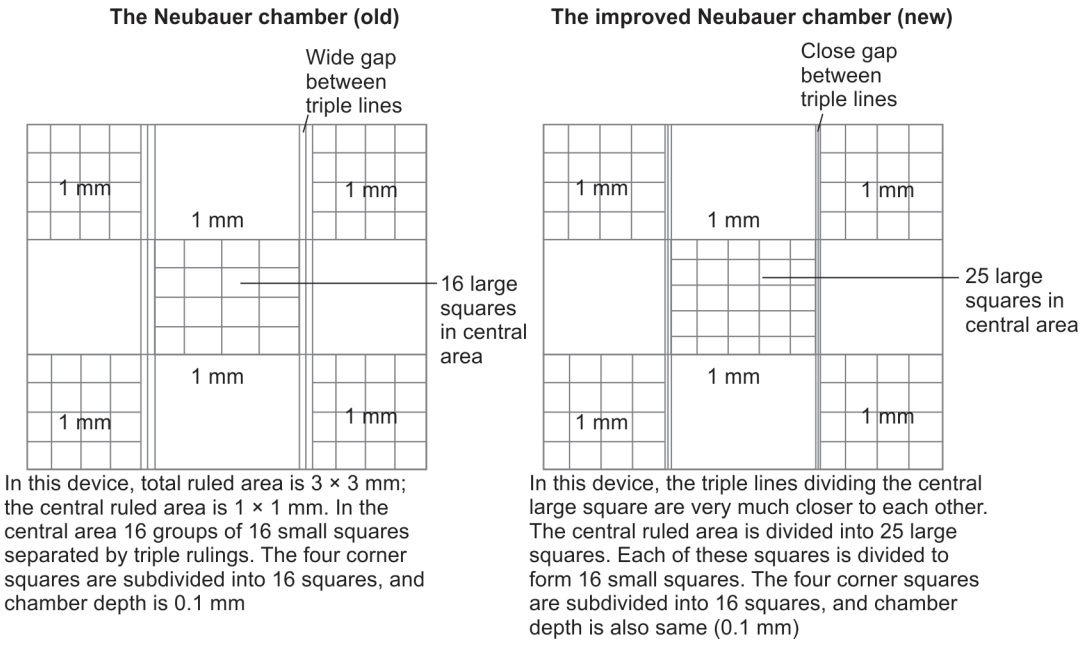


Fig. 4.2: Old and improved Neubauer chamber

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.
- 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)

i. A counting chamber
ii. A WBC pipette
iii. A RBC pipette
iv. 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 × 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 squres 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 × 1/5 mm or 1/25 mm^2. For counting

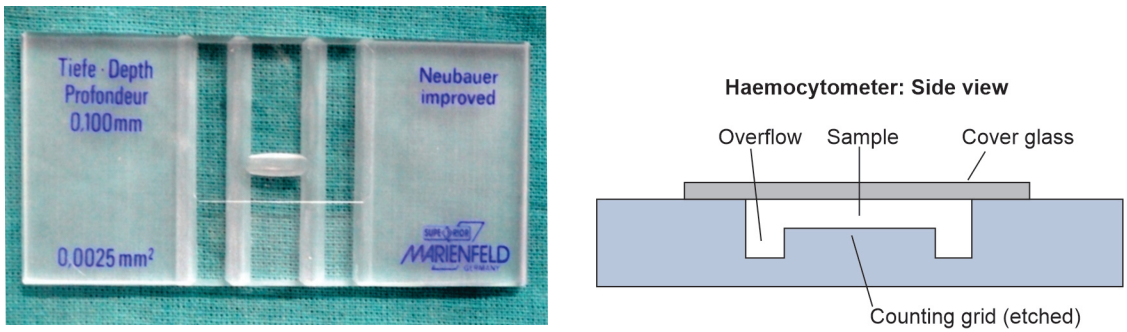


Fig. 4.3: Improved Neubauer's chamber

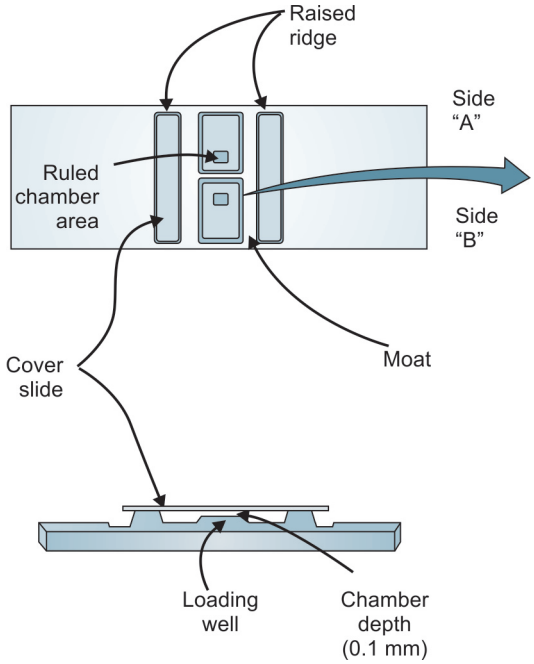


Fig. 4.4: Schematic diagram of using improved Neubauer's chamber

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

i. Total WBC count of blood (TLC).
ii. Total cell count from different body fluids like pleural fluid, CSF (cerebro-spinal fluid, peritoneal fluid).
iii. Total platelet count, if the platelet count is very low.
iv. 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

i. Total RBC count of blood
ii. Total platelet count of blood
iii. Total sperm count from seminal fluid
iv. 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.

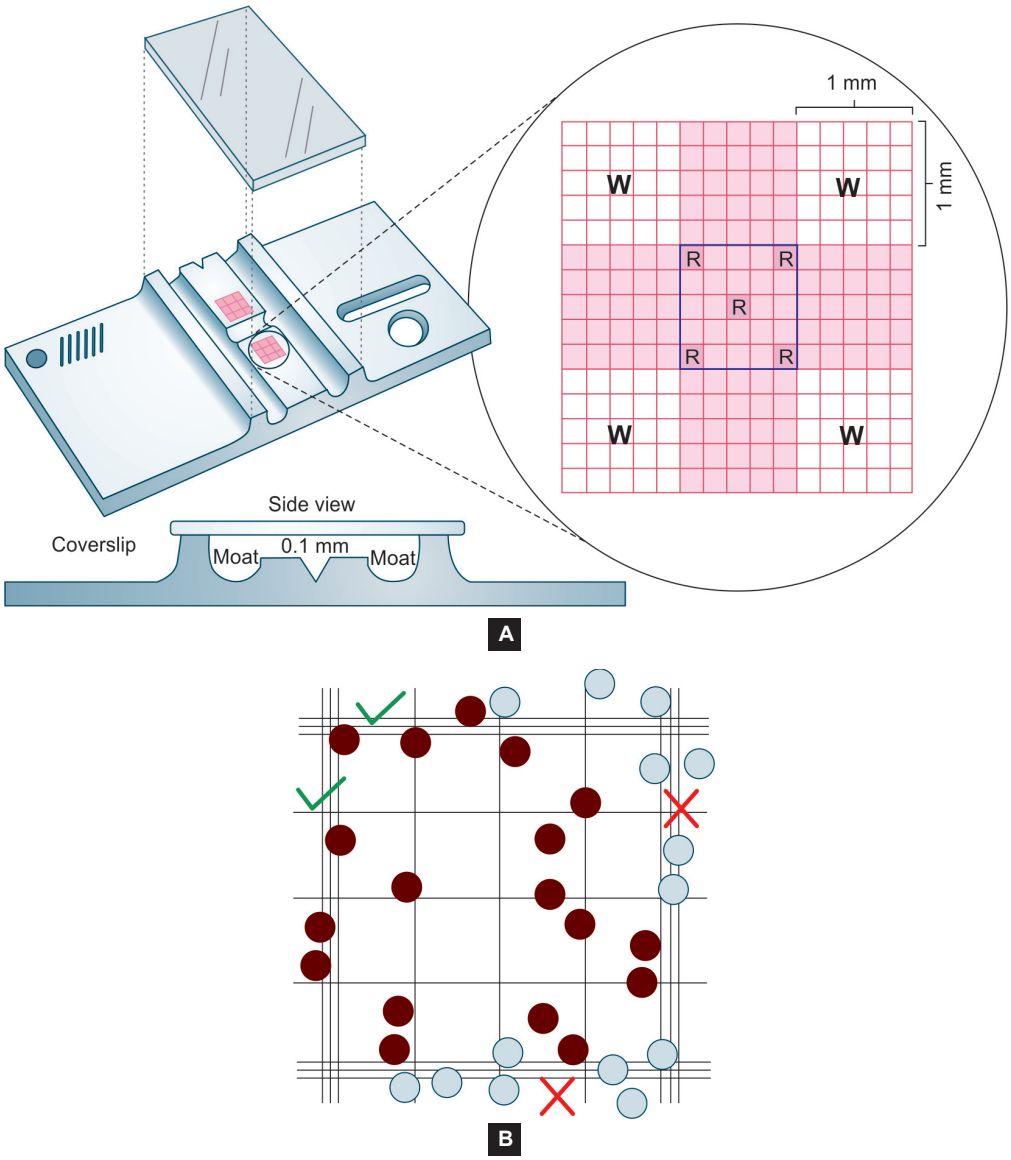


Fig. 4.5A and B: (A) Counting of WBC and RBC in the chamber; (B) Right way of counting cells. Cells on the upper and left triple lines are counted (black-coloured circles) while on right and lower triple lines are not counted (white-coloured circles)

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

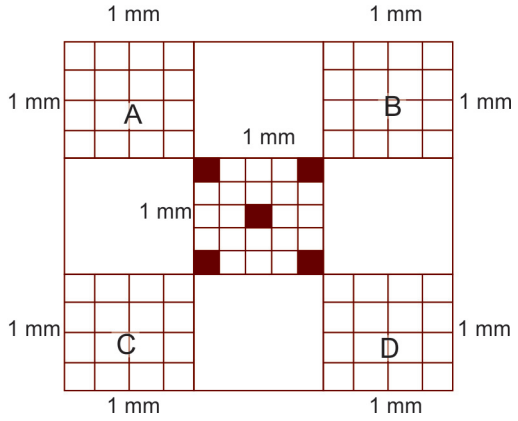


Fig. 4.6: Counting chamber in improved Neubauer's chamber

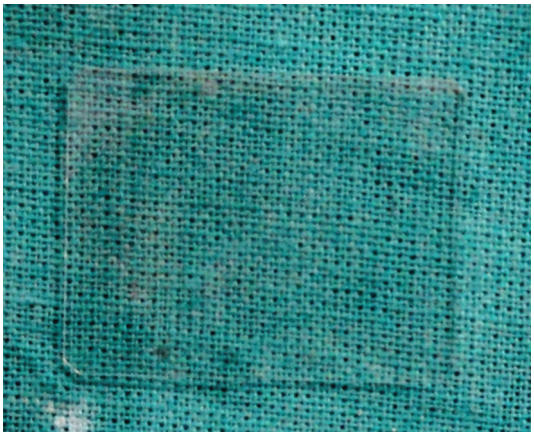


Fig. 4.9: Thick coverslip

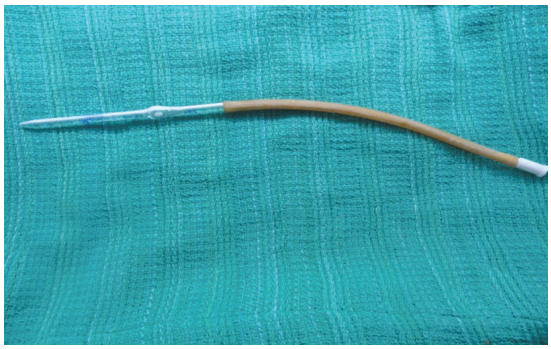


Fig. 4.7: WBC pipette

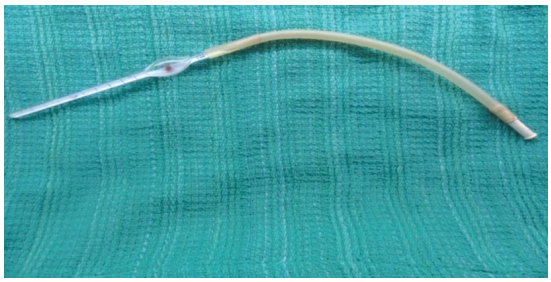


Fig. 4.8: RBC pipette

(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 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.

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

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 × 1 × 1/10 mm (depth)

So, the volume of four large squares = 1 × 1 × 1/10 × 4 mm^3 or cu mm = 2/5 mm^3

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

Then, 2/5 mm^3 of volume of blood contains leukocytes = N × dilution factor

Or, 2/5 mm^3 blood contains leukocytes = N × 20

Or 1 mm^3 blood contains leukocytes = N × 20 × 5/2 = N × 50.

Note

i. 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%.

ii. 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).

iii. Filing or charging defects leading to error:
   a. Chamber area incompletely filled
   b. Air bubble anywhere in the chamber area
   c. Any dirt or debris in the chamber (unclean or moist chamber).
   d. Overflow of the counting and ruled area.

iv. 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 most 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 gm
- 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 gm
- Sodium sulphate: 5 gm
- Mercuric chloride: 0.5 gm
- 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 rouleax. Hence, not recommended for use.

Counting of RBCs

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

Volume of 5 small squares = 1/5 × 1/5 × 1/10 × 5
(depth is 0.1 mm or 1/10 mm)
= 1/50 cu mm or mm^3

So, 1/50 mm^3 blood contains RBCs
= N × dilution factor
= N × 200 (dilution of blood in RBC diluting fluid)

Then, 1 mm^3 blood contains RBCs
= N × 200 × 50/1 = N × 10,000

Note

i. Like WBC pipette, RBC pipettes are also not used nowadays. Instead micropipette or Pasteur pipettes are used.
ii. Error in counting RBC may be due to filing or charging defects (vide WBC count).
iii. 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 mm^3 blood contains platelets
= N × dilution factor
= N × 200

Then, 1 mm^3 blood contains platelets
= N × 200 × 50/1 = N × 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 × 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 electromagnetic current, high frequency electromagnetic 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.

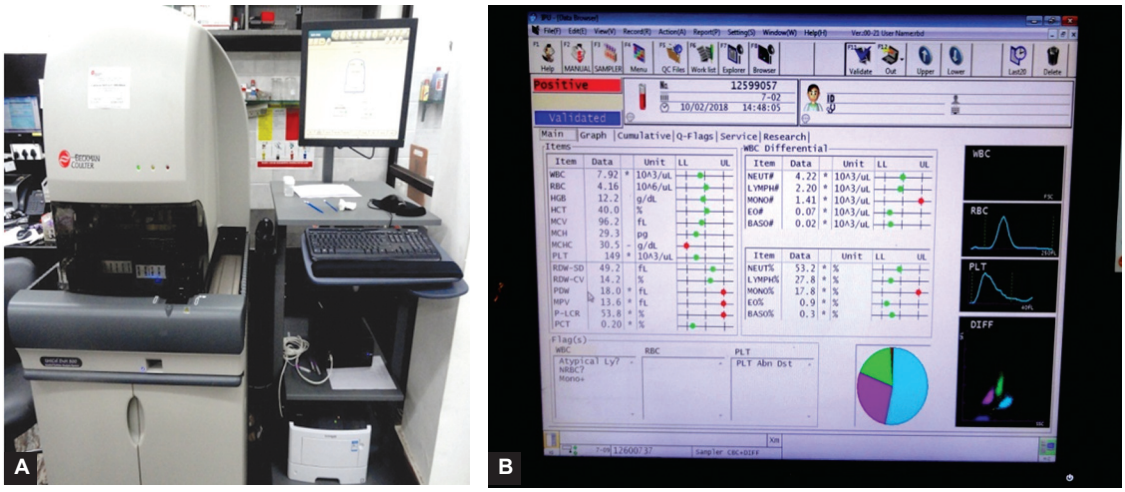


Fig. 4.10A and B: Automated blood cell counters

VIVA VOCE

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/µl), 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 eosiniphil 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)
- Wrong 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 gm, 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 millions/ml. Count below 20 millions/ml is considered abnormal.

Chapter 5

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.

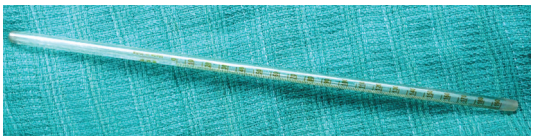


Fig. 5.1: Westergren pipette

Anticoagulant used: 3.8% trisodium citrate solution (no other anticoagulant with this method), 109 mmol/L (32 gm/L, $Na_3Ca_6H_5O_7.2H_2O$). 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 3.8% 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.

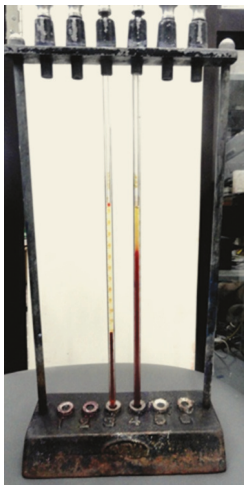


Fig. 5.2: Westergren or ESR stand (rack)

Mean ESR = (1st hour result + ½ of 2 hours result) ÷ 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,

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.

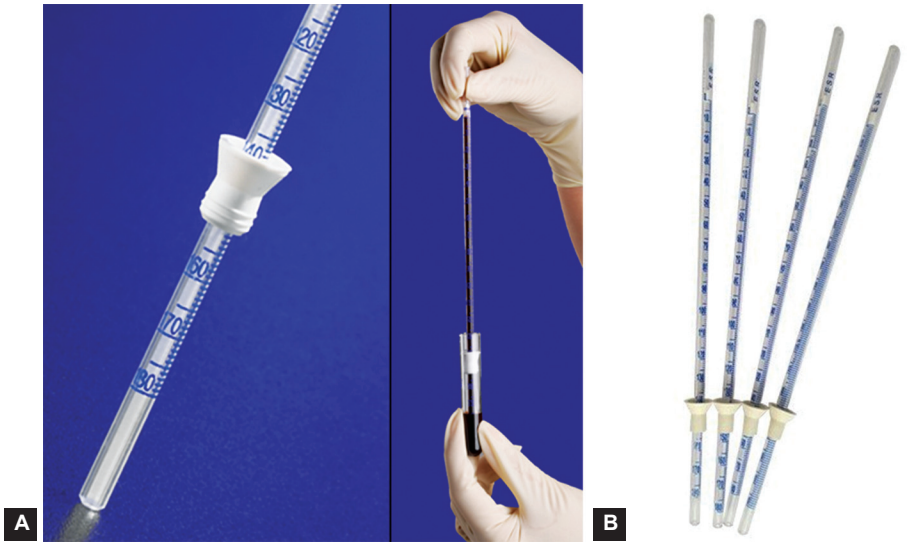


Fig. 5.3A and B: ESR estimation with disposable plastic pipettes (modified Westergren method)

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.
- A9/120 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

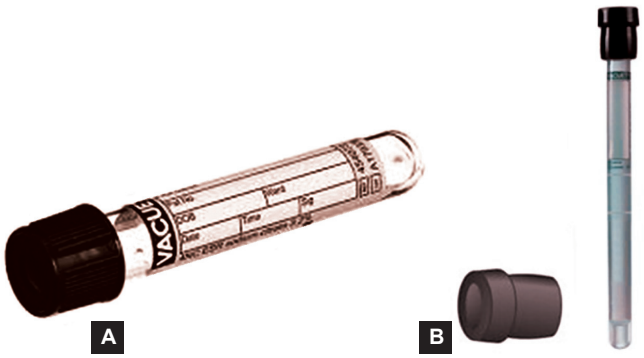


Fig. 5.4A and B: VACUETTE® ESR system

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 1hour 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.

Causes of Slow or Decreased ESR

- Polycythaemia vera
- Sickle 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.
- 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°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.

Different Automated Methods of ESR

(Fig. 5.5A and B)

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

Advantages of Automated Methods

- Provide more rapid results
- Use small sample volumes
- Save technician time
- Provide increased safety because the need for sample manipulation is decreased.

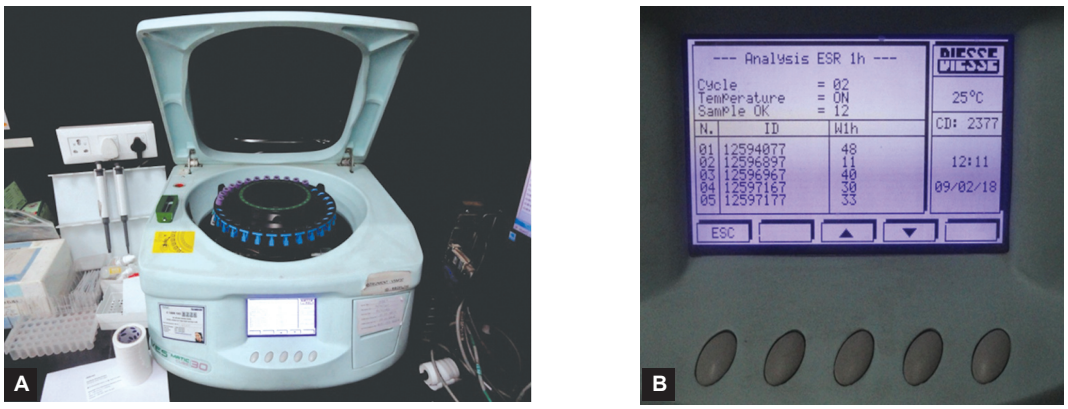


Fig. 5.5A and B: (A) Automated ESR machine; (B) Recording of ESR result

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 to 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 ± 5%, i.e. 40 to 50%
- Women: 41± 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 ½ to 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 ± 7%
- Female: 43 ± 5%

Advantages of Micro-method

- Time requirement for centrifugation is short.
- 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 × 3 = 45% (approx).

Table 5.1: Normal levels of packed cell volume (PVC) 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

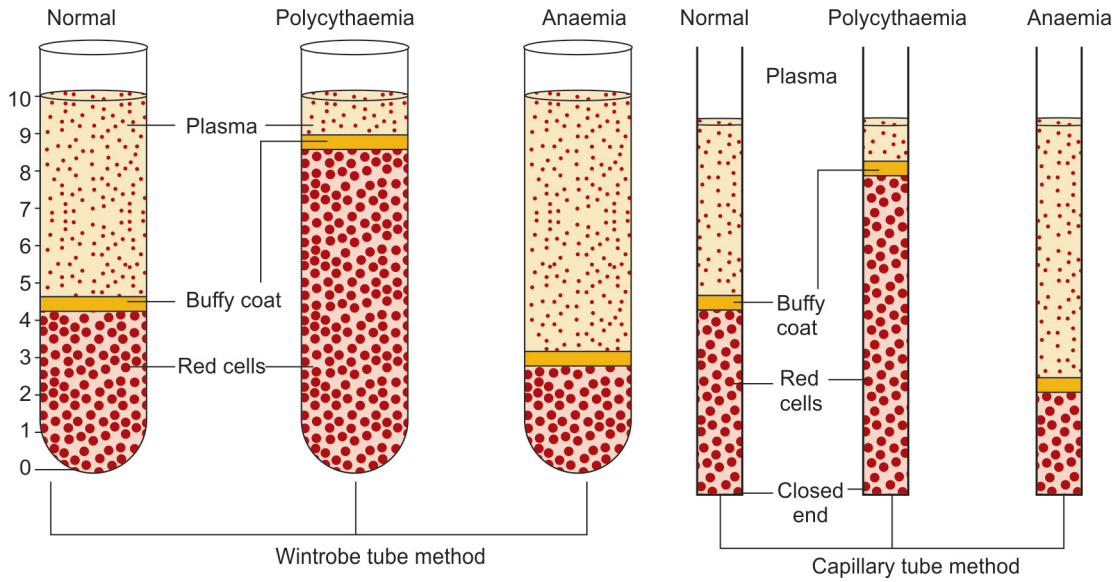


Fig. 5.7: Packed cell volume (PCV) shows comparison of normal, polycythaemia and anaemic blood samples by two different methods (Wintrobe and capillary tube)

VIVA VOCE

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 in L/L ÷ RBC 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 ÷ RBC 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 ÷ PCV 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.

Chapter 6

Reticulocyte Examination

RETICULOCYTE COUNT

Reticulocytes are immature red blood cells (young or juvenile RBCs). In the process of erythropoiesis (red blood cell formation), reticulocytes develop and mature in bone marrow and then circulate for about a day in the blood stream before developing into mature red blood cells. Like mature RBCs reticulocytes also do not have a cell nucleus. They are called reticulocytes because of a reticular (mesh-like) network of ribosomal RNA that becomes visible under a microscope with supravital stains like **brilliant cresyl blue** or **new methylene blue (best stain)**. These stains are called supravital stains because they stain ribonucleoprotein or RNA protein which is a living material. This reaction takes place only when the reticulocytes are still alive (without fixing the cells). Largest amount of dark blue network, or reticulin is found in the most immature reticulocytes, whereas only a few dots of short strands are seen in the least immature reticulocyte.

Reticulocytes can also be stained by Romanowsky stain (Fig. 6.1). But with Romanowsky stain, reticulocytes appear larger and slightly bluer than other RBCs. A reticulocyte stain measures an aggregate of residual ribosomes and mitochondria that form clumped granular material called reticulum.

Properties of Reticulocytes

In comparison to mature erythrocytes (RBCs)

- Greater volume: Approximately 24%
- Slightly higher Hb content: Approximately 13%
- Lower Hb concentration: Approximately 16.7%
- The constant volume ratio between reticulocytes and erythrocytes: Approximately 1.24 (Fig. 6.1).

Methods of Reticulocyte Count

There are two methods:

- Visual method
- Automated method

Visual Method

Principle of the test: As already said the supravital stains reticulocytes in living state. They stain ribosomes and RNA of reticulocytes in living state and unfixed state. When blood is incubated with these stains for a short period, the RNA is precipitated as a dye–ribonucleoprotein complex. On microscopy, the complex appears as a dark blue network (reticulum, or filamentous strand or granular material). Reticulocytes are stained polychromatic with Romanowsky stains (Fig. 6.1). Hence, they are called "polychromatophil" when present in peripheral blood.

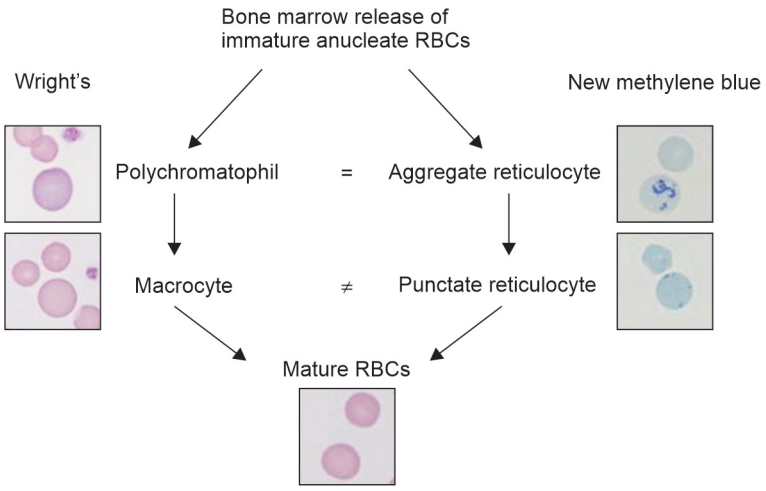


Fig. 6.1: Staining of reticulocytes

Isotonic Solution

Contents	Quantity	Action
• Brilliant cresyl blue or new methylene blue	• 1 gm	• Stains reticulocyte RNA and ribosomes
• Sodium citrate solution (3%)	• 20 ml	• Acts as anticoagulant
• Sodium chloride solution (0.9%)	• 80 ml	• Isotonic solution

When EDTA mixed blood is to be examined, sodium citrate solution is not required.

Procedure of Staining

- Take 2–3 drops of blood and supravital stain (staining solution in a test tube, and mix well)
- Keep it at room temperature or incubate at 37°C for 15–20 minutes.
- Cells are resuspended after gentle mixing
- Take a drop of mixture on the slide and prepare a thin-smear.
- The smear is air dried.
- View under the microscope with an oil immersion lens. At least 1000 red cells are counted and reticulocyte percentage is calculated (Fig. 6.2). A Miller disk ocular inserted into the eyepiece of microscope allows rapid estimation and reduces error.
- An area of film where cells are undistorted with good staining is chosen.

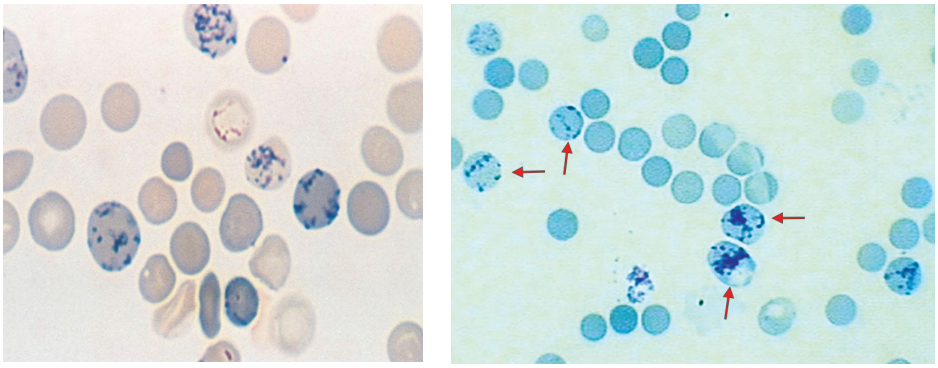


Fig. 6.2: Supravital stain shows reticulocytes

Normal Reticulocyte Count

Reticulocyte count is expressed as percentage of total red cells.

- Normal : 0.5–2.5%
- Newborn (cord blood): 1–7%
- Absolute reticulocyte count = Reticulocyte percentage × red cell count
  Normal is 50,000–85,000/mm^3

Notes (other Uses of Supravital Stains)

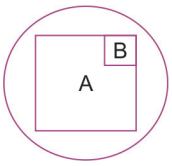
- **HbH inclusions:** Supravital stains also demonstrate HbH inclusions. HbH inclusions represent free β chains and are seen in α-thalassaemia. It appears as regular, tiny, multiple inclusion in the red cells and gives **golf ball-like appearance**.
- **Heinz bodies:** These bodies are aggregates of denatured haemoglobin. They appear as single or multiple rounded inclusions. They are found in G6PD deficiency after exposure to oxidizing drugs.

Miller Disc Method for Reticulocyte Counting

1. Use a 100X objective and 10X ocular secured with a Miller disc.
   a. The Miller disc imposes two squares (one 9 times the area of the other) onto the field of view. That means square B is 1/9th area of square A.
   b. Find a suitable area of the smear. A good area will show 3–10 RBCs in the smaller square of the Miller disc.
2. Count the reticulocytes within the entire large square including those that are touching the lines on the left and bottom of the ruled area. Count RBCs in the smaller square whether they contain stained RNA or not. A retic in the large square should be counted. Record RBC count and retic count separately.
3. Continue counting until a minimum of 111 RBCs have been observed (usually 15-20 fields). This would correspond to 999 RBCs counted with the standard procedure.

The Miller disc may be placed in one of the ocular lenses to aid in the counting of the reticulocytes (Fig. 6.3).

$$\% \text{ Reticulocyte} = \frac{\text{Total reticulocyte in square A} \times 100}{\text{Total RBC in square B} \times 9}$$



Suppose in square A, you get 100 reticulocytes and in square B, you get 500 RBCs. Then,

$$\text{reticulocyte\%} = \frac{100 \times 100}{500 \times 9} = 2.2\%$$

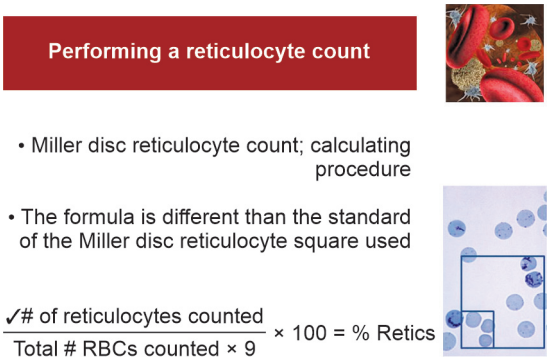


Fig. 6.3: Manual reticulocyte count

Comments and sources of technical error

- When using the Miller disk, failure to follow the "edge" rule may yield to erroneous results; that is, counting reticulocytes touching all 4 lines of either squares. Only reticulocytes touching the top and the left lines should be counted.
- The use of Romanowsky counterstain is no longer advised because it may obscure the supravitally stained granulofilamentous material of the retic.
- Refractive artifacts in erythrocytes caused by moisture in the air and poor drying of the blood film must not be confused with RNA filaments, which do not appear refractory when adjusting the fine focus

on the microscope. The RNA filaments simply disappear when out of focus.

- Blood and stain must be *well mixed* before making films, because *reticulocytes have a lower specific gravity* than mature erythrocytes and thus goes on the top of the mixture during incubation.
- Increased levels of *glucose in the blood may inhibit* staining of reticulocytes

Pappenheimer. Howell-Jolly and Heinz bodies will also stain supravitally. Howell-Jolly bodies stain supravitally as a deep purple and appear singly or in pairs. If *Pappenheimer bodies and Heinz bodies* are suspected, examination of Romanowsky stained peripheral blood film will confirm their presence, because these inclusions will stain. Whereas reticulocyte granulofilamentous material will not. Pappenheimer bodies must also be confirmed by iron staining; they are the most difficult to distinguish from reticulocytes. Heinz bodies does not stain with Romanowsky. However, on supravitally stained films, they stain light blue-green and they may be differentiated from retic because they are always found on the periphery of the erythrocyte and are larger than ribosomal RNA. Heinz body often make the cell look like a pitted golf ball.

Calculation of Different Ways of Reticulocyte Count

1. **Reticulocyte count:** This is the number of reticulocytes counted amongst at least 1000 red cells and expressed as a percentage.

$$\text{Reticulocyte} = \frac{\textit{Reticulocyte counted}}{\textit{Number of red cells}} \times 100$$

Suppose the number of reticulocytes in 200 oil immersion fields = 50
Total number of red cells in 200 field = 3000
Thus, reticulocyte percentage

$$= \frac{17}{1000} \times 100\% = 1.66\% = 1.7\%$$

Here, reticulocytes present per 1000 red cells are 50 ÷ 3 = 16.66 = 17. If someone calculate 1000 red cells and reticulocytes, then reticulocyte percentage will be

$$\frac{17}{1000} \times 100\% = 1.7$$

2. **Corrected reticulocyte count:** This is the reticulocyte count corrected for the degree of anaemia.
Corrected reticulocyte

$$= \frac{\textit{Reticulocyte counted} \times \textit{PCV\% of patient}}{\textit{Average PCV for corresponding age}}$$

This corrected reticulocyte count is also known as **Reticulocyte Index**. Normal reticulocyte index is 1–3%. In patients with anaemia, the reticulocyte count does not reflect the true bone marrow response. So, the reticulocyte count obtained should be corrected for the severity of anaemia. It is calculated by multiplying the reticulocyte percentage by a factor adjusting for the degree of anaemia (i.e. actual PCV divided by normal PCV for age).

Corrected reticulocyte count >2% suggests that the reticulocyte release is appropriate to the degree of anaemia and <2% is inappropriate.

3. **Reticulocyte production index (RPI) (Table 6.1):** After their formation in bone marrow the reticulocytes normally spend about 2 days in bone marrow and one day in peripheral blood before they become fully mature red cells. But in severe haemolytic anaemia and acute blood loss, reticulocytes are released prematurely in peripheral circulation where they require more time (2 days) for maturation. So, the number of reticulocytes in blood becomes double. In such cases, to avoid the overestimation of daily red cell production and to get idea about actual erythropoietic activity, reticulocyte production index is used.
Reticulocyte production index

$$= \frac{\textit{Corrected reticulocyte count}}{\textit{Maturation time in days}}$$

Table 6.1: Classification of anaemia according to reticulocyte production index (reticulocyte response)

Reticulocyte response	Reticulocyte production index (absolute reticulocyte count)	Causes
1. Appropriate for the degree of anaemia	≥2% (>100,000/µl)	Blood loss, haemolytic anaemias, i.e. hyperproliferative anaemias
2. Inappropriately low for the degree of anaemia	<2% (<75,000/µl)	Hypoproliferative anaemias like iron deficiency anaemias, megaloblastic anaemia, thalassaemia, anaemia of chronic disease, aplastic anaemia, sideroblastic are myelodysplasia, endocrine diseases

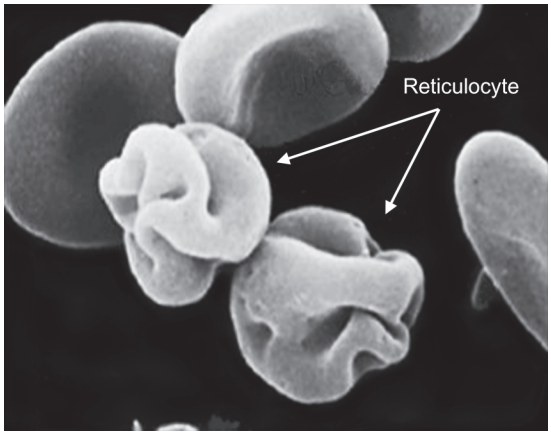


Fig. 6.4: Scanning electron micrograph shows normal reticulocyte shapes; the lobular membrane of the reticulocyte is shown by the arrows

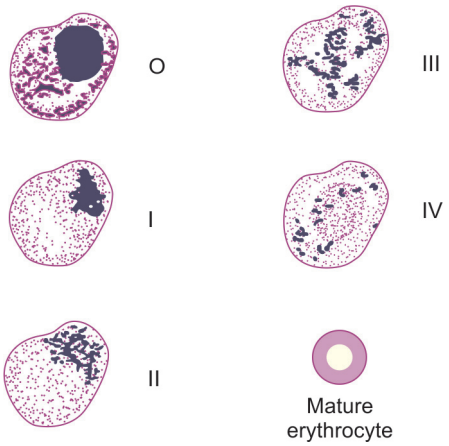


Fig. 6.5: Maturation stages of reticulocytes according to Heilmeyer classification

Group 0: Nucleated erythrocyte (orthochromatic normoblast), stained strongly for reticuling and the nucleus. This cell type is not included in the reticulocyte count.

Group I: Non-nucleated red cells, appearing with a densely clumped reticulum; they comprise 0.1% of the population of reticulocytes in normal individuals.

Group II: An extended network of loose reticulum; they comprise 0.7% of the reticulocyte population in normal individuals.

Group III: Scattered granules with residual reticulum network; they comprise 32% of the reticulocyte population in normal individuals.

Group IV: Scattered granules; they comprise 61% of the reticulocytes in normal individuals.

Maturation time in days according to PCV are:

- PCV >35%: 1 day
- PCV 25–35%: 1.5 day
- PCV 15–25%: 2 days
- PCV 5–15%: 2.5 days

AUtomated Method

Reticulocyte count can also be performed in automated cell counter or by a flow cytometer. It is superior and more accurate compared to manual method. But, Heinz bodies, Pappenheimer bodies and giant

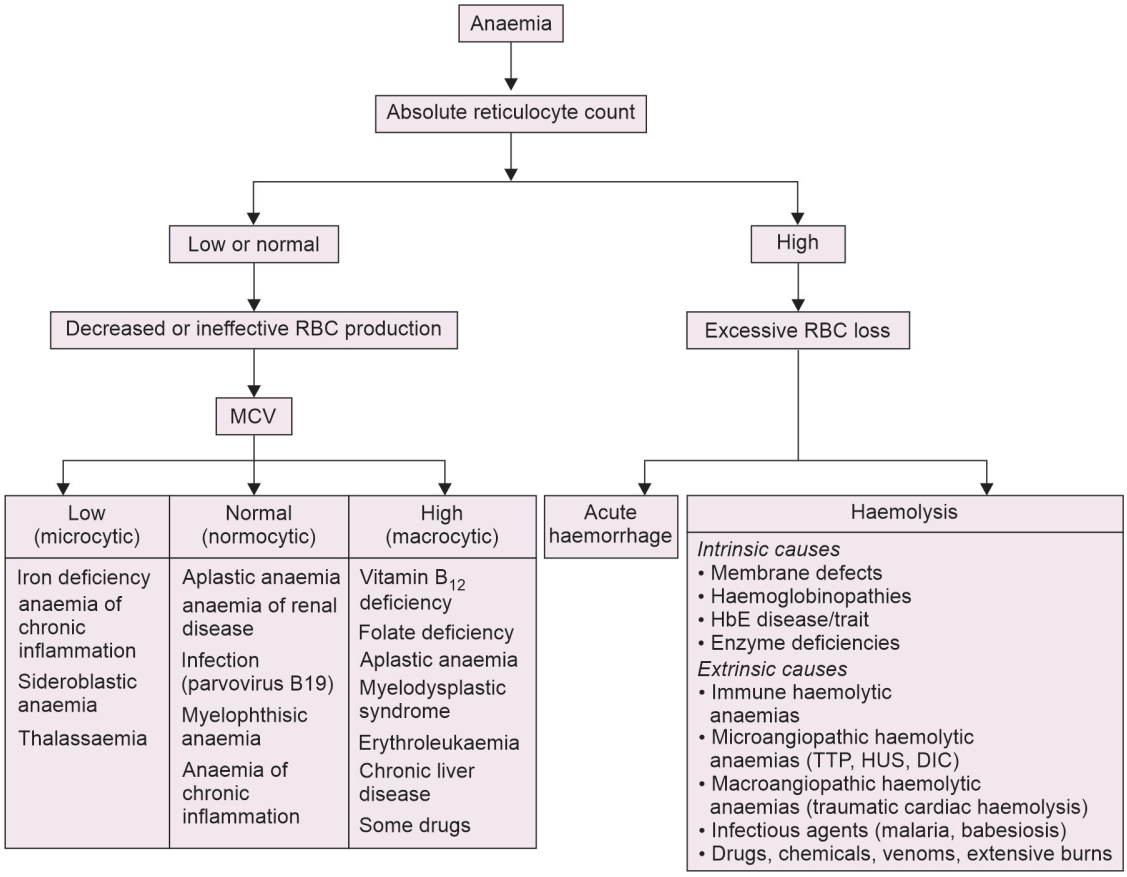


Fig. 6.6: Diagnostic use of absolute reticulocyte count

platelets if present are also counted as reticulocytes (hence give erroneous result).

Available automated reticulocyte analyzers include flow cytometry systems such as the FACS system from Becton Dickinson or the Coulter EPICS system; the Sysmex R-3500, R-500, XE-2100, XE–5000, and XN series system; the CELL–DYN 3500 R, 3700 and 4000 systems; the Coulter LH 750 systems and the Unicel DxH800; and the Siemens ADVIA 2120, 2120i and 120. All these analyzers evaluate reticulocytes based on optical scatter or fluorescence after the RBCs are treated with fluorescent dyes or nucleic acid stains to stain residual RNA in reticulocytes.

The Sysmex R-3000/3500 reticulocyte analyzer (stand-alone) which uses auramine O, a supravital fluorescent dye, and measures forward scatter and side fluorescence as the cells, in a sheath-stream, pass through a flow cell by an argon laser.

The CELL–DYN 3500 R performs reticulocyte analysis by measuring 10° and 90° scatters in the optical channel (MAPSS technology). The RBCs are stained with the thiazine dye new methylene blue N in an off-line sample preparation before the specimen is introduced to the instrument.

Beckman Coulter (LH700 and UniCel D × H800) uses a new methylene blue stain and the VCS technology. Volume is plotted against light scatter and against conductivity, which correlates with opacity of the RBC.

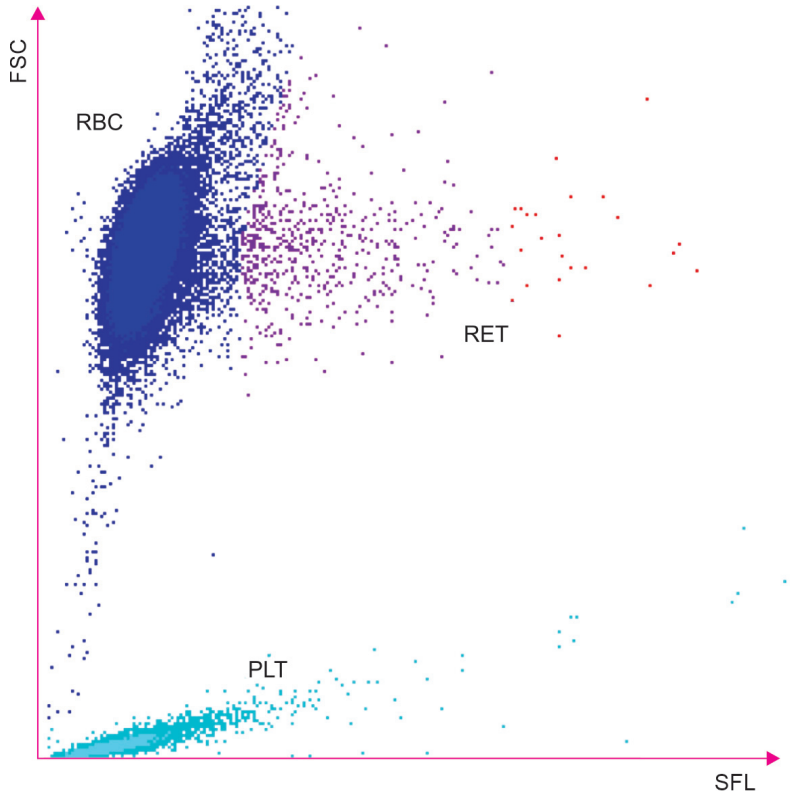


Fig. 6.7: Automated method of reticulocyte counting (scatterogram); SFL: Side fluorescent, FSC: Forward scattered light

Table 6.2: Causes of increased and decreased reticulocyte count

Increased count (reticulocytosis)	Decreased count (reticulocytopenia)
• Acute blood loss (haemorrhage) • Haemolytic anaemia • Haemolytic crisis • Response to specific therapy in nutritional anaemias (Following treatment in iron/vitamin B_{12}/folic acid. Highest counts are found on 6th /7th day of treatment and indicate response to haematinics)	Due to deficient RBC production • Aplastic anaemia • Iron deficiency anaemia • Anaemia of chronic disease • Anaemia due to marrow infiltration or myelophthisic anaemia (metastatic cancer, leukaemia, lymphoma) • Pure red cell aplasia • Myelofibrosis • Fanconi anaemia • Ineffective erythropoiesis • Megaloblastic anaemia
Criteria • Reticulocyte count >1.5% • Reticulocyte index or corrected reticulocyte count >3%	**Criteria** • Reticulocyte count <0.5% • Reticulocyte index <1%

- **Absolute reticulocyte count (ARC):** It is the number of reticulocytes present in 1 mm^3 of blood.
  ARC = (reticulocyte %) × RBC count/100.
- **Reticulocyte index (RI):** This index adjust reticulocyte count for haematocrit. It reflects bone marrow activity and is also regarded as "Poor man's bone marrow aspirate". Normal reticulocyte index is 1–3%.
  RI = Reticulocyte count × (Hb/age and sex adjusted normal Hb)

MATURATION OF RETICULOCYTES

Identified by their morphological features (Figs 6.4, 6.5 and 6.8):

1. Most immature reticulocytes: Large clumps of reticulin
2. Most mature reticulocytes: Few granules of reticulin

Maturation stage	Morphological descriptions
Stage I:	Reticulin consists of dense clots
Stage II:	Loosely arranged reticulum
Stage III:	Diffusely arranged reticulum
Stage IV:	Some scattered granules

AUTOMATED RETICULOCYTE COUNT (Fig. 6.9)

Method of detection: Fluorescence (forward light scatter and side fluorescent scatter)

Reagent used

i. Diluent: Tricine buffer
ii. Dye: Polymethine dye with methanol in ethylene glycol.

Principle: Nucleic acids remaining in immature erythrocytes are stained with a fluorescent dye RET search (II). Reticulocytes are

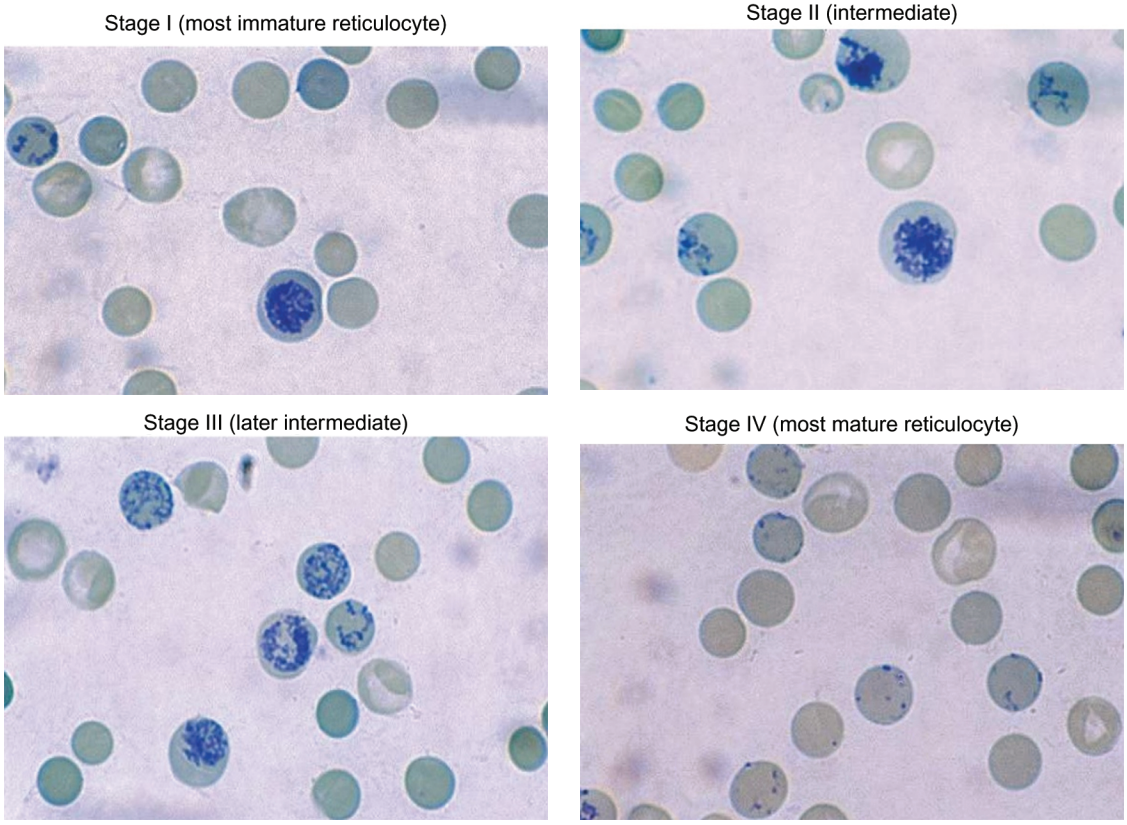


Fig. 6.8: Four stages (stages I, II, III and IV) of reticulocyte maturation.

measured based on the principle of flow cytometry. The fluorescence-stained reticulocytes are divided into 3 fractions by the intensity of fluorescence (Table 6.3).

Table 6.3: Different reticulocytes based on fluorescence

HFR	MFR	LFR
High fluorescent reticulocyte	Medium fluorescence reticulocytes	Low fluorescent reticulocyte
Little RNA	More RNA	High level of RNA
Mature reticulocytes	Semi-mature reticulocytes	Immature reticulocytes
Reference range: 86.5–98.5%	Reference range:1.5–11.3%	Reference range: 0–14%

Immature

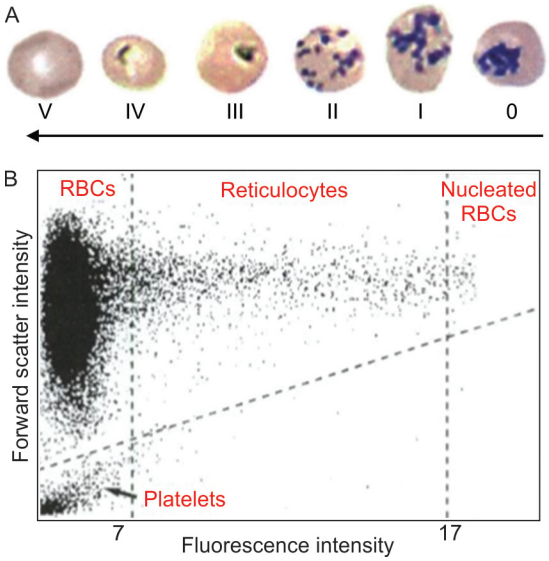


Fig. 6.9: Correlations of morphologic and flow cytometric reticulocyte counting techniques are indicated by vertical orientation of the three panels. A. Heilmeyer classification of erythropoietic cells. Maturation proceeds from right to left. B. Characteristic of flow cytometric scattergram demonstrates 200 × 103/jxL (6%) reticulocyte count and 0.33 immature reticulocyte fraction

IMMATURE RETICULOCYTE FRACTION (IRF)

IRF is the sum of MFR and HFR, i.e. IRF = MFR + HFR

Reference Range

Male: 1.5–13.7%

Female: 1.1–15.9%

Indications of Measuring IRF

- The IRF value is an early marker for evaluating the regeneration of erythropoiesis.
- The IRF percentage increases after only a few hours, the reticulocyte count increases after 2–3 days.
- If the IRF value does not increase during the treatment of deficiency anaemias with erythropoietin or vitamins, this indicates a lack of response to therapy (Fig. 6.10).

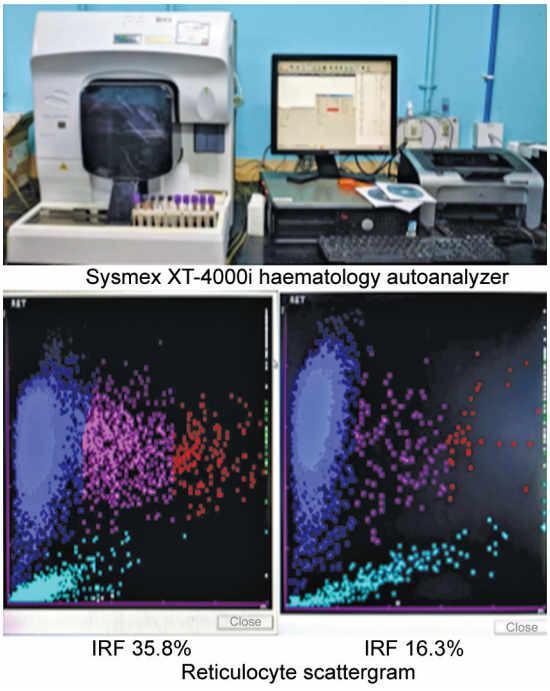


Fig. 6.10: Reticulocyte scattergram shows immature reticulocyte fraction or IRF

SOURCES OF ERROR DURING RETICULOCYTE COUNT

1. A refractile appearance of erythrocytes should not be confused with reticulocytes.
2. Filtration of the stain is necessary when precipitated material is present which can resemble a reticulocyte.
3. Erythrocyte inclusions should not be mistaken for reticulocytes.
   a. Howell-Jolly bodies appear as one or sometimes two, deep purple dense structures.
   b. Heinz bodies stain a light blue-green and are usually present at the edge of the erythrocyte.
   c. Pappenheimer bodies are more often confused with reticulocytes and are the most difficult to distinguish. These purple staining iron deposits generally appear as several granules in a small cluster. If Pappenheimer bodies are suspected, stain with Wright-Giemsa to verify their presence. Haemoglobin H inclusions will appear as multiple small dots in every cell.
4. Falsely decreased reticulocyte counts can result from under staining the blood with new methylene blue. Be sure the stain/blood mixture incubates the full 10 minutes.
5. High glucose levels can cause reticulocytes to stain poorly.
6. There is high degree of inaccuracy in the manual reticulocyte count owing to error (± 2%) in low counts and (± 7%) in high counts, and a lack of reproducibility because of the inaccuracy of the blood film. This inaccuracy has been overcome by the use of **automated instruments** using a **flow cytometry.**
7. If no reticulocytes are observed after scanning at least two slides, report "none seen".
8. Recent blood transfusion can interfere with accurate count.
9. Mishandling, contamination, or inadequate refrigeration of the sample can interfere and cause inaccurate test results.

Immature Reticulocyte Fraction

Definition: It is calculated as a ratio of immature reticulocytes to the total number of reticulocytes. It is the sum of the fraction of high-fluorescence intensity regions plus the fraction of middle-fluorescence intensity regions.

In healthy individuals, reticulocyte circulate in the peripheral blood for 1–2 days after being released from the bone marrow, before they lose sufficient RNA to become red blood cells. During periods of increased erythropoietic demand, reticulocyte life span in peripheral blood increases to 3 or more days, as a result of premature release of immature or "stress" reticulocytes from the bone marrow. The recent developmet of automated (rather than manual) reticulocyte counts has permitted more precise counting of reticulocytes, as well as an objective measure of reticulocyte maturity, based on their RNA content. It is now possible to precisely quantitate the proportion of all reticulocytes that is immature. This is termed **" immature reticulocyte fraction" or IRF** (Fig. 6.11). This parameter provides a very early and sensitive index of marrow erythropoietic activity.

The IRF result is always reported together with the reticulocyte count (both percentage and absolute value). The reference range is 0.11–0.38. Specimen requirement is one 5 ml EDTA (lavender-top) tube of blood. IRF can

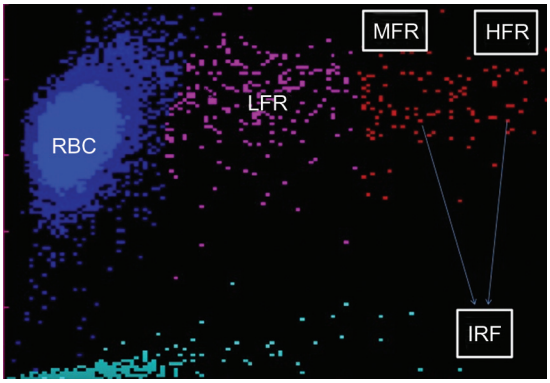


Fig. 6.11: IRF (immature reticulocyte fraction) in reticulocyte scattergram

be measured on the same tube used for the reticulocyte count.

Immature reticulocyte fraction (IRF): Measured as a ratio of immature reticulocytes to the total amount of reticulocytes. It was first used to determine the regenerative activity of the bone marrow during and after chemotherapy for leukaemia.

Reticulocyte haemoglobin content (CHr): It measures the amount of the iron available in the bone marrow that can be used by the reticulocyte for the production of haemoglobin. Multiple studies have shown a Chr of < 28 pg is a good predictor for iron deficiency anaemia.

Clinical Use of IRF (Tables 6.4 and 6.5)

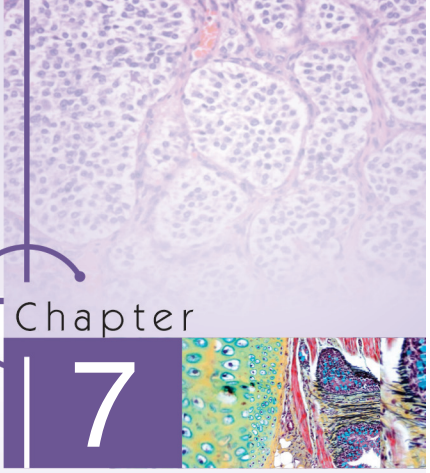
- Diagnostic applications: Aplastic anaemia and crisis, chronic renal disease, haemolytic anaemia.
- Bone marrow hypoplasia or aplasia: Primary or secondary to chemotherapy or radiation ablation.
- Myelodysplasia
- Therapeutic monitoring: Post-chemotherapy recovery, response to anaemia therapy (erythropoietin, iron, vitamin B_{12}).
- Transplant engraftment: Bone marrow, kidney, stem cell.

Table 6.4: Reticulocyte count and IRF (immature reticulocyte fraction) in different haematological diseases

Clinical condition	Reticulocyte count	IRF (immature reticulocyte fraction)
• Aplastic anaemia/crisis	Low	Low
• Hypoplastic anaemia	Low	Low
• Bone marrow regeneration	Low	High/within normal limits (WNL)
• Chronic disease	Low/WNL	WNL
• Iron deficiency	Low/WNL	High
• Thalassaemia	WNL/high	WNL/high
• Folate/Vit B_{12} deficiency	Low/WNL	High
• Myelodysplasia	Any level	WNL/high
• Haemolytic anaemia	High	High
• Blood loss/ anorexia	WNL/high	High

Table 6.5: IRF (immature reticulocyte fraction) in different categories and its diagnostic use

Category	Criteria	Causes
Category 1	Reticulocyte count: Low, IRF: Zero	Aplastic anaemia
Category 2	Reticulocyte count: Normal, IRF: Marginally high	Megaloblastic anaemia, MDS (myelodysplastic syndrome)
Category 3	Reticulocyte count: Normal to low, IRF: High	Early recovery from chemotherapy, megaloblastic anaemia
Category 4	Reticulocyte count: Normal to high, IRF: Zero	Malignancy, hypersplenism, autoimmune haemolytic anaemia
Category 5	Reticulocyte count: Very low, IRF: Low	Parvovirus B_{12} infection



Chapter 7

Haemoglobin Estimation

INTRODUCTION

Hemoglobin (American) or haemoglobin (British); abbreviated as Hb or Hgb, is the iron-containing oxygen-transporting **metalloprotein** 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 O_2 per gram. The mammalian Hb can bind up to four oxygen molecules. Hb also transports other gases like carbon dioxide (CO_2) in respiratory system, some of which form carbaminohaemoglobin in which CO_2 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 methaemoglobinaemia. 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.

Sulfhaemoglobin (SHb)

Sulfhaemoglobin 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 sulfhaemoglobin (SHb). Further oxidation of this sulfhaemoglobin will result in the denaturation and precipitation of haemoglobin known as **Heinz bodies**. Unlike methaemoglobin, sulfhaemoglobin cannot be reduced back to haemoglobin (irreversible) and it remains within the cells until they break down.

Sulfhaemoglobin (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% sulfhaemoglobin. Sulfhaemoglobinaemia 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 fomed due to acute carbon monoxide posisoning. 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 polycythaemia 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. 7.1). 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.

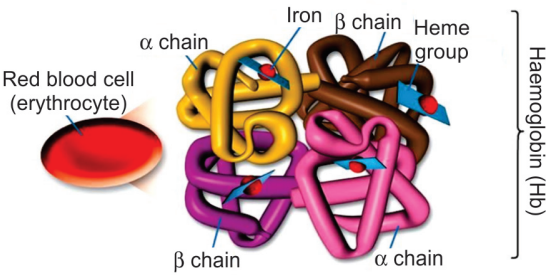


Fig. 7.1: Structure of haemoglobin

In the first three months of embryonic development, when blood cells are produced in the yolk sac, embryonic haemoglobins such as Hb Gower 1 ($\zeta_2 \varepsilon_2$), Hb Gower 2 ($\alpha_2 \varepsilon_2$) and Hb Portland ($\zeta_2 \gamma_2$) are produced. As erythropoiesis shifts to the liver and spleen, the foetal haemogloblin, (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 HbA_2 ($\alpha_2 \delta_2$) begin to be produced.

In the embryo

- Gower 1 ($\zeta_2 \varepsilon_2$)
- Gower 2 ($\alpha_2 \varepsilon_2$)
- 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 A_2 ($\alpha_2 \delta_2$)**: 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 sicke 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 HbA_2.
- **β-Thalassaemia minor:** Moderate to high rise of HbA_2, 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, HbA_2 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 (HbO_2) 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,

Table 7.1: Different methods of haemoglobin estimation and principle of the method

Method	Principle of the method
1. Colorimetric 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
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	Specific gravity
3. Gasometric method	Oxygen combining capacity of haemoglobin
4. Chemical method	Iron content of the haemoglobin

other methods are being replaced in modern laboratories as it is accurate, reliable and fast.

Acid Haematin or Sahli's Method (Fig. 7.2)

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.

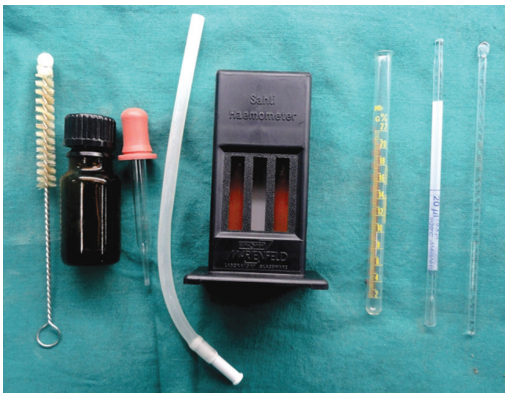


Fig. 7.2: Reagents and equipment for Sahli's method

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 gm 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 piptte 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 gm/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 anticoagulant (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**

(sulfhaemoglobin 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 gm
- 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 µl) 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 × **concentration standard (mg/100 ml) × 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 sulfhaemoglobin)
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 (KiCN) 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 (KiCN), lauryl sulphate has been proposed as it has similar properties to HiCN.
- Dilution factor is 200 if 20 µl blood is mixed with 4 ml of Drabkin's solution (i.e. 1:200). But when 20 µl 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 (HbO_2) 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 sulfhaemoglobin.
2. **Alkaline haematin method:** There are two methods which follows 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 sulfhaemoglobin is present. Plasma proteins and lipids have a little effects on the development of colour. Fetal haemoglobin (HbF) and Bart haemoglobin (g_4) cannot be estimated by this method as they are resistant to alkali denaturation normally. But this problem can be overcomed 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 sulfhaemoglobin; 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. 7.3)**:** 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.

- Oxygen carrying capacity measured by van Slyke apparatus
- Based on formula, 1 gm of Hb carries 1.34 ml of oxygen
  It does not measure carboxyhaemoglobin
- Sulfhaemoglobin
- Methaemoglobin
- Time-consuming and expensive
- Result is 2% less than other methods

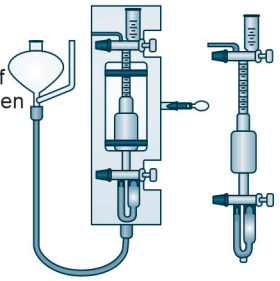


Fig. 7.3: Gasometric method

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
- Chidren: 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 B_{12}, 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 gm/dl and in women is 16 gm/dl or haematocrit >49% in men and >48% in women).

Limitations of Accurate Hb Estimation

(Table 7.2)

- 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 7.2: 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 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 7.2: 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. 7.4)

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).

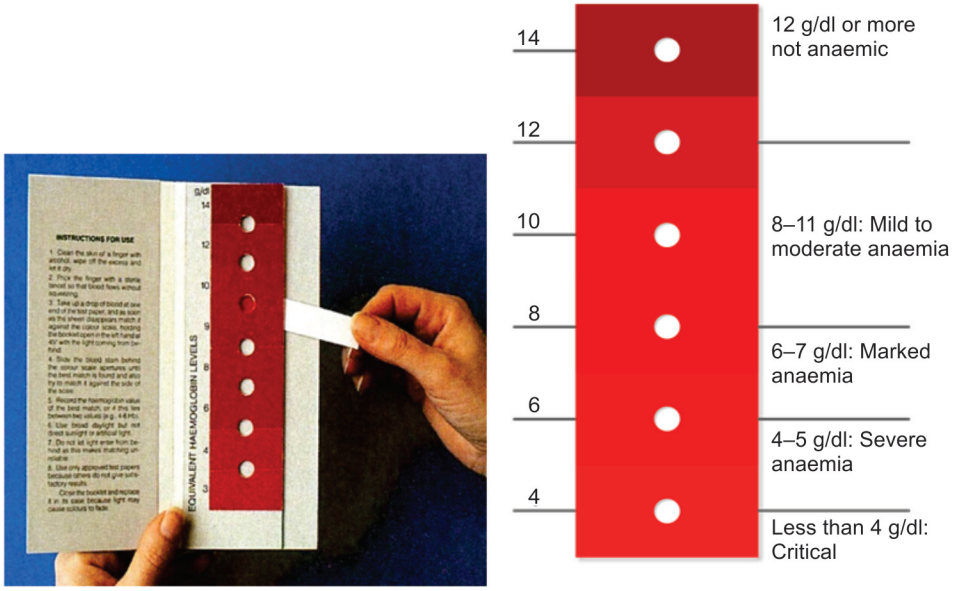


Fig. 7.4: Haemoglobin colour scale (HCS)

VIVA VOCE

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:

i. 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.

ii. Almost all haemoglobin except sulfhaemoglobin is converted to cyanomethaemoglobin. So, this method can calculate almost all haemoglobins.

iii. This is a colorimetric method, so personal error like matching colour as in Sahli's method is absent.

iv. A stable reference standard is available.

v. 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:

i. For RBC count: 4 ml RBC fluid + 0.02 ml blood (dilution 1:200).

ii. 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).

iii. For platelet count: 0.4 ml platelet count fluid + 0.02 ml blood (dilution 1:20).

iv. For eosinophil count: 0.2 ml of Dunger's fluid + 0.02 ml blood (dilutions 1:20).

v. 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 gm% Hb. Test sample is compared against this standard sample.

Q4. In automated cell counter, what chemical regent 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: i. 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.

ii. Hb raises as increase with altitude.

iii. Hb value highest in the morning and lowest in the evening.

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

Ans: i. When blood is drawn for Hb estimation during intravenous blood transfusion or iron containing drugs.

ii. 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:
i. In 3rd trimester of pregnancy, due to increase in plasma volume, Hb concentration falls (1–2 gm/dl).
ii. Splenomegaly due to pooling of red cells in spleen.
iii. Congestive heart failure due to fluid retention.
iv. Multiple myeloma/paraproteinaemia.
v. 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.

Chapter 8

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 protrudes 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/mm^3). When platelet counts are low, expected bleeding time can be predicted with the following formula:

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

  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/mm^3):** It may be primary (essential) or secondary. When the platelet count is <50,000/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 subendothelium as there is absence/defective von Willebrand factor.
4. **Vascular abnormalities:** As found in Ehlers-Danlos syndrome, or in pseudoxanthoma elasticum.
5. **Deficiency of clotting factors:** Occasionally sever deficiency or 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. 8.1) 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

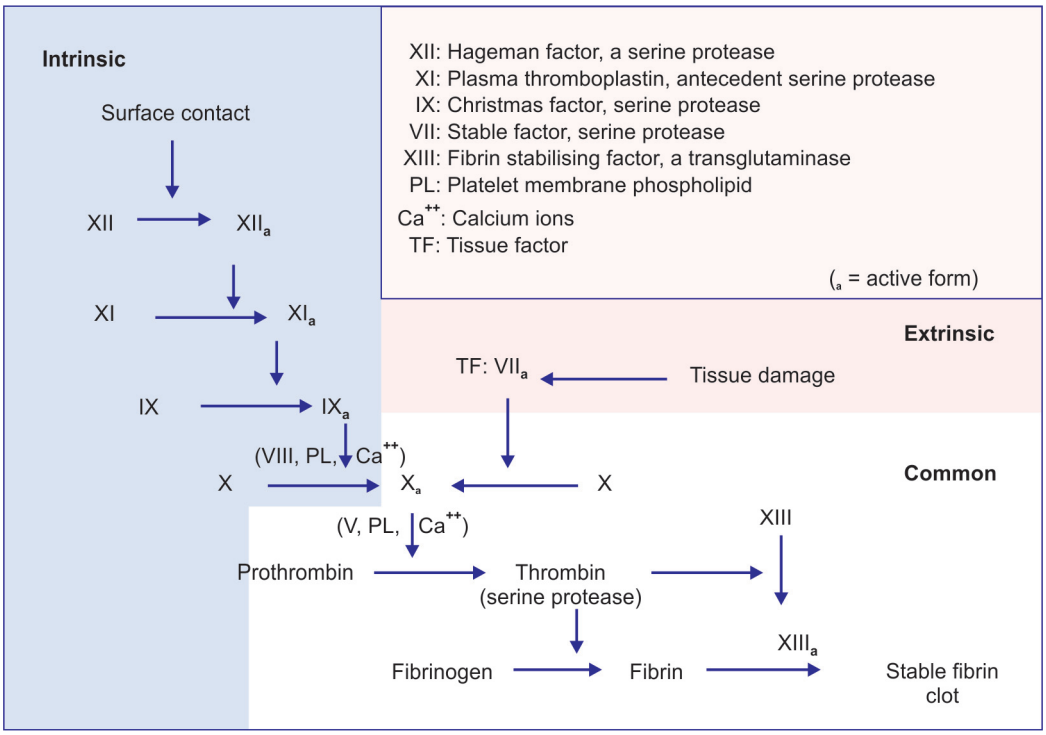


Fig. 8.1: The three pathways that makeup the classical blood coagulation pathway

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. 8.2)

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

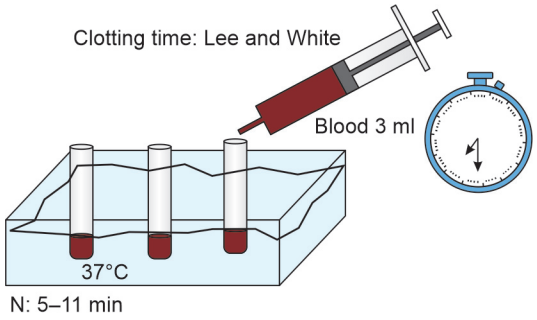


Fig. 8.2: Lee and White method of clotting time

Note

i. Rise of temperature will speed up the coagulation process, so the test should be done at a particular temperature.

ii. 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.

iii. 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

1. **Haemophilia A** (deficiency of factor VIII. and **haemophilia B** or Christmas disease (deficiency of factor IX).
2. **Anticoagulant therapy** (hyperheparinemia or warfarin therapy).
3. **Hypoprothrombinaemia:** Seen in cirrhosis of liver, obstructive jaundice, vit K deficiency, malignancy of liver.
4. **Fibrinogen deficiency:** When fibrinogen level falls below 50 mg/dl in blood.
5. **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. 8.3).

Reagents

i. **Patients control plasma samples**: Patients control plasma samples (preferably platelet poor) are prepared from whole blood and citrate anticoagulant 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.

ii. **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.

iii. **Calcium chloride ($CaCl_2$):** 0.025 mol/litre. The test reagent is prepared fresh before use.

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. 8.3)

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)

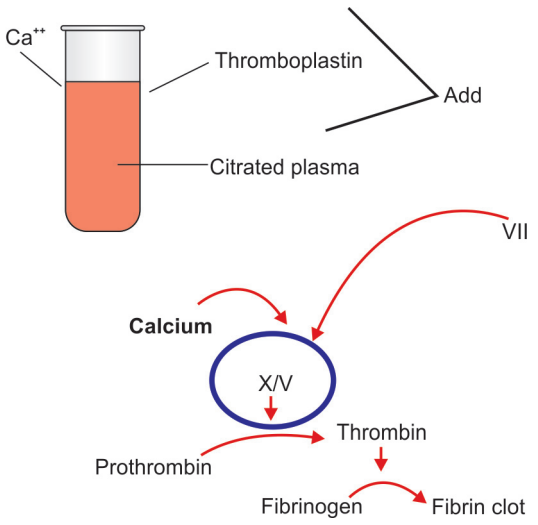


Fig. 8.3: Schematic diagram of prothrombin time (PT)

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:

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 × 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 ($CaCl_2$):** 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 $CaCl_2$ 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.

Normal range: 30–40 seconds.

Causes of Prolonged APTT (Fig. 8.4)

- **Inherited deficiency** of factor VIII and factor IX. Also prolonged in inherited deficiencies of other coagulation factors in intrinsic pathway and common pathway.

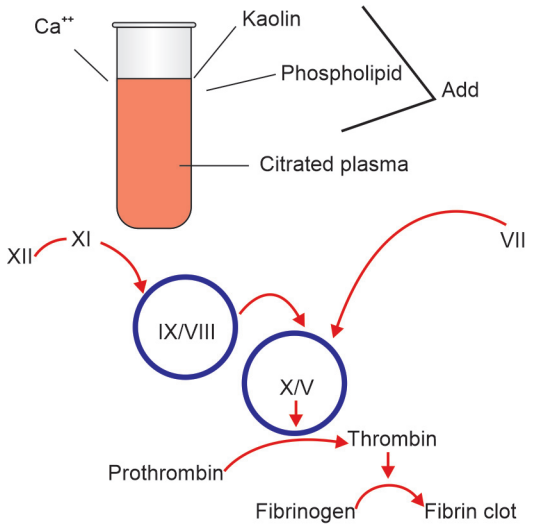


Fig. 8.4: Schematic diagram of activated partial thromboplastin time (APTT)

- **Circulating inhibitors**: Inhibitors may be of two types—specific and nonspecific. **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

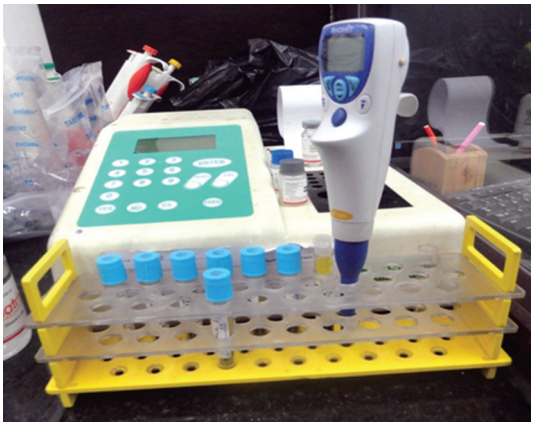


Fig. 8.5: Coagulometer (coagulation analyzer) to determine PT and APTT

Points to Remember (Fig. 8.6)

- **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 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 cause 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.

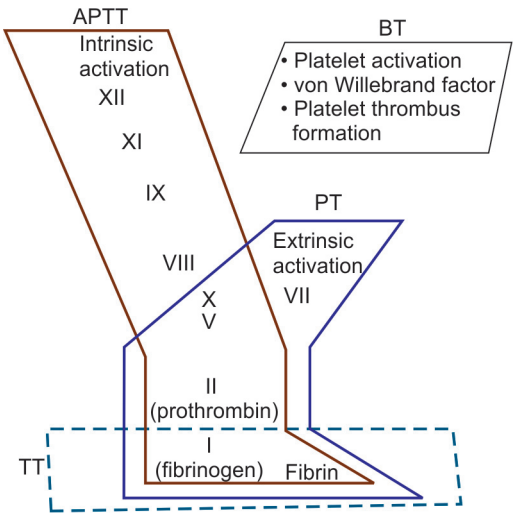


Fig. 8.6: Basic test for haemostasis
BT: Bleeding time, APTT: Activated partial thromboplastin time, PT: Prothrombin time, TT: Thrombin time

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:
i. 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).
ii. Deficiency of von Willebrand's factor (vWF) in von Willebrand's disease (also prolonged bleeding time).
iii. 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:
i. Congenital deficiency of factor VII, which is very rare.
ii. 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 APPTT).

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 aqequate 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 prolongation 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) × (100 – Haematocrit) × 0.00185

Chapter 9

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 A_1 and A_2. The much rarer A subgroups are A_3, A_x and A_m. But the A_1 and A_2 subgroups are important only. So, ABO system increases to six: A_1, A_2, B, A_1B, A_2B and O. About 80% of blood group A and blood group AB belong to A_1 or A_1B respectively. ABO antigens are found predominantly on erythrocyte membrane protein band 3 and 4.5, membrane glycophorin 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. 9.1, Tables 9.1 and 9.6). 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,

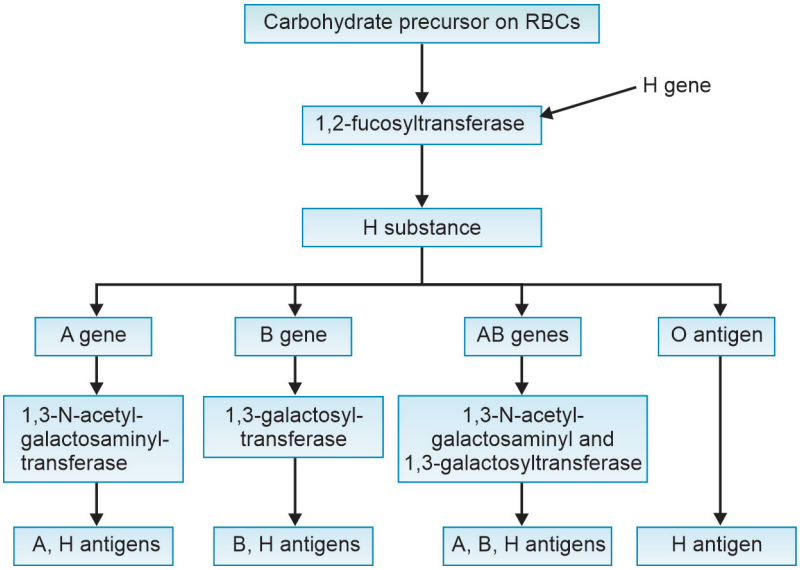


Fig. 9.1: Formation of A, B and H antigens on RBCs

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.

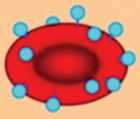
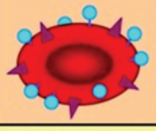
Differences between A_1 and A_2 Subgroups of Blood Group A and AB

Subgroups of A are distinguished by their lack of agglutination with anti-A_1 lectin prepared from *Dolichos biflorus* or with anti-A_1 reagent derived from serum of group O or B persons that has been absorbed with A_2 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 *Dolichos biflorus* (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 9.1: 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 A_1) 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.

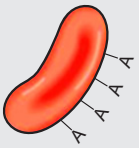
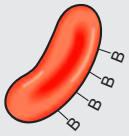
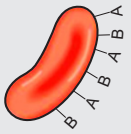
Preparation of 2% red cell suspension

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,

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		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
	Antigens A and B	Makes neither anti-A nor anti-B		
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

	Donor							
Recipient	**O–**	**O+**	**A–**	**A+**	**B–**	**B+**	**AB–**	**AB+**
O–	✓	×	×	×	×	×	×	×
O+	✓	✓	×	×	×	×	×	×
A–	✓	×	✓	×	×	×	×	×
A+	✓	✓	✓	✓	×	×	×	×
B–	✓	×	×	×	✓	×	×	×
B+	✓	✓	×	×	✓	✓	×	×
AB–	✓	×	✓	×	✓	×	✓	×
AB+	✓	✓	✓	✓	✓	✓	✓	✓

in small 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. 9.2A to D and Fig. 9.4)

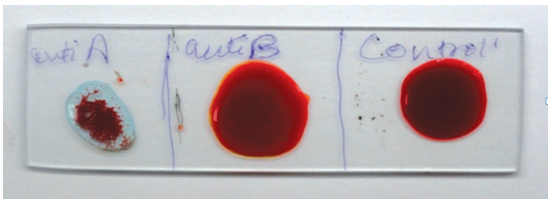
1. Take a clean glass slide. Mark anti-A on left side corner and anti-B on middle and control on right side corner of that slide.
2. Two drops of blood or 20–40% red cell suspension are placed by a Pasteur pipette at each three demarcated areas.
3. On the left side a drop of anti-serum, on the middle 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:

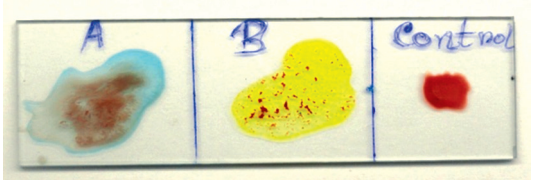
i. Blood group A: Agglutination with anti-A serum (left corner) but not with anti-B serum (middle).
ii. Blood group B: Agglutination with anti-B (middle) but not with anti-A (left corner).
iii. Blood group AB: Agglutination on both left and middle portions.
iv. Blood group O: No agglutination on left 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.

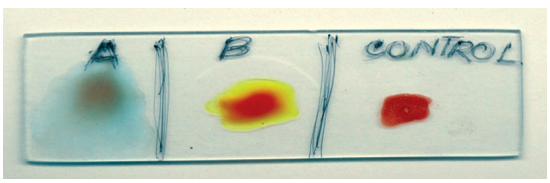
A



C



B



D

Fig. 9.2A 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. 9.3A and B, and Table 9.2)

i. 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.
ii. Put 1 drop of 2–5% of red cell suspension to each tube.
iii. Mix the suspension by tapping the tubes and leave them undisturbed for 15–30 minutes. Check agglutination.

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.

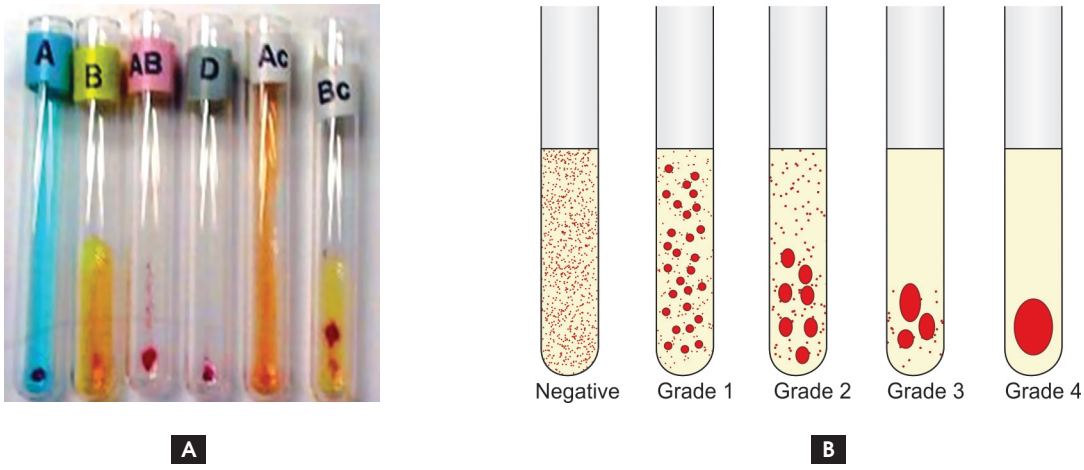


Fig. 9.3A and B: (A) Test tube methods for blood grouping; (B) Scoring system (grades 1 to 4) in test tube method of blood grouping

Table 9.2: 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 9.3). 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, A_1 subtype is commonly used. A_2 cells should be used if there is suspected presence of anti-A_1.

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-P_1 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 9.3: 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 i*n 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 antiserum 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 Rh_o, rh′, rh″hr′, hr″. While Fisher postulated that Rh antigens (C, c, D, d, E, e) are determined by three pairs of closed linked allelomorphic genes which are located on chromosome 1 (Table 9.4). 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 9.5).

Table 9.4: 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 9.5: 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. 9.4)

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

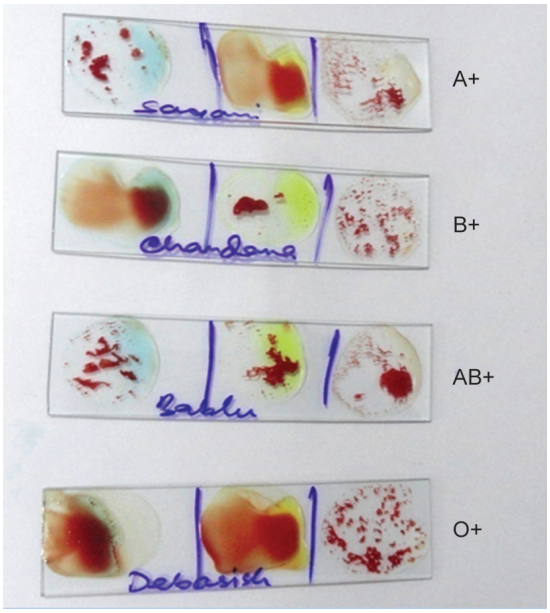


Fig. 9.4: Three compartments. First drop of blood on left side mixed with anti-A serum, middle drop of blood mixed with anti-B serum and last drop of blood on right side mixed with anti-D (Rh) serum. All are Rh group positive with ABO blood grouping of A, B, AB and O respectively

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 9.6).

Table 9.6: 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 9.5 and 9.6). 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

i. Blood grouping (ABO and Rh typing) and cross-matching
ii. Coombs' antiglobulin test
iii. For diagnosis of sickle cell anaemia, PNH (paroxysmal nocturnal haemoglobinuria)
iv. For diagnosis of different infective organisms (diphtheria, syphilis, measles, parvovirus, etc.)
v. Antibody identification

Advantages of gel card method

i. This method is easy, accurate, standardized.

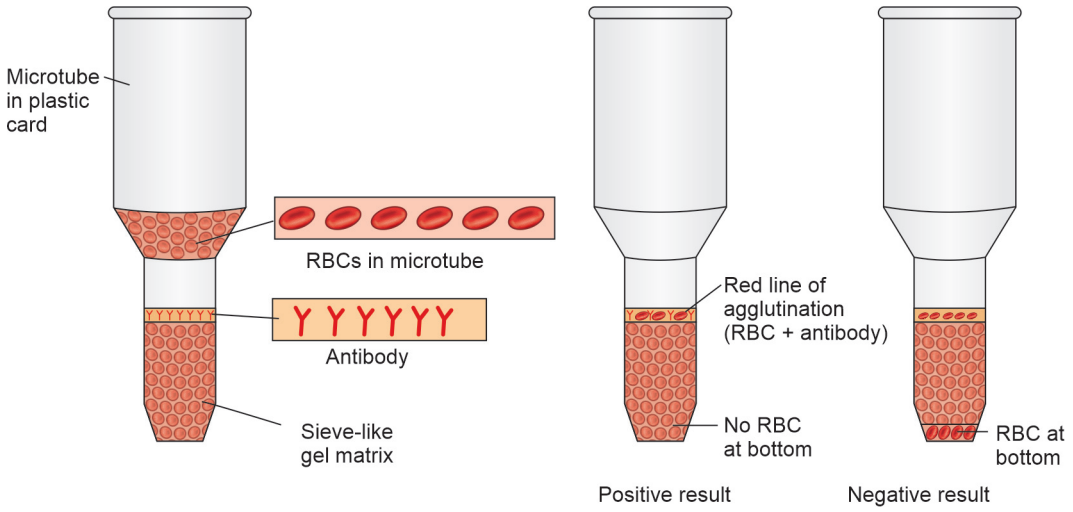


Fig. 9.5: Gel card method of blood grouping (column agglutination or microtyping system)

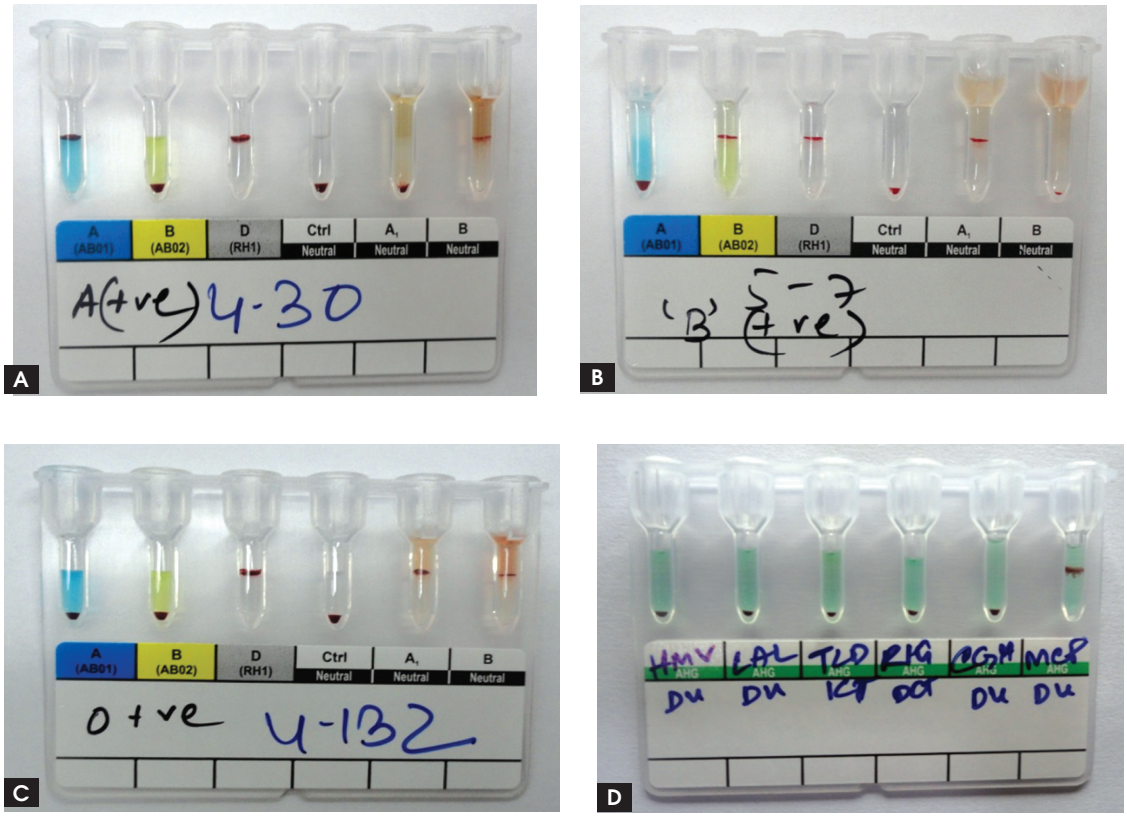


Fig. 9.6A to D: (A to C) showing ABO blood grouping in gel card method. (A) Blood group A+; (B) Blood group B+, (C) Blood group O+. There are six tubes. First one contains anti-A serum, second one contains anti-B serum, third tube contains anti-D serum, fourth tube is control, fifth tube contains antigen A_1, 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).

Table 9.7: 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 is leukaemia or cancer	i. Polyagglutinable RBCs ii. Acquired B antigens, seen in gastric or colon cancer, intestinal obstruction iii. Foetomaternal haemorhage iv. Wrong (out-of-group) transfusion v. RBCs coated by Wharton's jelly of umbilical cord vi. Bone marrow transplantation

Table 9.8: 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

VIVA VOCE

Q1. What is Bombay blood group?

Ans: Both antigens A and B or 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—A_1 and A_2 in blood group A. A_1 subgroup is 80% and A_2 subgroup is 20% based on the presence of A_1 antigen or A_2 antigen on RBCs. A_1 has greater number of antigenic site than A_2 (10^6 site vs. 250,000 site). A potent anti-A_1 serum (anti-A_1) can agglutinate A_1 red cells but not A_2 red cells. There is no specific anti-A_2 serum. So, A_2 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 A_1 blood cells and A_1B blood cells.

Saline extract of seeds of *Ulex europaeus* is used to agglutinate A_2, A_2B 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 A_2 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*.

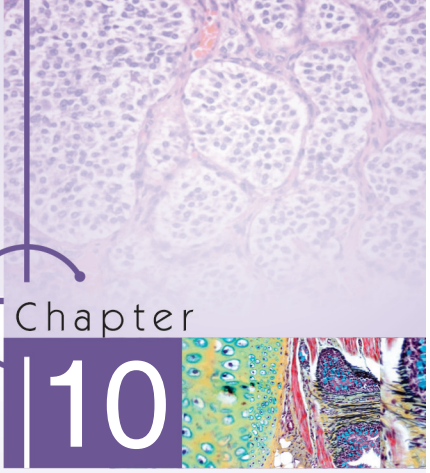
Q13. How different blood groups are associated with disease?

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.



Chapter 10

Normal and Abnormal Blood Cell Morphology

Blood smear examination is an essential part of haematological investigation.

After selecting a suitable area, a high power (40X) objective or 60X oil immersion objective is used first. A much better appreciation of variation of RBC size, shape and staining can be visualized than with 100X oil immersion lens. Oil immersion (100X) with preferable 6X eyepiece (or alternatively 10X eyepiece) is reserved for final examination of unusual cells and to look for fine details such as cytoplasmic granules, punctate basophilia, etc.

Blood Stains

For staining blood smears usually, aniline dyes are used which are of two general classes: Basic dyes such as methylene blue, azure B and acid dyes, such as eosin.

Acid substances stain with basic stain (methylene blue, azure B) and are called **basophilic,** e.g. nuclei and nucleic acids. Basic substances are stained with acid stains and are called **acidophilic,** e.g. haemoglobin stains which are composed of both acid and basic dyes are called Romanowsky stains which make subtle distinctions in staining of cell and staining of the granules differentially.

- Neutrophilic granules in cytoplasm: stained by azure complexes
- *Eosinophilic granules:* Stained by acid dye (eosin)
- *Basophil granules:* Stained by basic dye as it contains acid heparin.

Structures which are stained by a combination of the two are called **neutrophilic.**

The thiazine's basic components consist of methylene blue (tetramethylthionine) and, in varying proportions, its analogues produced by oxidative demethylation: Azure B (trimethylthionine); Azure A (asymmetric dimethylthionine); Symmetric dimethylthionine ; and Azure C (monomethylthionine). The acidic compoent, eosin is derived from a xanthene skeleton.

Most Romanowsky stains are dissolved in methyl alcohol and combine fixation as well as staining. Common stains are Wright's stain, Giemsa stain and Leishman stain.

Staining Problems

- **Excessive blue stain:** Thick blood smears, prolonged staining time, inadequate washing on too high an affinity of stain or diluent tends to cause excessive basophilia or blue staining.

  In this condition, the RBCs appear blue or green, the nuclear chromatin is deep blue to black, and the granules of the neutrophils are deeply overstrained and become large and prominent. Eosinophil granules are blue or gray.

Solution to this problem: Staining the blood smears for less time or using less stain and more diluent may correct this. If not corrected then the buffer is very alkaline. A new buffer with lower pH should be prepared.

- **Excessive pink stain:** Insufficient staining, prolonged washing time, mounting the coverslips before the smears are dry, or using stain or buffer which has highly acidic pH (low pH).

  In this condition, the RBCs are bright red or orange, the nuclear chromatin is pale blue, and the granules of eosinophils are bright red.

  Solution to this problem: The problem may be due to low pH of the buffer or it may be due to transformation of methyl alcohol, present in stain to formic acid as a result of oxidation on standing. In the first case, change the buffer. In the next case prepare fresh stain with methyl alcohol.
- **Other staining problems:** Inadequately stained RBCs, nuclei, or eosinophilic granules may be due to understanding or excessive washing. Prolonging the staining or reducing the washing may solve this problem.

  Precipitate on the films (blood smears): Precipitate may be due to unclean slides; drying during the period of staining; inadequate washing of the slide at the end of staining period (especially failure to hold the slide horizontally during initial washing), inadequate filtration of the stain; or dust settled on the slide on smear.

ERYTHROCYTES (RBCs)

In blood smear from a healthy person, the RBCs when not crowded together appear as circular, homogeneous biconcave discs of nearly uniform size, ranging from 6 to 8 µm in diameter. As a rough guide, normal RBC appears to be about the size of the nucleus of a small lymphocyte on the dried film.

The RBCs stain quite deeply with the eosin component of Romanowsky dyes, particularly at the periphery of the cell as a consequence of RBC's normal biconcavity. A small portion (<10%) of RBCs is oval rather than round. The centre of RBCs is somewhat paler than the periphery (central pallor 1/3rd of the diameter of red cell).

COLOUR

Haemoglobin Content

The depth of staining gives a rough guide to the amount of haemoglobin in red cells and the term normochromic, hypochromic and hyperchromic are used to describe this feature of red cells.

- **Normochromia:** It means normal intensity of staining (red colour with central 1/3 of pallor in Romanowsky stain) of red cells. It is seen in RBCs of healthy persons without having anaemia or disease.
- **Hypochromia:** Presence of RBCs with larger than normal central pallor (>1/3rd of central pallor) due to low Hb content in a RBC or abnormal thinness of RBCs (Fig. 10.1).

  Causes: Iron deficiency anaemia, thalassaemia, sideroblastic anaemia, chronic infections, chronic inflammation.
- **Hyperchromia:** It refers to presence of RBCs having a smaller than normal area of central pallor; causing the RBCs to take on excessive staining and demonstrate higher than normal haemoglobin pigmentation.

Causes

i. In megaloblastic anaemia, the RBCs are larger and hence thicker, may stain deeply and have less central pallor. These cells are hyperchromatic because they have an increased Hb content (MCH) but the Hb concentration (MCHC) is normal.

ii. In hereditary spherocytosis, the RBCs are also hyperchromatic. Here Hb content (MCH) is normal and Hb concentration, (MCHC) is usually slightly increased due to reduced surface/volume ratio and increased thickness of red cell.

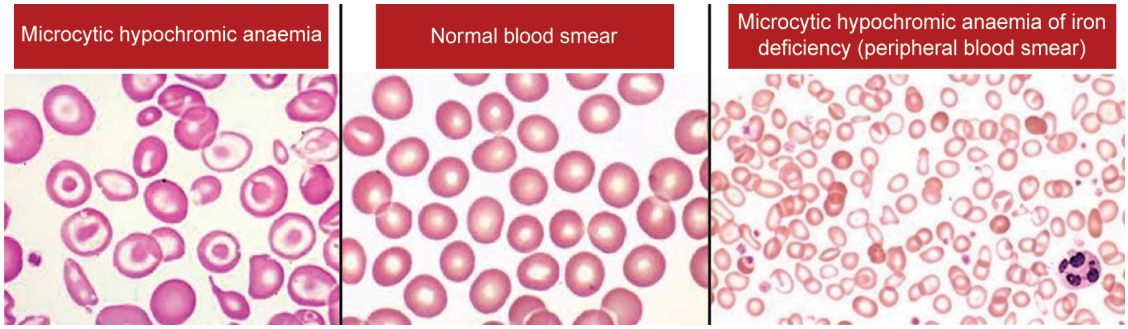


Fig. 10.1: RBCs show hypochromasia in peripheral blood smear

- **Anisochromia:** Variable staining intensities indicating unequal Hb content due to multiple populations of red blood cells (RBCs). Sometimes, it is called **dimorphic anaemia** there are two populations of RBC like hypochromic as well as normochromic RBCs.

Causes

i. Iron deficiency anaemia responding to iron therapy.
ii. Transfusion of normal blood to a patient with hypochromic anaemia.
iii. Sideroblastic anaemia.

Polychromatophilia: It means the RBCs are stained by many colours such as stain with both an acid and basic dye (components). They are demonstrated by a bluish pink tinge caused by the presence of both haemoglobin stained by acid dye (red colour) and RNA in cytoplasm stained by basic component (blue colour). The presence of residual RNA in the RBC indicates that it is a young red cell in the blood for 1–2 days. These cells are larger than mature RBC and may lack central pallor. Cells staining shades of blue, 'blue polychromasia' are unusually young reticulocytes (Fig. 10.2). The reticulocyte count is usually twice that of polychromatic cells because most immature reticulocytes (stage 1) represent polychromatic cells.

Causes

i. Blue polychromasia is most often seen in extramedullary erythropoiesis, e.g. in myelosclerosis or carcinomatosis.
ii. Haemolysis and in acute blood loss.

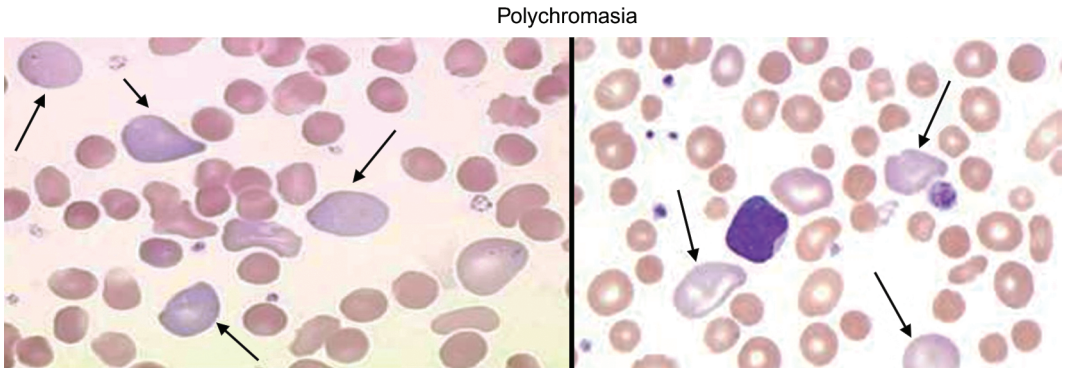


Fig. 10.2: RBCs show polychromasia in peripheral blood smear

SIZE OF RBC

- **Normocytes:** Normal size of RBC (6–8 µm in diameter) which is equal to the size of nucleus of small lymphocyte.
- **Microcytes:** RBC diameter is less than normal, i.e <6 µm; MCV <80 fl, MCHC <27%. Increased amount of microcytes are called microcytosis.

  Causes

  i. Iron deficiency anaemia
  ii. Sideroblastic anaemia
  iii. Thalassaemia
  iv. Anaemia of chronic disease
- **Macrocytes:** RBC diameter is greater than normal, i.e. >8 µm. MCV is >100 fl and MCHC is also usually increased (Fig. 10.3).

  Causes

  i. Megaloblastic anaemia (folic acid and vitamin B_{12} deficiency)
  ii. Liver disease
  iii. Hypothyroidism
  iv. Cytotoxic drugs like methotrexate or other drugs like phenytoin, zidovudine (HIV drug)
  v. Myelodysplastic syndrome
  vi. Alcoholism
  vii. Red cells of neonate and in pregnancy (physiologic cause)

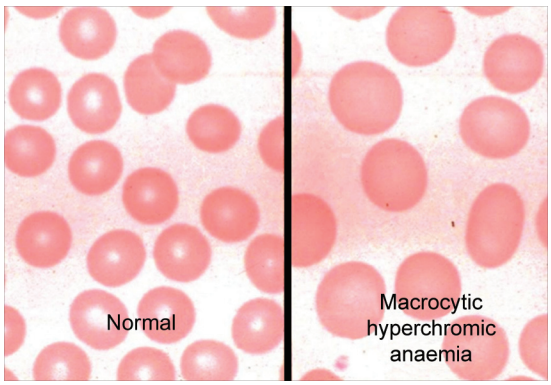


Fig. 10.3: Macrocytes in peripheral blood smear

- **Anisocytosis:** Variation of size of RBCs that means presence of different sizes of RBCs.

SHAPE OF RBC

- **Poikilocytosis:** Variation in shape of RBC is called poikilocytosis.
- **Spherocytes:** These are nearly spherical (round) RBC in contrast to normal biconcave discs (Fig. 10.4). Their diameter is smaller than normal (<8 µm), hence called **microspherocytes**. They lack central pale area or have a smaller often eccentric, pale area (because the cell is thicker than normal). Tiny bits of cell membrane of RBC (in excess of Hb) are removed from the red cells, leaving the cell with a decreased surface/volume ratio. Spherocytes are best detected in the area of blood smear where red cells are just touching one another.

  Causes

  i. Hereditary spherocytosis
  ii. Autoimmune haemolytic anaemia (warm antibody type)
  iii. ABO haemolytic disease of newborn
  iv. Direct physical or chemical injury like heat
  v. Bacterial toxins like *Clostridium perfringens* (Fig. 10.4)
- **Target cells (codocytes, mexican hat cells or leptocytes):** These are thinner than normal red cells and when stained show a peripheral rim of haemoglobin with a dark, central, haemoglobin-containing area (Bull's eye appearance) (Fig. 10.5). Target cells are presumed to result from cells having a surface which is disproportionately large compared with their volume.

  Causes

  i. Thalassaemia, haemolytic anaemia
  ii. Obstructive jaundice, chronic liver disease
  iii. Haemoglobin disease, haemoglobin SS disease
  iv. Following splenectomy (Fig. 10.5)

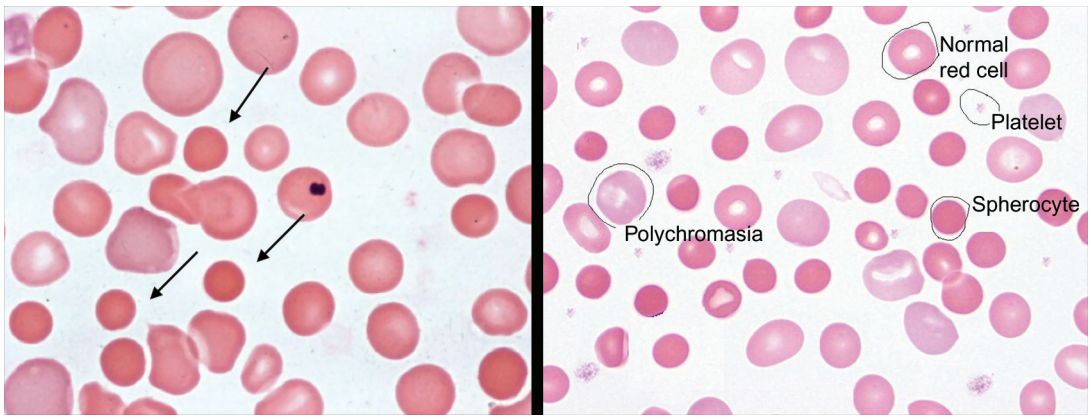


Fig. 10.4: Spherocytes in peripheral blood smear

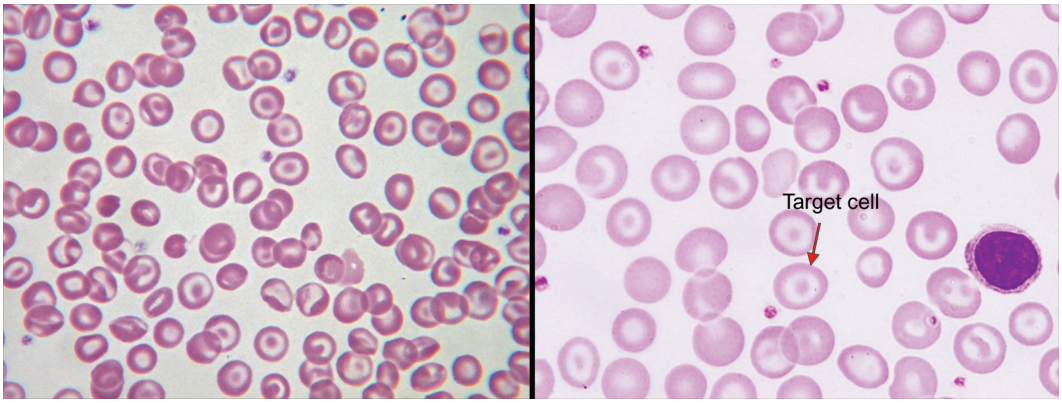


Fig. 10.5: Target cells in peripheral blood smear

- **Schistocytes (helmet cell, fragmented cell):** These are fragmented red cells, which take various shapes like helmet triangle, crescent, etc.

  Causes

  i. Microangiopathic haemolytic anaemia
  ii. Cardiac value prosthesis
  iii. Severe burns

- **Acanthocytes (spurr cells):** These are irregularly spiculated red cells in which the ends of the spicules are bulbous and rounded. These spicules of sharp projections have variable length on surface.

  Causes

  i. Abetalipoproteinemia (hereditary or acquired)
  ii. Liver diseases
  iii. Following splenectomy
  iv. McLeod phenotype caused by lack of Kell precursor (Kx).

- **Echinocytes (burr cells):** These are also called crenated cells which have regularly placed small projections on surface. It may also occur as an artifact during preparation of blood films or may be due to hyperosmolarity or to the discocyte-echinocyte transformation. *In vivo*, the latter may be associated with decreased red cell ATP as a result of many causes.

  Causes

  i. Uraemia
  ii. Artifact due to water in methyl alcohol fixative.

- **Sickle cells (drepanocytes):** These are narrow and elongated red cells with one or both ends pointed (sickle shaped). RBC becomes sickle form when a RBC containing HbS is deprived of oxygen. But sickle cells are not seen on PBS in neonates with sickle cell disease because high percentage HbF (fetal haemoglobin) during first 4–6 months prevents sickling.

  Causes

  i. Sickle cell anaemia (HbSS disease)

- **Teardrop cells (dacrocytes):** RBCs have tapering drop-like shape, and look like a teardrop (Fig. 10.6).

  Causes

  i. Myelofibrosis and myelophthisic anaemia

  ii. Also in megaloblastic anaemia and in thalassaemia major.

- **Elliptocytes** (Fig. 10.7): These are elongated rod-like RBCs with haemoglobin concentrated at both ends. Ovalocytes are elongated oval-shaped red cells. According to some authors, a RBC with its long axis more than twice its short axis should be called **elliptocyte,** whereas when the long axis less than twice its short axis should be regarded as an **ovalocyte**.

  Causes

  i. Hereditary elliptocytosis (numerous elliptocytes)

  ii. Iron deficiency anaemia, megaloblastic anaemia, thalassaemia, myelodysplastic syndrome or MDS (both elliptocytes and ovalocytes).

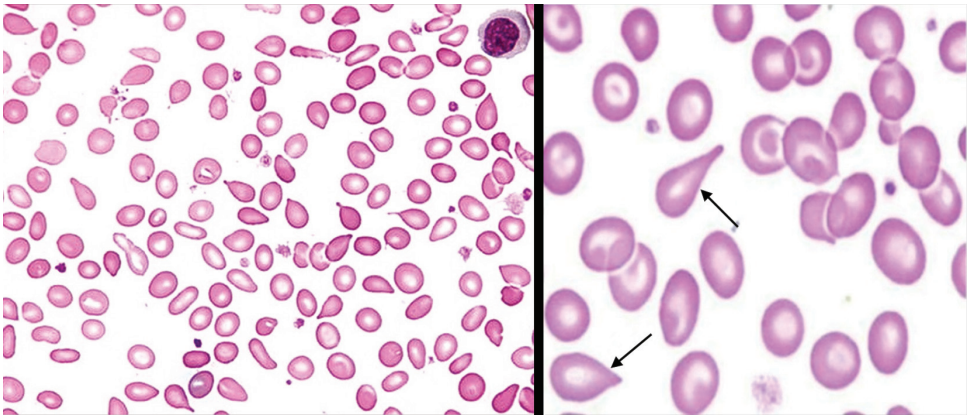


Fig. 10.6: Teardrop cells in peripheral blood smear

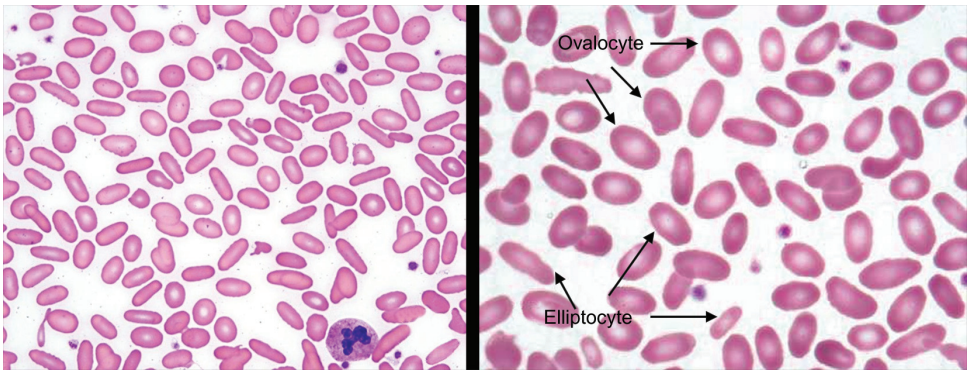


Fig. 10.7: Elliptocytes in peripheral blood smear

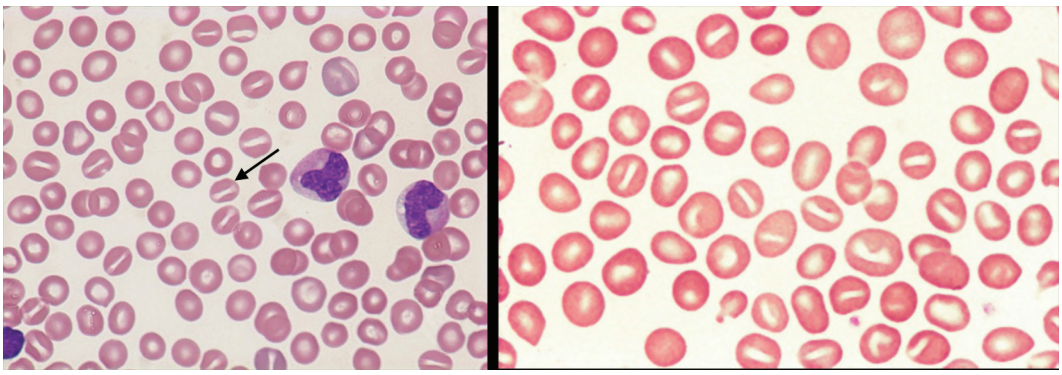


Fig. 10.8: Stomatocytes in peripheral blood smear

- **Stomatocytes (mouth cell)** (Fig. 10.8): These are soap cup-shaped RBCs with a central slit or stoma. These cells are produced by antibodies or hydrocytosis.

  Causes

  i. Alcoholic liver disease
  ii. Hereditary stomatocytosis
  iii. Rh null disease
  iv. As an artifact due to the change produced by decreased pH and as the result of exposure to cationic detergent like compounds and non-penetrating anions (Fig. 10.8).

Bite cells and Heinz bodies: These are seen in G6PD deficiency (Fig. 10.9).

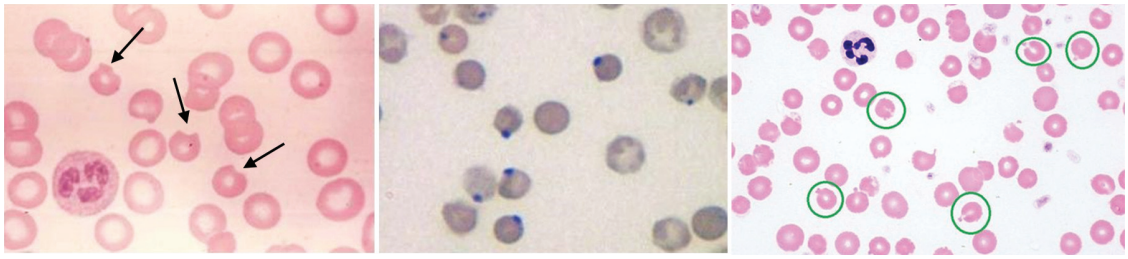
Bite cells: Abnormally shaped red blood cells with one or more semicircular portions removed from the cell margin, appearing as though the cells have had "bite" or "bites". The bites result from the removal of haemoglobin with an altered structure (denatured) by special cells (macrophages) in the spleen.

Heinz bodies: These are indicative of oxidative injury to the erythrocyte. They are clumps of irreversibly denatured haemoglobin attached to the erythrocyte cell membrane. Although the precise mechanism is not entirely understood, the presumption is that amino acid substitutions in the beta chains of the haemoglobin polypeptides allow them to precipitate within the red blood cell and form Heinz bodies.

- **Leptocyte (thin cell, water cell):** These are thin, flat RBCs with haemoglobin at the periphery.

  Causes

  i. Thalassaemia
  ii. Obstructive liver disease



Bite cells Heinz bodies

Fig. 10.9: Bite cells and Heinz bodies in peripheral blood smear

RED CELL INCLUSIONS

- **Basophilic stippling (punctate basophilia)** (Fig. 10.10)**:** It refers to the presence of numerous irregular basophilic granules (deep blue in Wright's stain) which are uniformly distributed within RBCs. The granules may be fine or coarse. These granules represent aggregates of RNA or ribosomes. Their presence indicates ineffective erythropoiesis. They do not give +ve Perls' reaction for ionized iron.

  Causes

  i. **Coarse granules:** Lead poisoning, megaloblastic anaemia and pyrimidine–5–nucleotidase deficiency:

  ii. **Fine granules:** Increased red cell production with increased polychromatophilia (Fig. 10.10).

- **Pappenheimer bodies (siderocytes)** (Fig. 10.11)**:** These are small basophilic, iron containing granules with RBC. They are actually mitochondrial concentration of ferritin (nonhaemoglobin iron) deposits and are stained by Perls' stain (unlike basophilic stippling which is not stained). Pappenheimer bodies are few in number (unlike basophilic stippling which are numerous in number) and located in the periphery of cells.

  Causes

  i. Sideroblastic anaemia

  ii. Thalassaemia

  iii. Following splenectomy

- **Cabot rings** (Fig. 10.12): They are ring-shaped, figure of eight or loop-shaped

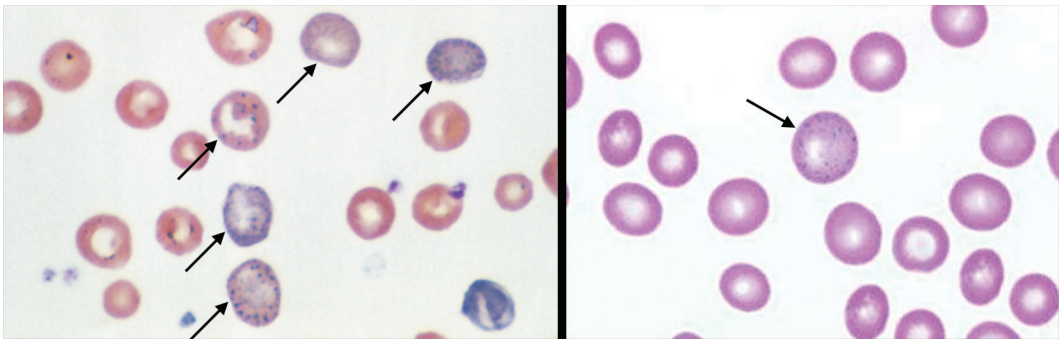


Fig. 10.10: Basophilic stippling or punctate basophilia within RBCs in peripheral blood smear

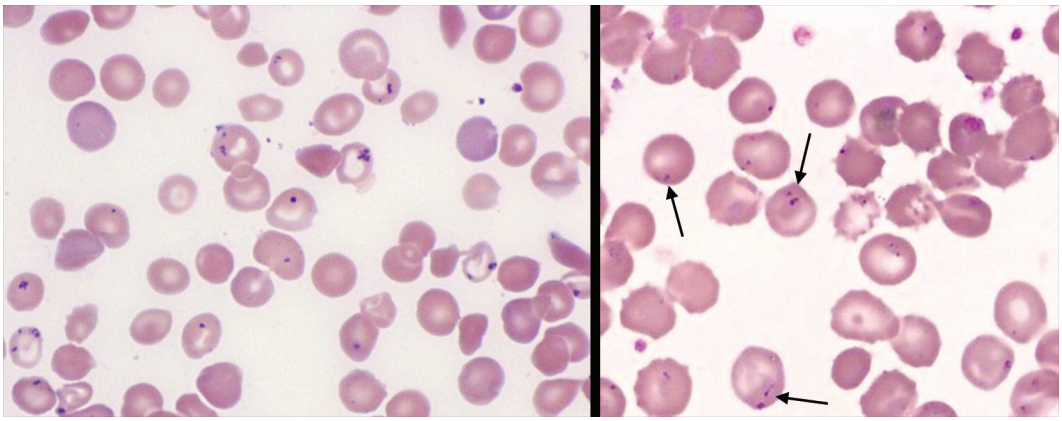


Fig. 10.11: Pappenheimer bodies (siderocytes) within RBCs in peripheral blood smear

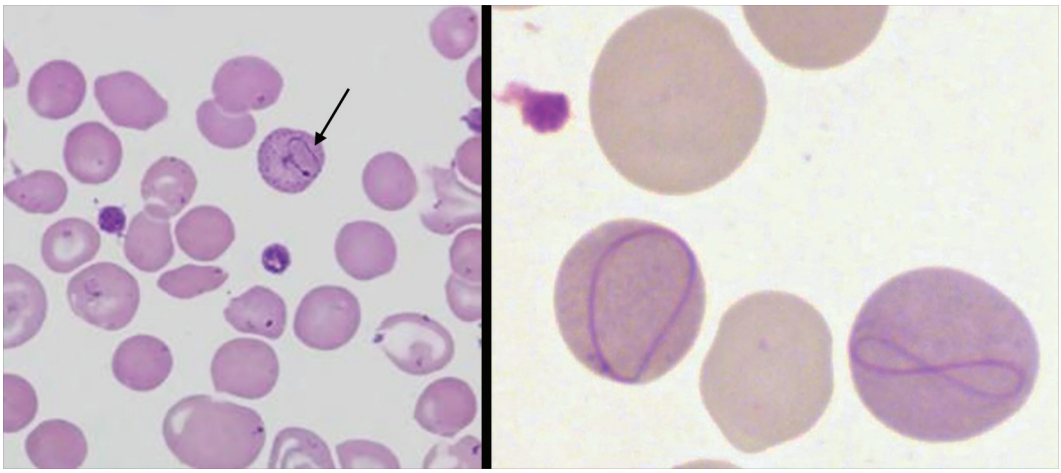


Fig. 10.12: Cabot rings within RBCs in peripheral blood smear

structures. They indicate impaired erythropoiesis. These rings are probably microtubules remaining from a mitotic spindle (due to defective erythropoiesis). They stain red on reddish purple.

Causes

i. Pernicious anaemia, megaloblastic anaemia
ii. Lead poisoning (Fig. 10.12)

- **Howell-Jolly bodies** (Fig. 10.13)**:** These are small, round, purple coloured remnants of nuclear chromatin, located at the periphery of red cell. Mostly these bodies are seen singly within RBC.

Causes

i. Single, Howell-Jolly bodies → Megaloblastic anaemia, haemolytic anaemia and after splenectomy.
ii. Multiple Howell-Jolly bodies → Megaloblastic anaemia, steatorrhoea with splenic atrophy (Fig. 10.13).

- **Malarial stippling:** Pigments of different four species of malaria may be seen in the red cells. Schuffner dots are seen in P. vivax, Maurer's dots are seen in *P. falciparum*, Ziemann's dots are seen in *P. malariae* and Jame's dots are seen in *P. ovale* infection.

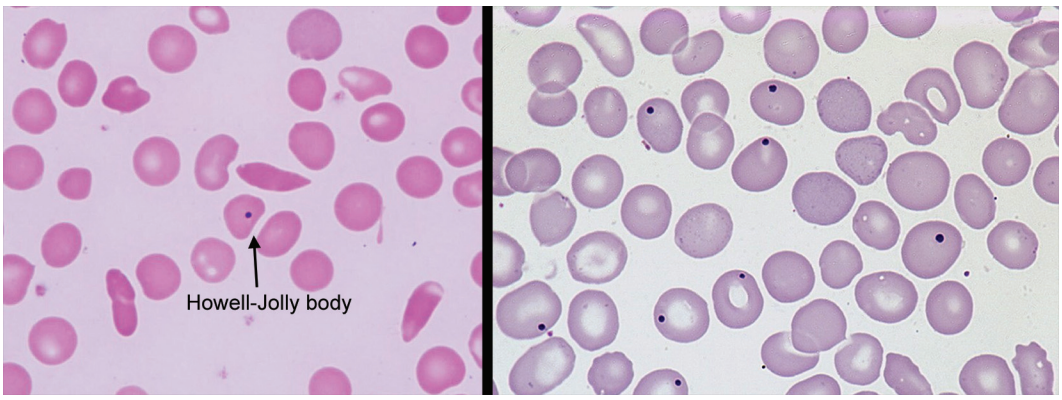


Fig. 10.13: Howell-Jolly bodies within RBCs in peripheral blood smear

P. vivax: Fine granular, yellow-brown pigments
P. falciparum: Dark to black clumped mass of pigments
P. ovale: Coarse light brown pigments
P. malariae: Dark, prominent at all stages.

Rouleaux Formation and Autoagglutination

Rouleaux formation (Fig. 10.14) is the alignment of red cells on one another so that RBCs resemble stacks of coins. Elevated plasma fibrinogen or globulin cause rouleaux to form and also increases the ESR. Rouleaux formation is decreased by presence of abnormally shaped RBCs, e.g. hereditary spherocytosis, sickle cell disease.

Causes of rouleaux formation

i. Multiple myeloma
ii. Waldenström's macroglobulinaemia, paraproteinaemia
iii. Hypergammaglobulinaemia
iv. Intravenous administration of dextran (Fig. 10.14).

Autoagglutination (Fig. 10.15) refers to the clumping of red cells in large, irregular groups on blood smear. It tends to show more irregular and tight clumps than the linear rouleaux do. But sometimes massive rouleaux formation may be confused with autoagglutination. This is called **pseudo-agglutination**. It can be do distinguished from true agglutination by two observations:

1. By noting that RBCs, although forming parts of larger clumps, are mostly arranged side by side as in typical rouleaux.
2. By adding 3–4 volumes of normal saline (9 g/L NaCl) to the preparation. Pseudo-agglutination due to massive rouleaux formation should either disperse completely or transform itself into typical rouleaux.

But true agglutination will not disperse or break up unlike rouleaux.

Autoagglutination is a reason for false reporting during ABO blood grouping. Presence of IgM autoantibodies reactive at room temperature in patients' serum can lead to autoagglutination. If autocontrol is not used, it will be wrongly reported as AB blood group.

Causes of autoagglutination

i. Cold agglutination disease (Fig. 10.15)

NUCLEATED RBCs IN PERIPHERAL BLOOD

In contrast with erythrocytes of lower vertebrates and with most mammalian cells, the human erythrocyte lacks a nucleus. Nucleated RBCs (normoblasts) are precursors of the non-nucleated mature red cells in the blood. In human, normoblasts are usually present only in bone marrow. The stages in

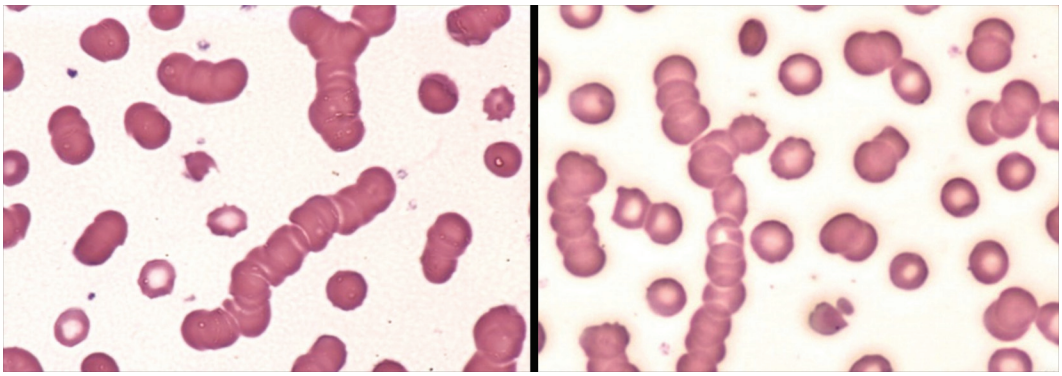


Fig. 10.14: Rouleaux formation in peripheral blood

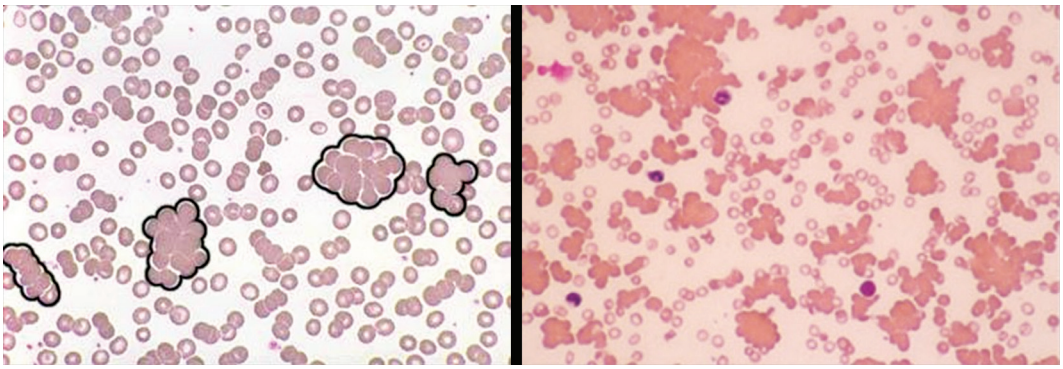


Fig. 10.15: Peripheral blood smear shows autoagglutination

their production are proerythroblast, basophilic or early normoblast, polychromatophilic or intermediate normoblast and orthochromatic or late normoblast.

- **Proerythroblast (pronormoblast):** A large (15–20 µm) cell with a fine, uniform chromatin pattern, one or more conspicuous nucleoli and dark blue cytoplasm.
- **Basophilic normoblast or early normoblast:** Smaller in size (12–16 µm) and has a coarser chromatin with a inconspicuous nucleoli. The cytoplasm is deeply basophilic.
- **Polychromatophilic normoblast or intermediate normoblast:** The cell size is 12–15 µm. The nuclear size is smaller and the chromatin becomes clumped. Polychromasia of cytoplasm results from admixture of blue ribonucleic acid and pink haemoglobin.
- **Orthochromatic normoblast on late normoblast:** The cell size is 8–12 µm. The nucleus size is small, dense and pyknotic. The nucleus is commonly eccentrically located. The cytoplasm stains mostly pink due to haemoglobinzation. It is called cytoplasmatic because cytoplasmic staining is largely similar to erythrocytes (Fig. 10.16).

The nucleus of orthochromatic normoblast is expelled and reticulocyte (young RBC) is formed. The reticulocyte still has remnants of ribosomal RNA in the form of a cytoplasmic reticulum. After 1–2 days in the bone marrow and 1–2 days in peripheral blood; reticulocytes lose RNA and become mature pink staining RBCs (erythrocytes).

Significance: Normoblasts though normally present in the bone marrow, may be released in peripheral blood prematurely. This is a normal finding in cord blood and peripheral blood of newborns. These nucleated RBCs (nRBCs) may persist longer than a week in immature infants. The average number 3–10/100 WBCs in a healthy full term infant to 25/100 WBCs in a premature infant.

In peripheral blood smear, the normoblast count is calculated relative to 100 WBCs. If >3–5 normoblasts per 100 WBCs are found, the total count of WBC must be corrected accordingly.

Large number of nRBCs (nucleated RBCs) or normoblasts in PBS is seen in immature infants, haemolytic diseases of newborn, haemolytic anaemia, leukaemias, myelophthisic anaemia, and myelofibrosis (Fig. 10.17).

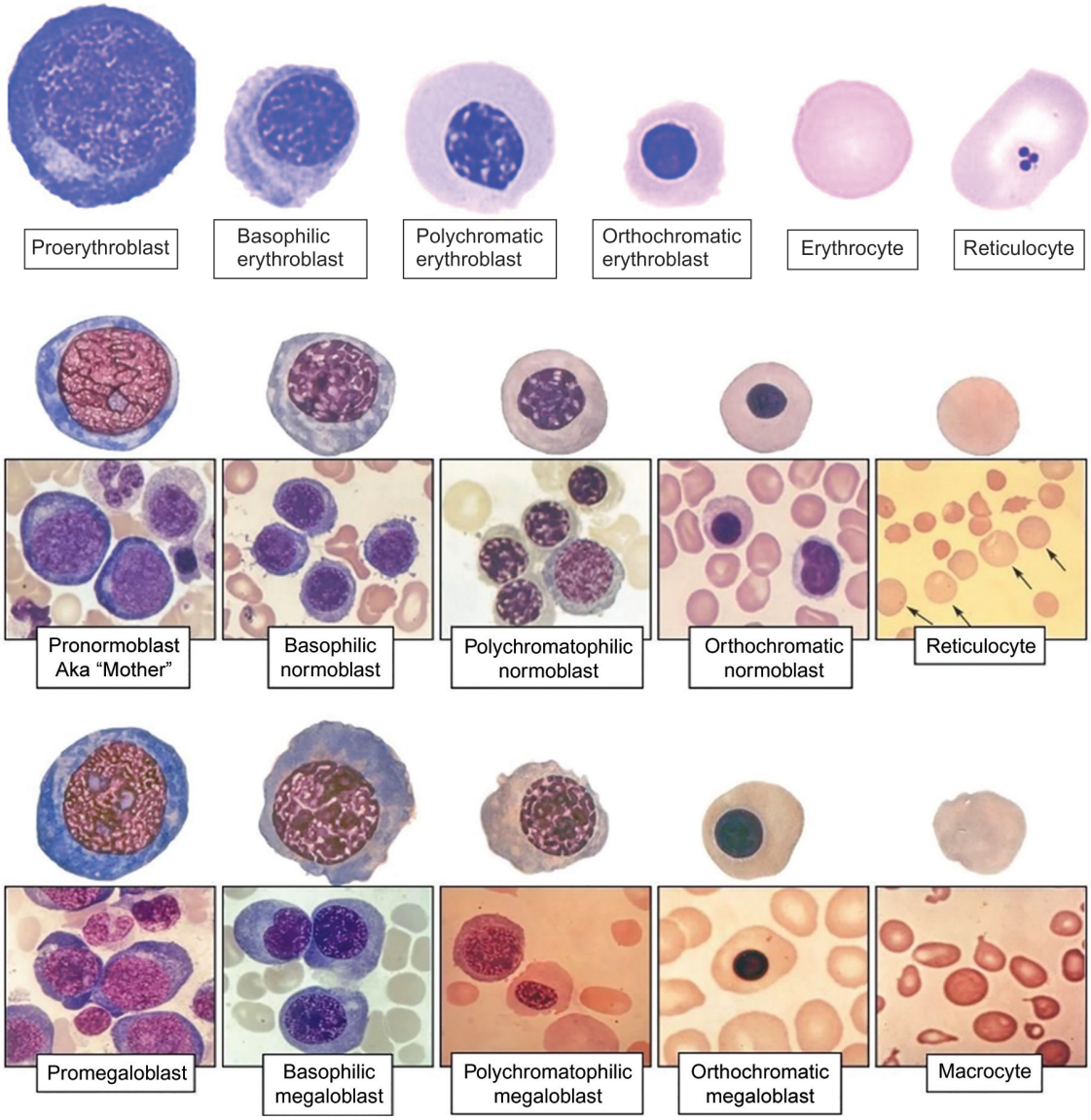


Fig. 10.16: Proerythroblast or pronormoblast, basophilic normoblast, polychromatophilic normoblast, orthochromatic normoblast

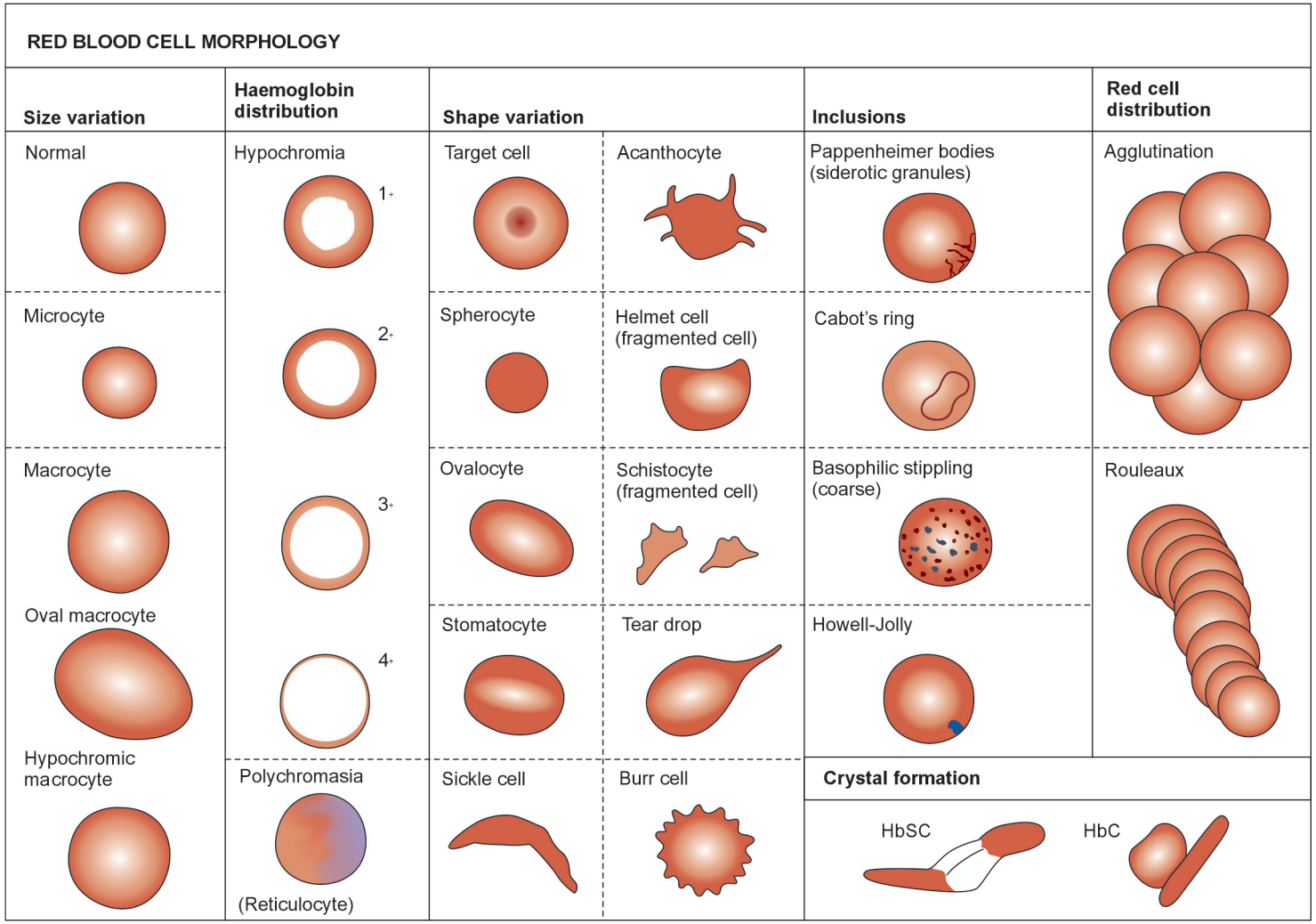


Fig. 10.17: Abnormal RBCs

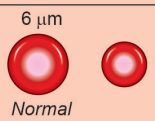
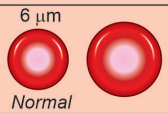
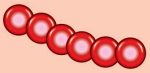
Abnormal RBC morphology	Cartoon image	May be associated with
Microcytic RBC		Pyridoxine deficiency Thalassaemia Iron deficiency anaemia Chronic disease anaemia (sometimes) Sideroblastic anaemia (sometimes)
Macrocytic RBC		Vitamin B_{12} or folate deficiency Liver disease MDS Chemotherapy (e.g. methotrexate)
Spurr cell RBC (acanthocyte)		Abetalipoproteinaemia, liver disease, McLeod blood group phenotype, post-splenectomy, etc.
Burr cell RBC (echinocyte)		Artifact, uraemia, liver disease, etc.
Schistocyte		Microangiopathic haemolytic anaemia, mechanical valve induced, etc.
Bite cell RBC		G6PD deficiency Unstable haemoglobin disorders Oxidative drugs
Elliptocyte		Hereditary elliptocytosis Severe iron deficiency anaemia
Spherocyte		Hereditary spherocytosis Autoimmune haemolytic anaemia
Stomatocyte		Hereditary stomatocytosis Liver disease
Target cell RBC		Thalassaemia Haemoglobinopathies Post-splenectomy Liver disease Artifact
Sickle cell RBC		Haemoglobin SS disease Haemoglobin SC disease Haemoglobin SD disease S-beta-thalassaemia
Teardrop		Myelofibrosis, underlying marrow process/infiltrate, etc.
Haemoglobin C crystals		Haemoglobin C disease Haemoglobin SC disease
Red cell agglutinate		Cold autoimmune haemolytic anaemia Paroxysmal cold haemoglobinuria IgM associated lymphoma Multiple myeloma
Rouleaux		Chronic liver disease Malignant lymphoma Multiple myeloma Chronic inflammatory diseases

Fig. 10.18: Abnormal RBC morphology and their causes

Leucocytes (WBCs) (Fig. 10.19)

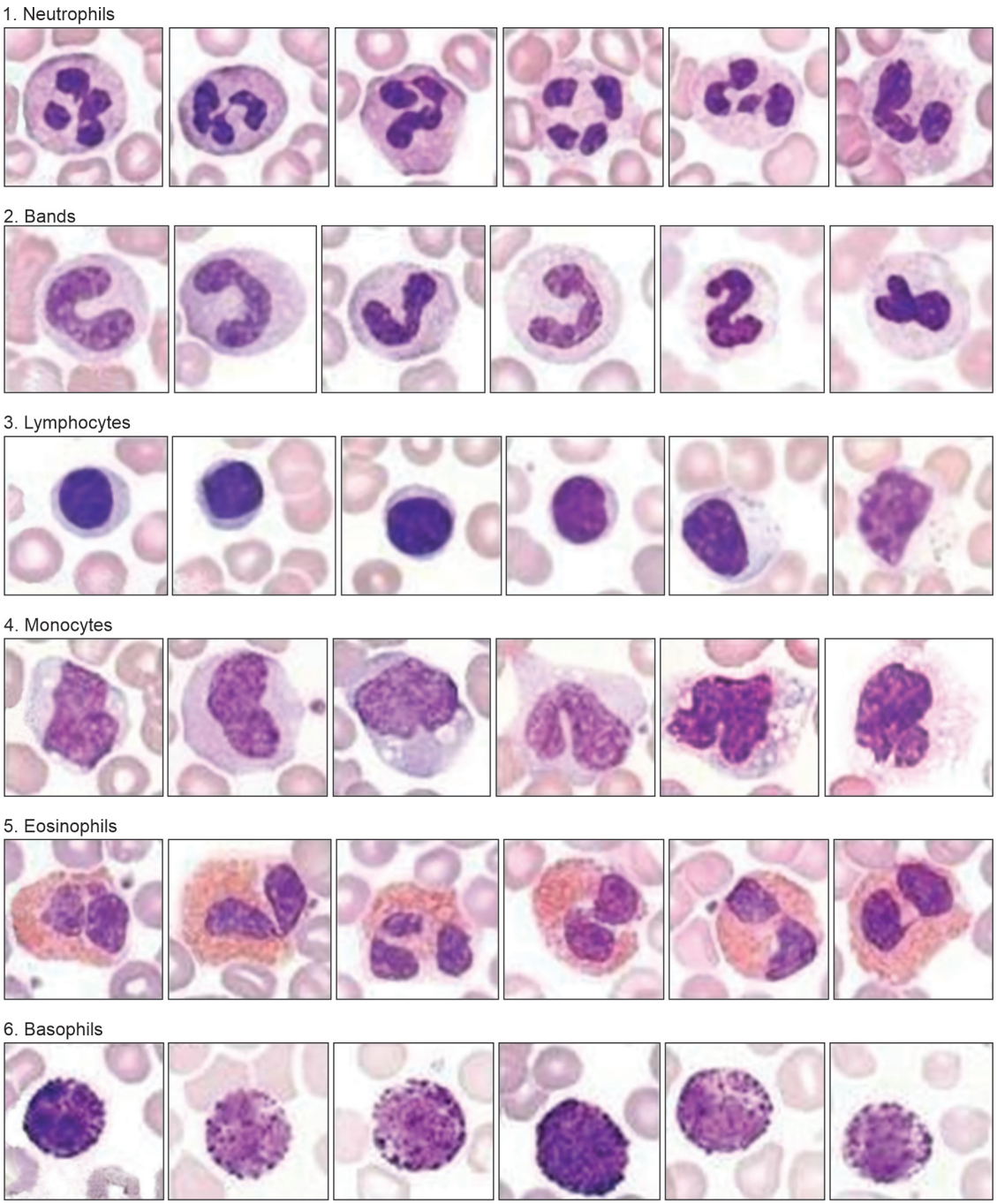


Fig. 10.19: Different types of normal leucocytes

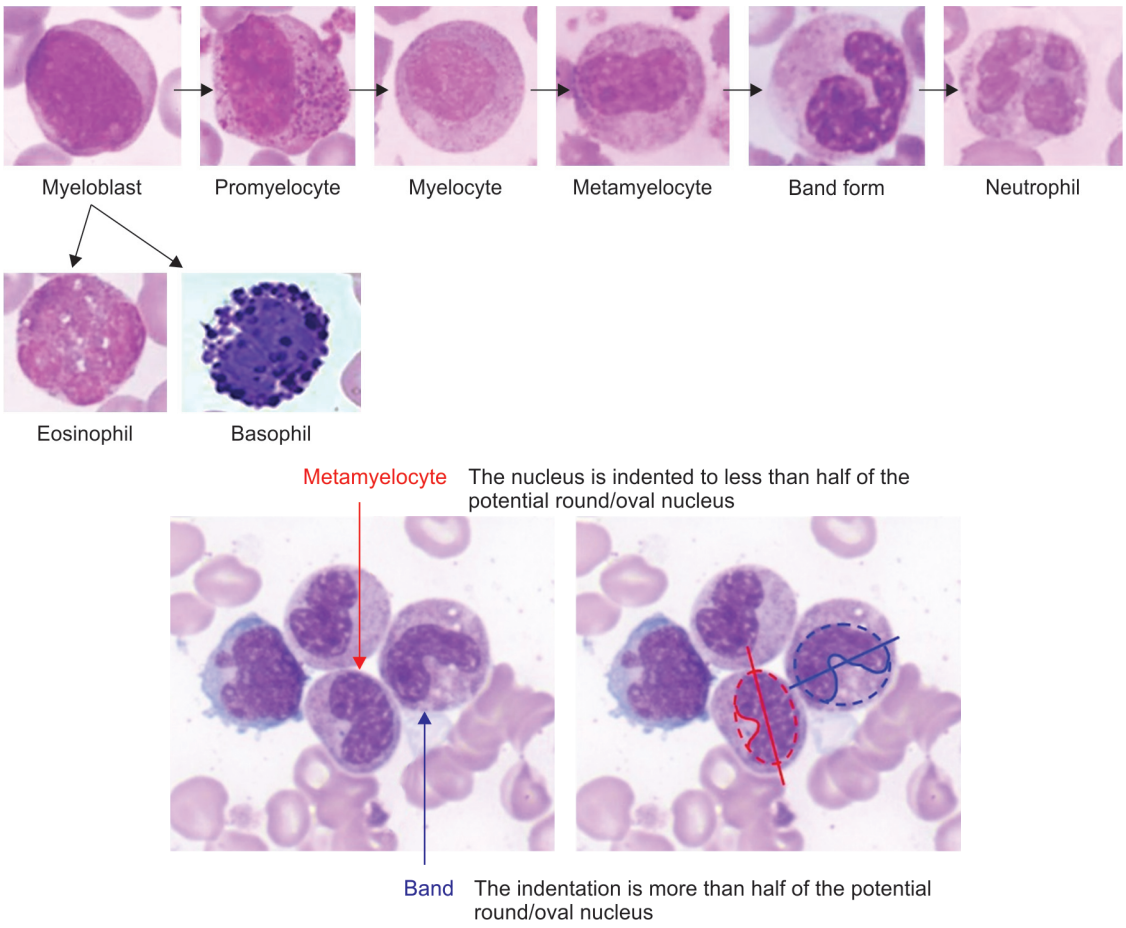


Fig. 10.20: Maturation of myeloid cells

Morphology of abnormal leucocytes

- **Toxic granules** (Fig. 10.21)**:** These are dark blue or purple coarse granules in the cytoplasm of segmented neutrophils, band forms, and metamyelocytes. They represent **azurophil or primary granules** and probably result from impaired cytoplasmic maturation while generating large number of neutrophils. These granules are myeloperoxidase positive but less peroxidase activity is seen than in normal neutrophils.

  Increased basophilia of azurophil granules in normal neutrophils simulating toxic granules may occur if blood smear is stained for a prolonged time or decreased (low) pH of staining reaction (low buffer pH).

  Causes

  i. Severe bacterial infection, sepsis
  ii. Other causes of inflammation
  iii. Administration of granulocyte colony-stimulating factor or chemotherapy

  Note: Fractionally larger, coarser granules (not toxic granules) may be seen in aplastic anaemia and myelofibrosis (Fig. 10.21).

- **Döhle inclusion bodies** (Fig. 10.22)**:** These are small, oval, pale blue cytoplasmic inclusions in the periphery of neutrophils. They represent remnants of ribosomes and rough endoplasmic reticulum.

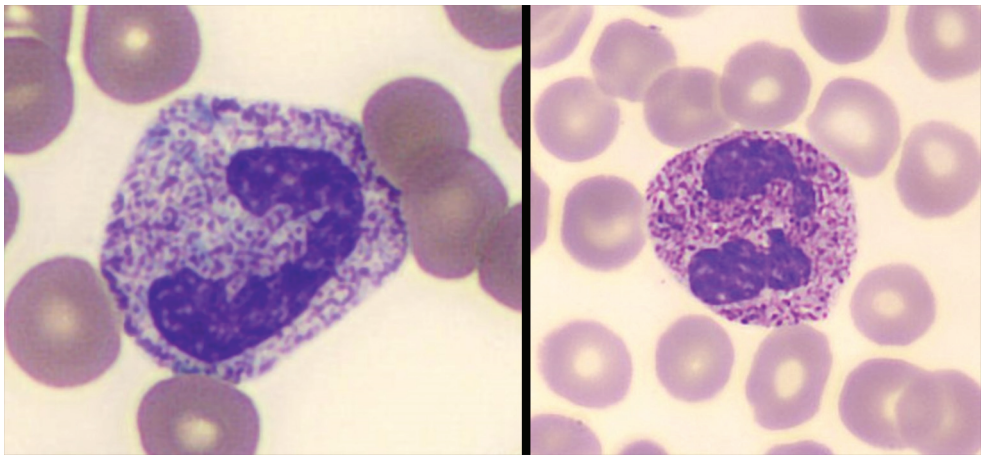


Fig. 10.21: Toxic granules in neutrophil

Causes

i. Originally it was described in scarlet fever
ii. Bacterial and other infections
iii. Burns, pregnancy
iv. Aplastic anaemia
v. Administration of toxic agents

- **Cytoplasmic vacuoles:** Vacuoles in neutrophils indicate phagocytosis and are seen in bacterial infections. This is a sign of toxic change in infections (Fig. 10.22).

Causes

i. Septicemia, bacterial infection
ii. As an artifact, if smears are made from stored blood in EDTA anticoagulant (Fig. 10.22).

- **Intracellular organism:** Bacteria, fungi (Histoplasma, Candida) and Ehrlichia may be seen in neutrophils.
- **Hypersegmented neutrophils** (Fig. 10.23)**:** If the PBS shows ≥5% of neutrophils having 5 lobes (segments) or 1% of neutrophils having 6 lobes, then the neutrophils are called hypersegmented.

Causes

i. Megaloblastic anaemia
ii. Uraemia
iii. Hydroxyurea treatment in CML and other diseases.
iv. Cytotoxic therapy especially with methotrexate treatment (Fig. 10.23).

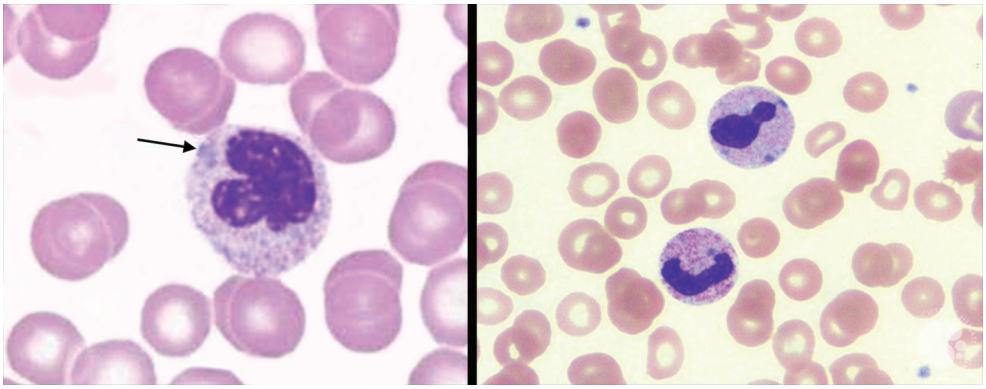


Fig. 10.22: Döhle bodies in neutrophils

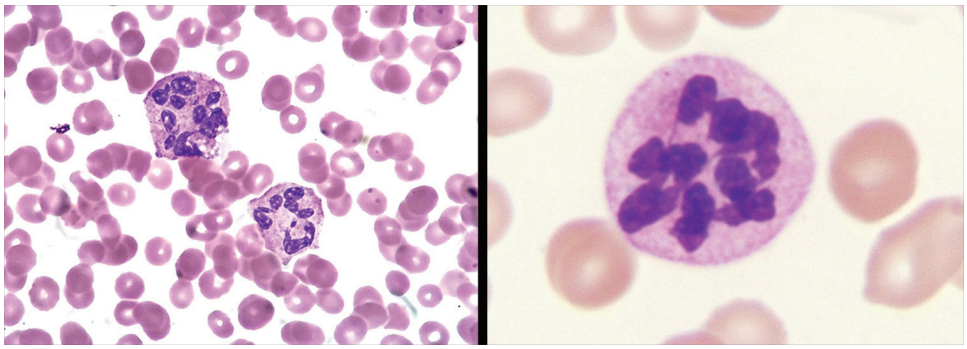


Fig. 10.23: Hypersegmented neutrophils

- **Hypogranular neutrophils:** Reduced number of cytoplasmic granules in neutrophils. May be seen in MDS (myelodysplastic syndrome) and AML (acute myeloid leukaemia).
- **Pelger-Huet cells** (Fig. 10.24)**:** In Pelger-Huet anomaly (a benign inherited, autosomal dominant), neutrophils fail to segment properly. The majority of circulating neutrophils have only two discrete, equal sized lobes connected by a thin chromatin bridge. The chromatin is coarsely clumped and cytoplasmic granules are normal.
- **Pseudo-Pelger cells:** The neutrophils have two lobes like Pelger-Huet cells but this condition is acquired. It is seen in MDS, AML and CML. Cytoplasmic granules are reduced in number (hypogranular) and they tend to have markedly irregular nuclear pattern (Fig. 10.24).
- **Atypical lymphocytes** (Fig. 10.25)**:** These lymphocytes are large, irregularly shaped with abundant cytoplasm and irregular nuclei. Cytoplasm shows deep basophilia at the periphery and scalloping of borders. Nuclear chromatin is open (unlike dense nuclear chromatin of mature lymphocyte). In infectious nucleosis (glandular fever) these lymphocytes have a tendency to adhere to adjacent erythrocyte.

  Causes

  i. Infectious mononucleosis (glandular fever)
  ii. Pertussis and respiratory syncytial virus infection: Atypical lymphocytes with clefted nuclei.

  Atypical lymphocyte can be classified into three types: Downey type I or leucocytoid lymphocytes, Downey type II or stress lymphocyte and Downey type III.

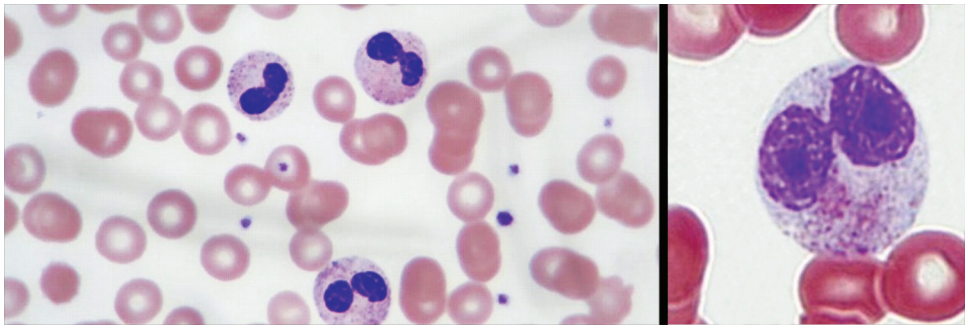


Fig. 10.24: Pelger-Huet and pseudo-Pelger cells

1. **Downey type I (atypical lymphocyte):** Nuclei may have so-called open chromatin and deep indentations. The cytoplasm shows basophilia, an increase of azurophil granules and frequently vacuoles. Some cells resemble plasma cells and some resemble monocytes.
2. **Downey type II (atypical lymphocyte):** These cells have a relatively smooth but still mature nucleus and abundant smooth cytoplasm with patchy peripheral and radial basophilia. They are often most numerous types.
3. **Downey type III (atypical lymphocyte):** These cells look like blasts, presumably in response to the viral stimulation. These immature lymphocytes, usually representing only a small percentage of total lymphocyte count are large reticular lymphocytes (nonleukaemic lymphoblasts) with a coarsely reticular nucleus and abundant, deeply basophilic cytoplasm (Fig. 10.25).

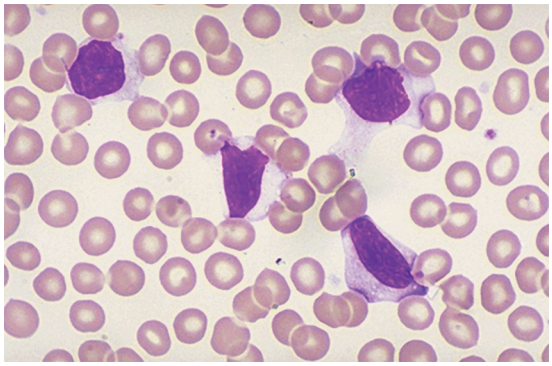


Fig. 10.25: Atypical lymphocytes are shown here. These WBCs are "atypical" because they are larger (more cytoplasm) and have nucleoli in their nuclei. The cytoplasm tends to be indented by surrounding RBCs. Such atypical lymphocytes are often associated with infectious mononucleosis from Epstein-Barr virus (EBV) infection

- **Blast cells** (Fig. 10.26)**:** These cells are immature precursor of myeloid cells (myeloblasts) or lymphoid cells (lymphoblasts). Morphology of the blasts in PBS or bone marrow is very important feature. These cells are seen in severe infections, infiltrative disorders, lymphoma and leukaemia. Presence of 20% of blasts in PBS or bone marrow suggests acute leukaemia. In some types of acute myeloid leukaemias (AML), Auer rods (rod-shaped structures in cytoplasm) are present in blasts and are pathognomonic of AML. Blast cells have following morphological features:
  - ✓ Larger/bigger than normal counterpart (15–25 µm)
  - ✓ High N:C (nucleocytoplasmic) ratio
  - ✓ Nucleus is large, immature having open chromatin and 1–5 nucleoli.
  - ✓ Scant to moderate amount of cytoplasm which often appear atypical (Fig. 10.26).

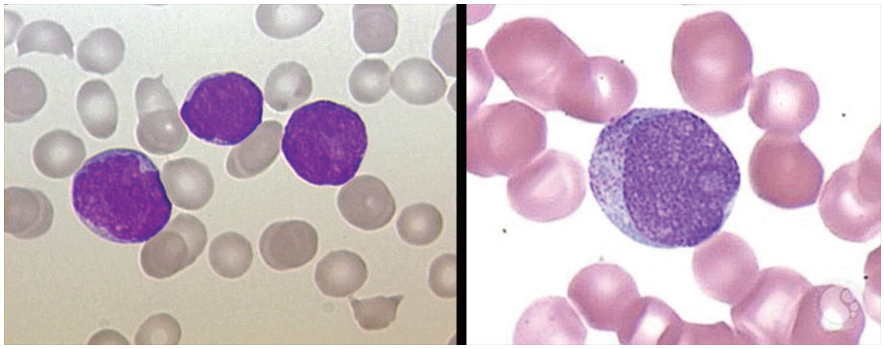


Fig. 10.26: Lymphoblast (left side) and myeloblast (right side)

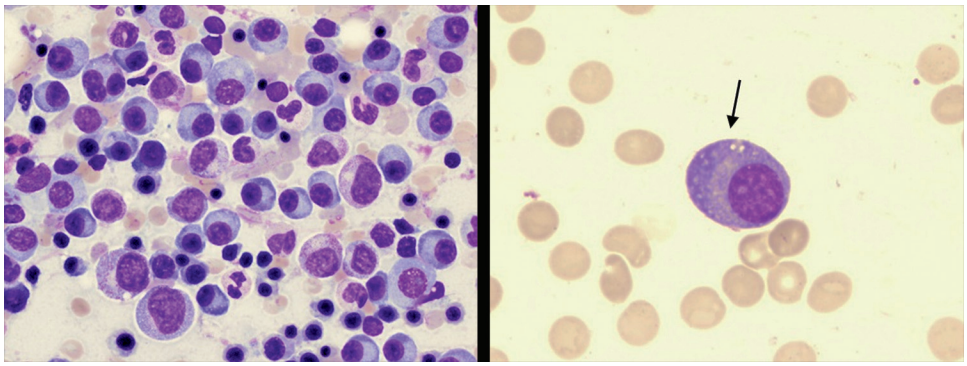


Fig. 10.27: Plasma cells

- **Plasma cells** (Fig. 10.27)**:** Plasma cells usually do not appear in PBS (present in bone marrow). Occasionally, few reactive plasma cells are seen in PBS in inflammation and infections. Neoplastic plasma cells in PBS are seen in plasma cell dyscrasias like multiple myeloma and plasma cell leukaemia (Fig. 10.27).
- **Abnormal lymphoproliferative disorder:** Abnormal (neoplastic) lymphocytes with characteristic morphology on PBS are seen in chronic lymphocytic leukaemia (CLL), prolymphocytic leukaemia, hairy cell leukaemia, splenic marginal zone lymphoma, large granular lymphocytic leukaemia and Sézary syndrome.
- **Inherited disorders**
  - i. **Alder-Reilly anomaly:** This is an autosomal recessive disorder. Large lilac inclusions (darkly staining azurophil granules) are seen in the cytoplasm of granulocytes, lymphocytes and monocytes.
  - ii. **May-Hegglin anomaly:** This is an autosomal dominant disorder. Large basophilic inclusions (Dohle-like) are seen in all leucocytes. Giant platelets and thrombocytopenia are also noted.
  - iii. **Chediak-Higashi anomaly:** This is an autosomal recessive disorder. Large gray blue granules in cytoplasm of monocytes and granulocytes (giant peroxidase-positive lysosomal granules) are seen.
- **Leukoerythroblastic reaction:** It is the presence of immature WBCs as well as nRBCs (nucleated RBCs) in peripheral blood.
- **Triad of features in PBS**
  1. Immature WBCs or myeloid cells
  2. nRBCs
  3. Teardrop cells.

  Causes
  - i. Haematological disorders: Myelofibrosis, multiple myelomas, lymphoma, severe haemolysis (erythroblastosis foetalis).
  - ii. Storage disorder: Gaucher's disease, Niemann-Pick disease
  - iii. Bone metastasis: Carcinoma of breast; prostate, lung, gastrointestinal tract
  - iv. Infectious disease: Miliary tuberculosis (Fig. 10.28).
- **Leukaemoid reaction** (Fig. 10.29)**:** This refers to the presence of markedly increase in total leucocyte count (≥50,000/mm^3) with immature white blood cells in PBS resembling leukaemia but occurring in a non-leukaemic conditions.

  Though by definition total WBC count is >50,000/mm^3 but practically many times the leukocytosis is usually <50,000/mm^3.

  Depending on the predominat cell in PBS, leukaemoid reactions may be neutrophilic (myeloid cells), lymphocytic, eosinophilic and monocytic. Neutrophilic leukaemoid reaction is most common type (Fig. 10.29).

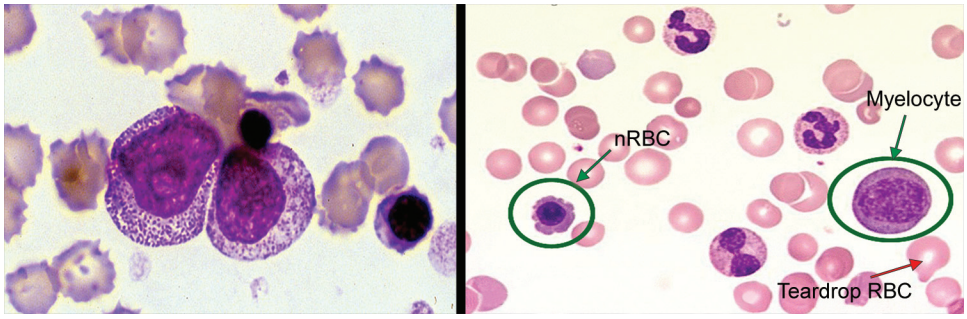


Fig. 10.28: Leukoerythroblastic reaction shows immature WBCs and nucleated RBCs

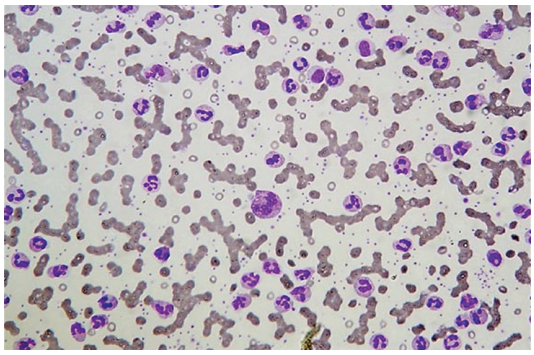


Fig. 10.29: Leukaemoid reaction with rouleaux formation

i. Myeloid (neutrophilic) and leukaemoid reaction (Table 10.1):
   - ✓ Severe bacterial infections like septicaemia, pneumonia
   - ✓ Severe hemorrhage and haemolysis
   - ✓ Malignancy like multiple myeloma, Hodgkin lymphoma, bone marrow metastasis
   - ✓ Intoxications like eclampsia, severe burns, mercury poisoning

ii. **Lymphoid leukaemoid reaction**
   - ✓ Viral infections like infectious mononucleosis, infectious lymphocytosis, cytomegalovirus infection
   - ✓ Bacterial infections like tuberculosis, whooping cough.

Table 10.1: Differences between leukaemoid reaction and chronic myeloid leukaemia (CML)

Parameters	Leukaemoid reaction	Chronic myeloid leukaemia
• Clinical presentation	Features of underlying disease, fever common	Splenomegaly, bone pain and lymphadenopathy also seen
• Examination of blood	Moderately increased, rarely exceeds 5000/mm^3	Markedly increased and usually >50,000/mm^3
i. TLC (WBC count) ii. DLC (differential leucocyte count)	Shift to left with few immature myeloid cells like myelocyte, metamyelocyte, band form and occasion myeloblasts (<5%)	Shift to left with numerous immature myeloblasts (<20%)
iii. Toxic granules and Döhle bodies	Present	Absent
iv. Eosinophilia and basophilia	May or may not be present (variable)	Present

Contd.

Table 10.1: Differences between leukaemoid reaction and chronic myeloid leukaemia (CML) (Contd.)

Parameters	Leukaemoid reaction	Chronic myeloid leukaemia
v. RBC (anaemia)	Usually mild or absent	Severe and progressive
vi. Platelet number	Variable	Normal or increased
• LAP (leucocyte alkaline phosphatase) score	High	Low or decreased
• Extramedullary myeloid tumours	Absent	Present
• Philadelphia chromosome	Absent	Present, t(9,22)
• Clonality	Polyclonal	Monoclonal

PLATELETS (THROMBOCYTES)

Platelets are derived from cytoplasmic fragmentation of bone marrow cells known as **megakaryocytes**. Platelets measure 1–3 µm in diameter. Normally, platelet count is 1.5–4 lacs/mm^3 in peripheral blood. They are viable for approximately 10 days in circulation. About 1/3rd of the total platelets in the body remain in the spleen (splenic reservoir) and rest in circulation of peripheral blood.

Under light microscope when stained with one of the Romanowsky stains, platelets appear as small, irregular with fine cytoplasmic processes. Cytoplasmic granules are often, visible. The peripheral cytoplasm looks clear (hyalomere) while central portion of platelets are packed with granules (granulomere).

Ultrastructurally (electron microscope), there are 3 zones in the platelets. (1) **Peripheral zone:** Exterior coat (glycocalyx), cell membrane, open canalicular system; (2) **Sol-gel zone:** Microfilaments, circumferential microtubules, dense tubular system; (3) **Organelle zone:** Alpha granules, dense granules, mitochondria and lysosome (Table 10.2).

If blood films are made from non-anticoagulated fingerstick specimens, some platelet clumping is an expected feature. If the platelet count is done by automated blood cell counter using EDTA as an anticoagulant, about 1% persons may show falsely low count due to presence of EDTA-dependent antiplatelet antibody in them.

Table 10.2: Platelet granules and their contents

Alpha (α) granules
• Coagulation system protein: von Willebrand factors, fibrinogen, high molecular weight kininogen
• Fibrinolytic system protein: Plasminogen, α_2-antiplasmin, PAI-1
• Platelet specific protein: Platelet factor 4, β-thromboglobulin, platelet derived growth factors, thrombospondin
• Others: Albumin, fibronectin
Dense (δ) granules
• Anions: ATP, GTP, ADP, GDP
• Cations: Calcium, serotonin

Examination of a parallel blood smear is useful to avoid false diagnosis of thrombocytopenia (low platelet count). Occasionally, platelets may show rosetting around neutrophils (platelet satellitism). This is due to platelet antibodies. Aggregation of platelets on blood smear may cause discharge of the granule contents, and platelets appear as pale blue mass.

Larger platelets (>3 µm in diameter) (Fig. 10.30) are seen when platelet production is increased. So, in severe immune thrombocytopenia, some large platelets will be

seen on the film due to compensatory increased platelet production.

Very high platelet count (thrombocytosis), particularly when associated with myeloproliferative disorders (CML, polycythaemia vera, myelofibrosis, etc.), platelet may show extreme anisocytosis with some platelets as large as RBC (6–8 µm in diameter). These large platelets are possibly arising from megakaryocyte cytoplasmic fragments.

The platelet count frequently rises with acute inflammatory stress or bleeding but rarely 10 lacs/mm^3. If the count is more than this, a myeloproliferative disorder should be suspected. Severe bacterial infections (sepsis or bacteremia), *E. coli* infection, dengue also cause low platelet count. Use of some drugs like heparin, quinine, sulfa-containing antibiotics and anticonvulsants also cause low platelet count.

Characteristic morphological features of platelets are seen in two inherited platelet disorders associated with bleeding (Fig. 10.30).

i. **Bernard-Soulier syndrome:** Giant platelets with defective-ristocetin response.

ii. **Grey platelet syndrome:** Platelets lack granules and have a ghost-like appearance on the blood smear.

Thrombocytopenia may appear in May-Hegglin anomaly, where the platelets will appear large and reddish.

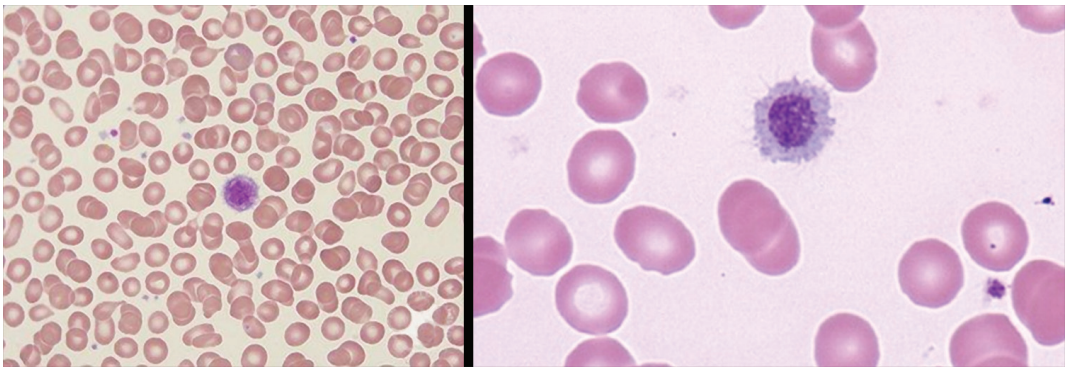
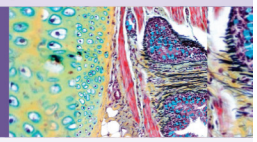


Fig. 10.30: Giant platelets



Chapter 11

Anaemia: Common Types and their Investigations

Definition of anaemia: It is defined as the decrease below normal limit (below the reference level for the age and a sex of the person) of the haemoglobin concentration, erythrocyte count or haematocrit.

Functionally, anaemia is defined as the decrease in the oxygen-carrying capacity of blood, which leads to tissue hypoxia.

WHO criteria for anaemia

Category	Hb level (g/dl)	MCHC	Mean normal Hb (g/dl)
• Adult male	<13	<34%	14.5
• Adult female	<12	<34%	13.5
• Pregnant female	<11	<34%	12.5
• Child <6 years	<12	<34%	12

Severity of Anaemia

- **Mild anaemia:** Hb 9.1–10.5 g/dl
- **Moderate anaemia:** Hb 6.0– 9.0 g/dl
- **Severe anaemia:** Hb <6 g/dl.

General approach for investigations of anaemia (Tables 11.1 and 11.2)

- Haemoglobin (Hb) and packed cell volume (PCV) estimation
- Total count of RBC, WBC and platelets estimation
- Peripheral blood smear (PBS) examination
- Erythrocyte sedimentation rate (ESR) estimation
- Red cell indices (MCV, MCH and MCHC) calculation
- Reticulocyte count
- Bone marrow examination.

RBC INDICES (Table 11.3)

- **Mean corpuscular volume (MCV):** Average volume of a red cell. Normal value 80–100 fl (femtoliter).
- **Mean corpuscular haemoglobin (MCH):** Average content (mass) of haemoglobin per red cell. Normal value 30 ± 3 pg (picogram). MCH = Hb/RBC count.
- **Mean corpuscular haemoglobin concentration (MCHC):** Average concentration of haemoglobin in a given volume of packed red cells. Normal value 34 ± 2 g/dl. MCHC = Hb/MCV.

RED CELL DISTRIBUTION WIDTH

Red cell distribution width (RDW) is the index to measure degree of anisocytosis (variation in size of red blood cells). It is also known as RDW–CV (coefficient of variation) and RDW–SD (standard deviation). Normal value of RDW is 11.5–14.5.

Table 11.1: Classification of anaemia according to morphology

Morphology of RBC	RBC indices	Common causes
• **Microcyitc hypochromic**	MCV <78 fl; MCH <26 pg	• Iron deficiency anaemia • Thalassaemia • Sideroblastic anaemia • Anaemia of chronic disorder
• **Normocytic (normochromic)**	MCV 80 –100 fl; MCH 30.3 pg	• Haemolytic anaemia • Acute blood loss • Aplastic anaemia • Secondary anaemia (renal cause, inflammation, liver disease, endocrine deficiency)
• **Macrocytic**	MCV > 100 fl	• Megaloblastic anaemia • Liver disease • Alcoholism • hypothyroid

Table 11.2: Classification of anaemia according to reticulocyte

Reticulocyte response	Reticulocyte production index (absolute reticulocyte count)	Causes
• In appropriately low for the degree of anaemia	<2% (<75,000/µl)	Hypoproliferative anaemias (iron deficiency anaemia, anaemia of chronic disease, endocrine disease, thalassaemia, sideroblastic anaemia, megaloblastic anaemia, aplastic anaemia, myelodysplasia)
• Appropriate for the degree of anaemia	≥2% (>100,000/µl)	Hyperproliferative anaemia like blood loss, haemolytic anaemias

Calculation of RDW

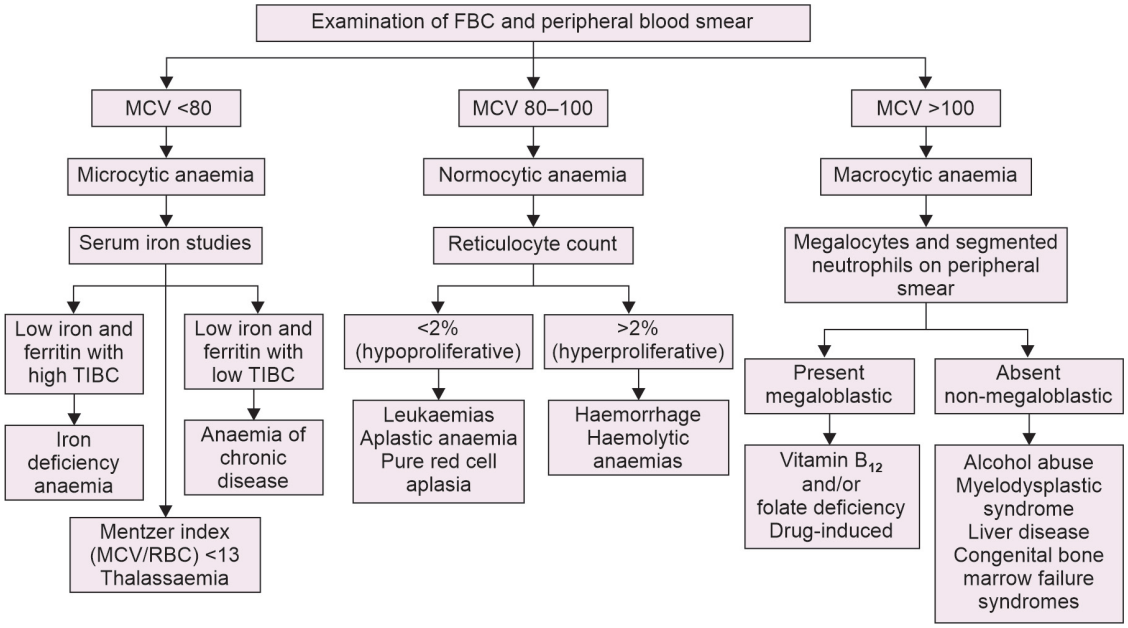
The "width" in RDW is sometimes thought to be "misleading" since in fact it is a measure of deviation of the volume of RBCs, and not directly the diameter. However, "width" refers to the width of the volume curve (distribution width, here presented as the coefficient of variation or CV) and not the width of cells. So, RDW–CV is more accurate term.

$$\text{RDW} - \text{CV} = \frac{\text{Standard deviation of MCV}}{\text{MCV}} \times 100$$

Significance of RDW

Usually RBCs have a diameter of 6–8 µm. Certain disorders, however, cause a significant variation in RBC size. Higher RDW values indicate greater variation in size of RBC. Value of RDW is often used together with MCV to determine the possible causes of the anaemia. It is mainly used to differentiate iron deficiency anaemia (RDW high) and thalassaemia especially thalassaemia trait or minor (RDW low). It is also used to differentiate an anaemia of mixed causes from an anaemia of a single cause.

Flowchart 11.1: Determining causes of different anaemia



High RDW (>14.5)

- Iron deficiency anaemia usually presents with high RDW and low MCV.
- Megaloblastic anaemia (high RDW and high MCV)
- Mixed deficiency (both iron deficiency and vitamin B_{12}/folic acid deficiency). Here RDW is high and MCV is variable.
- Recent haemorrhage (high RDW, normal MCV)
- Falsely high RDW: If EDTA blood is used instead of citrated blood.

Normal RDW (11.5–14.5)

- Thalassaemia (especially thalassaemia minor)
- Anaemia of chronic disease, hereditary spherocytosis, acute blood loss, aplastic anaemia.

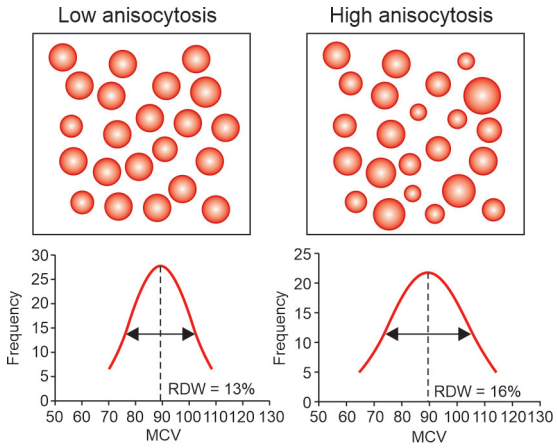
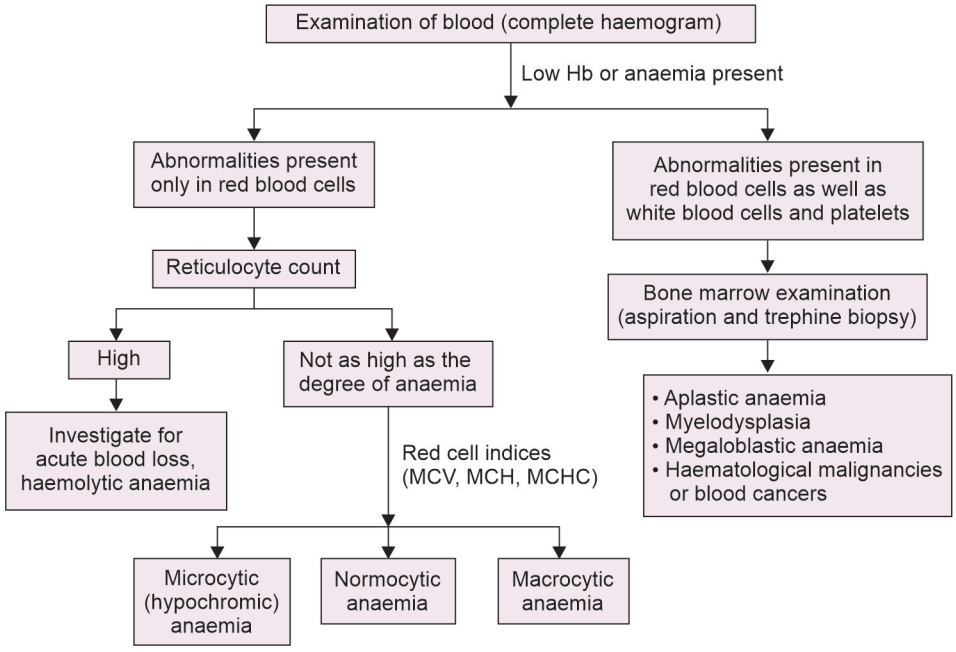


Fig. 11.1: Low RDW in low anisocytosis and high RDW in high anisocytosis

Table 11.3: Some hematological parameters and their significance

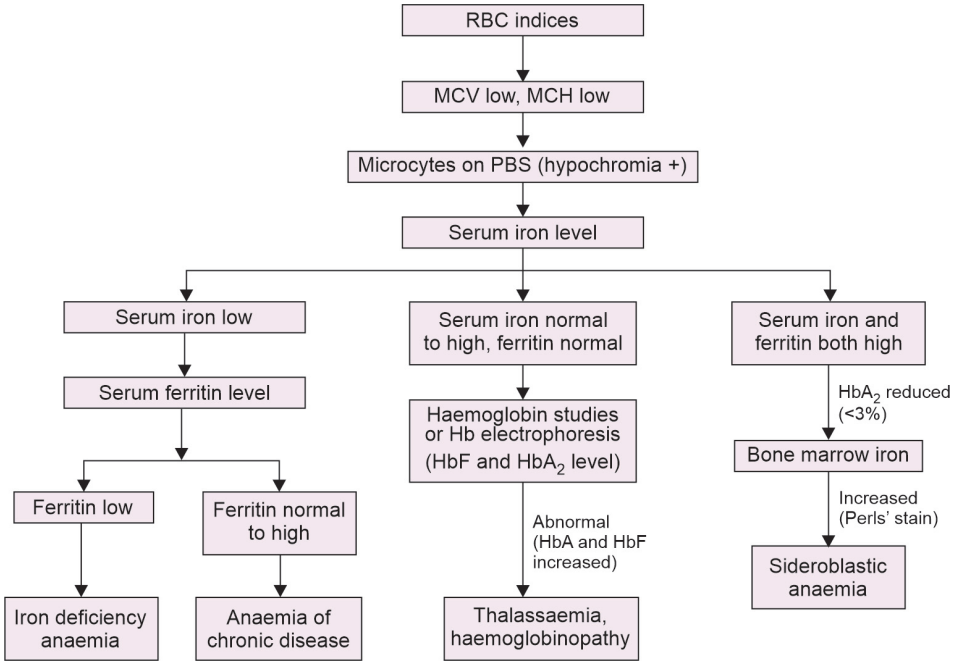
Parameter	Calculation	Normal range	Interpretations
MCV (mean corpuscular volume)	$MCV = \frac{PCV\%}{\text{Red cell count}} \times 10$ in million/mm^3. It is the average volume of a single RBC	80–100 fl	• Decreased in iron deficiency anaemia • Increased in megaloblastic anaemia
MCH (mean corpuscular Hb)	$MCH = \frac{Hb\,(g/dl) \times 100}{\text{Red cell count in mm}^3}$	27–32 pg	• Decreased in iron deficiency anaemia • Increased in megaloblastic anaemia
MCHC (mean corpuscular Hb concentration)	$MCHC = \frac{Hb\,(g/dl)}{PCV\,(\%)} \times 100$	32–36 g/dl	• Decreased in iron deficiency anaemia • Increased in hereditary spherocytosis • Normal in megaloblastic anaemia
PCV or haematocrit	The ratio of RBC to serum expressed in percentage	Men (39–45%) Women (33–43%)	• Decreased in anaemia (<30%) • Increased in severe dehydration, polycythaemia
RDW (red cell distribution width)	Index to measure degree of anisocytosis of RBC	11.5–14.5	• Increased in iron deficiency anaemia, megaloblastic anaemia, immune haemolytic anaemia • Normal in thalassaemia
ESR (erythrocyte sedimentation rate)	Measurement of sedimentation of RBC after 1 hour	Men: 4–14 mm/hr; women 6–20 mm/hr	• Very high (100 >mm/hr):TB, multiple myeloma, rheumatoid arthritis, macroglobulinaemia, Hodgkin disease • Very low (0–1 mm/hr): Polycythaemia, hypo- or afibrinogenaemia, congestive cardiac failure • Low: Sickle cell anaemia, spherocytosis

Flowchart 11.2: Simplified approach for evaluation of anaemia based on complete haemogram

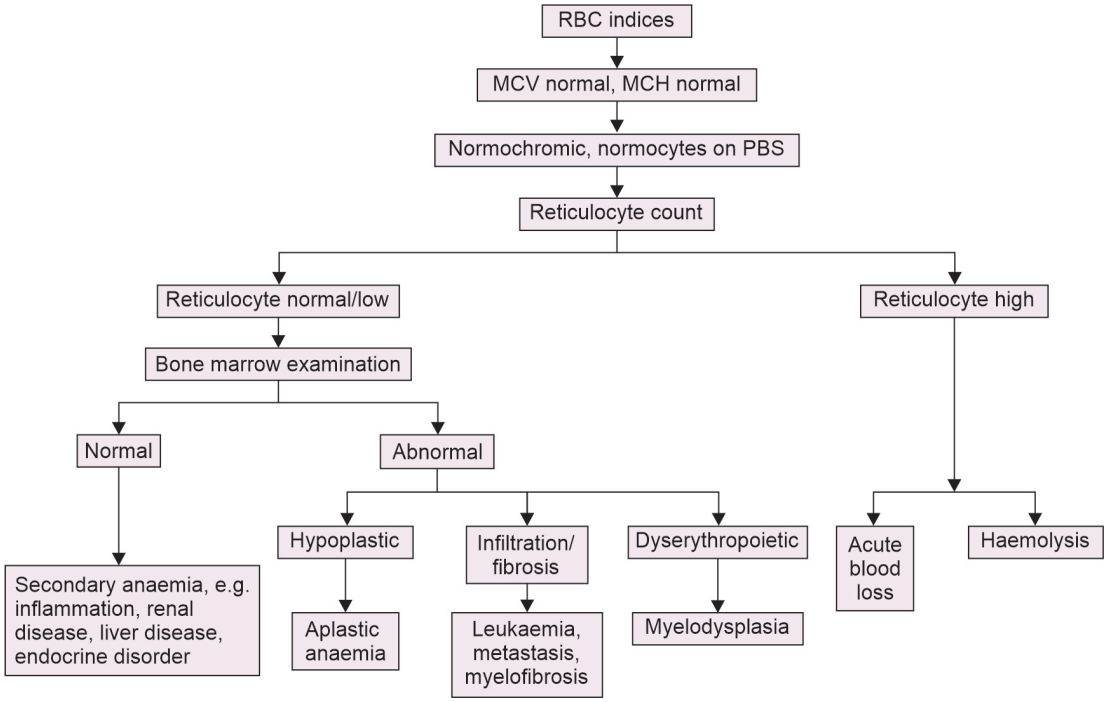


- **Complete haemogram tests:** Also known as complete blood count (CBC). It includes a wide array of tests: Total WBC count (TLC), total RBC count, haemoglobin (Hb), MCV, MCH, MCHC, platelet count, RDW, differential count (DLC), ESR, PCV and blood cell morphology on PBS.

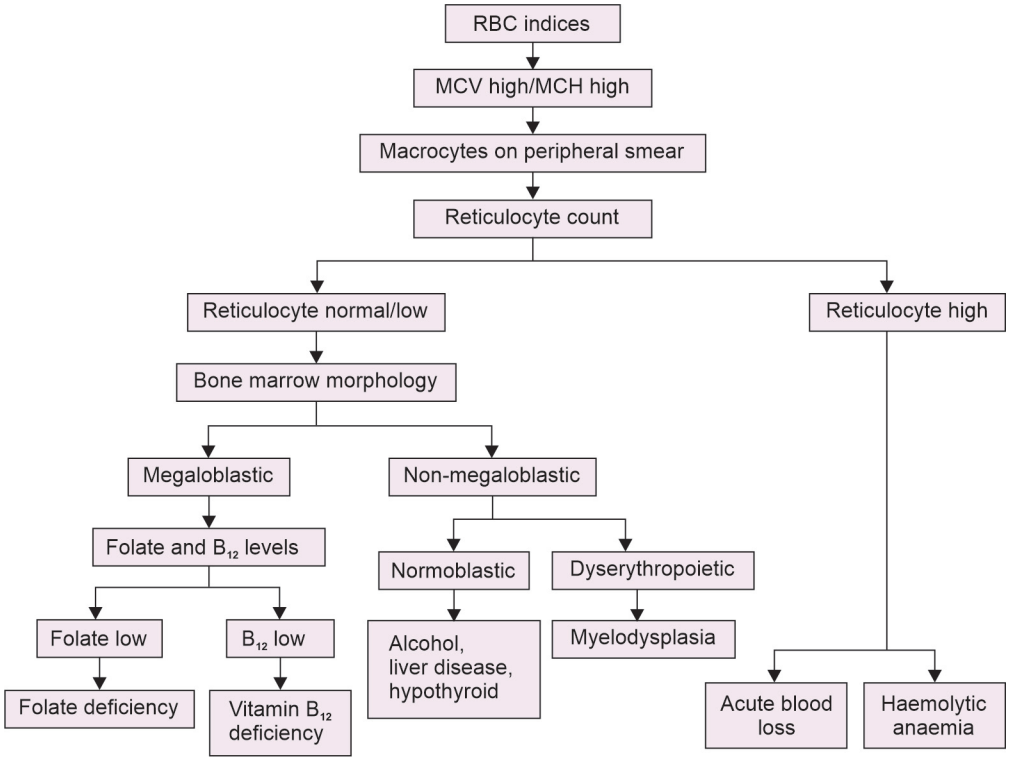
Flowchart 11.3: Investigation of microcytic hypochromic anaemia



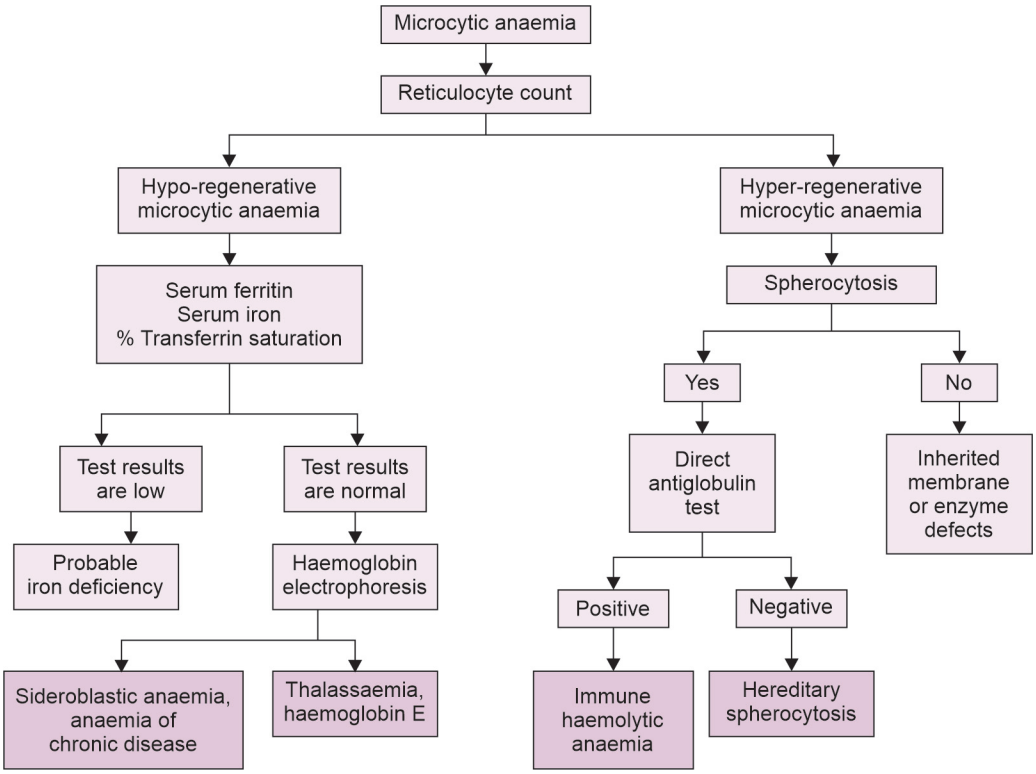
Flowchart 11.4: Investigation of a normocytic normochromic anaemia



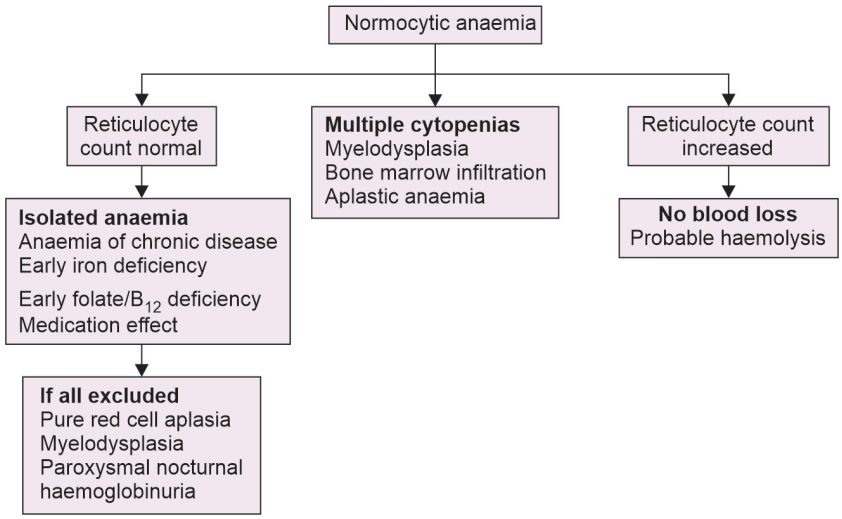
Flowchart 11.5: Investigation of a macrocytic anaemia



Flowchart 11.6A: Schematic diagram to investigate microcytic anaemia
(*Source:* Laboratory Medicine Diagnosis of Disease in Clinical Laboratory, 2/E (Lange)



Flowchart 11.6B: Schematic diagram to investigate normocytic anaemia



Flowchart 11.6C: Schematic diagram to investigate macrocytic anaemia

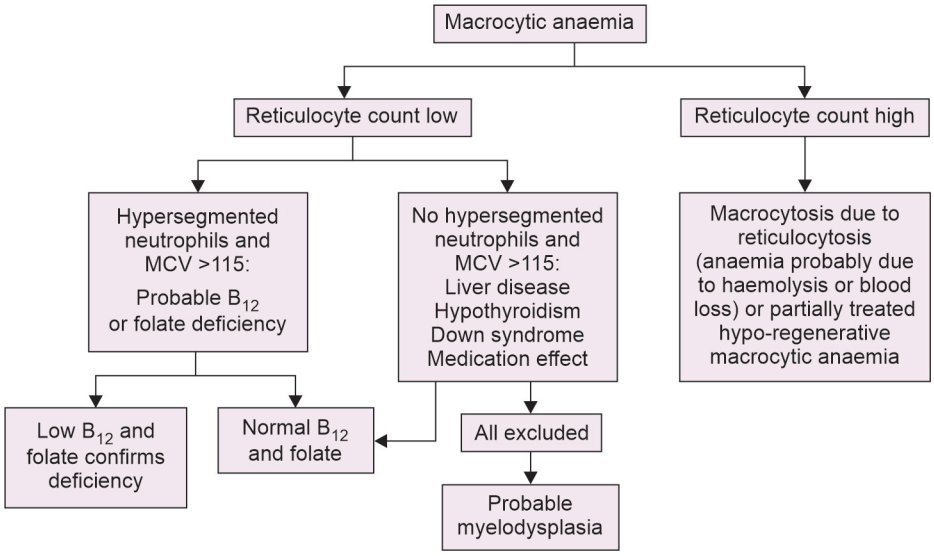
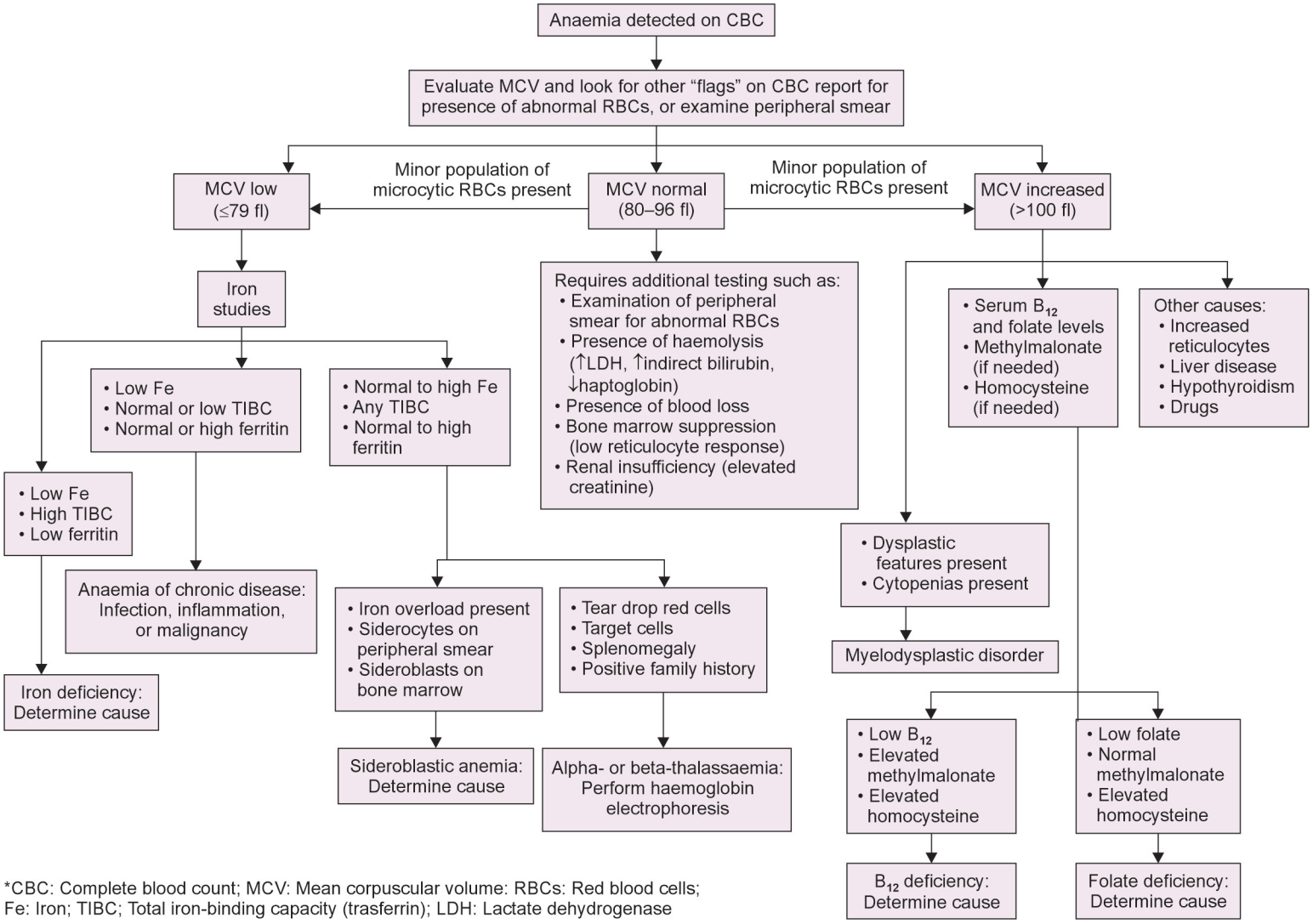


Table 11.4: Differential diagnoses of anaemia based on MCV and RDW

MCV	RDW	Causes
Normal	Normal	Anaemia of chronic diseases, sickle cell trait, hereditary spherocytosis
Normal	High	Early phase of iron deficiency or megaloblastic anaemia, sideroblastic anaemia, myelofibrosis, sickle cell anaemia
Low	Normal	Thalassaemia trait/carrier, anaemia of chronic disease
Low	High	Iron deficiency anaemia, sickle cell β-thalassaemia, haemoglobin H disease
High	Normal	Aplastic anaemia, myelodysplastic syndrome (MDS)
High	High	Megaloblastic anaemia, immune haemolytic anaemia

Flowchart 11.7: Simplified approach for evaluation of anaemia based on completed haemogram or complete blood count (CBC)



*CBC: Complete blood count; MCV: Mean corpuscular volume: RBCs: Red blood cells; Fe: Iron; TIBC; Total iron-binding capacity (trasferrin); LDH: Lactate dehydrogenase

HAEMATOLOGICAL INVESTIGATIONS IN MICROCYTIC HYPOCHROMIC ANAEMIA (IRON DEFICIENCY ANAEMIA)

The degree of anaemia in iron deficiency anaemia (IDA) may vary (Tables 11.5 and 11.6). Usually, anaemia is mild to moderate but sometimes it may be marked (Hb <6 g/dl) due to severe and persistent blood loss. Some of the relevant haematological investigations are:

- Low Hb level and low PCV (packed cell volume)
- Red cell indices: MCV less than <80 fl (usually <50 fl), MCH <27 pg (usually <15 pg), and MCHC <30 g/dl (usually <20 g/dl)

Table 11.5: Differentiating features of microcytic hypochromic anaemia

Parameter	Iron deficiency anaemia	β-Thalassaemia minor or trait	Anaemia of chronic disease
MCV	Decreased (<80 fl)	Markedly decreased	Normal or decreased
Red cell morphology	Microcytic hypochromic, pencil cells, anisocytosis	Microcytic hypochromic, basophilic stippling target cells, polychromasia	Microcytic hypochromic
RBC count	Decreased	Normal	Decreased
RDW (11.5–14.5)	Increased (>14.5)	Normal	Increased or normal
Serum iron	Decreased	Normal	Decreased
Serum ferritin	Decreased (<12 µg/L)	Normal	Normal or increased
TIBC	Increased	Normal	Decreased
Transferrin saturation	Decreased (16%)	Normal or increased	Normal or decreased
FEP or free erythrocyte protoporphyrin (30–50 µg/dl)	Increased	Normal	Increased
Hb electrophoresis	Normal	HbA_2 >3.5%	Normal
Bone marrow iron (haemosiderin)	Low or absent	Normal	Normal or increased

Table 11.6: Difference between iron deficiency anaemia and thalassaemia

Haematological parameters	Iron deficiency anaemia	Thalassaemia
Haemoglobin (Hb)	4–12 g/dl	Thalassaemia minor (trait): 9–14 g/dl Thalassaemia major: 2–7 g/dl
• MCV (fl)	74 (53–93)	Thalassaemia minor (trait): 68 (56–75); thalassaemia major: 64 (48–72)
• RBC count	Low	Usually normal
• Mentzer index (MCV/RBC count)	>13	Thalassaemia minor (trait): <13
• MCHC (g/dl)	28 (22–32)	Thalassaemia minor (trait): 31 (29–33) Thalassaemia major: 31 (23–32)

Contd.

Table 11.6: Difference between iron deficiency anaemia and thalassaemia (Contd.)

Hematological parameters	Iron deficiency anaemia	Thalassaemia
• RDW (11.5–14.5)	Markedly increased	Slightly increased
• Serum iron (50–150 µg/L)	Decreased	Usually normal (except in case of iron overload when it may increase)
Serum ferritin (15–300 µg/L)	Low (<12 µg/L)	Normal (except in case of iron overload when it may increase)
Transferrin saturation %	Low (<16%)	Normal (except in case of iron overload when it may increase)
Bone marrow iron	Low	Normal (except in case of iron overload when it may increase)
TIBC (300–400 mg/L)	Increased	Normal
Srivastava index (MCH/RBC)	>3.8	Thalassaemia minor (trait): <3.8
Shine and Lal index ($MCV^2 \times MCH/100$)	>1530	Thalassaemia minor (trait): <1530
England and Fraser index [MCV – (5 × Hb) – RBC]	> 0 (positive)	Thalassaemia minor (trait): <0 (negative)
Haemoglobin pattern on serum electrophoresis or HPLC	Normal	Abnormal. In thalassaemia minor: HbA_2 >3.5 % (3.6–8%), and HbF (1–5%); In thalassaemia major: HbA_2 <3.5% and HbF (10–95%).

- RDW: Value >14.5
- Reticulocyte count : It is normal or may be slightly reduced but may be slightly raised (2–5%) in cases of haemorrhage.
- **Red Blood cells (RBCs) or erythrocytes:** RBCs in PBS are microcytic and hypochromic. Anisocytosis and poikilocytosis are also present. Hypochromia generally precedes microcytosis. Poor filling of the RBCs with haemoglobin causes increased central pallor and hypochromia. In severe IDA, RBCs reveal only a thin rim of haemoglobin at the periphery (ring or pessary cells). Target cells, elliptical cells, pencil cells and polychromatic cells are often seen in PBS. Normoblasts are absent but may be seen in IDA with haemorrhage. RBC count is low but it is generally not proportionate to the fall in Hb level. When both iron deficiency and folate/vit B_{12} deficiency are present, a dimorphic blood picture (both microcytes and macrocytes).
- WBCs (leucocytes): The total leucocyte count (TLC) and differential leucocyte count (TLC) are usually normal. But if the IDA is due to parasitic infestations like hookworm infestation, then there will be eosinophilia.
- Platelets: Platelet count is usually normal but may be raised (mild to moderate degree) in patients who have recent bleeding.

Reticulocyte haemoglobin content (cHr):

This index is available in selected haematology analyzers. It measures the functional availability of iron during Hb synthesis in RBCs. A decrease in cHr is an early indicator of iron-deficient erythropoiesis in non - anemic patients. Multiple studies have shown a cHr <28 pg is a good indicator for iron deficiency anaemia.

Haematological Investigations in Macrocytic Anaemia

- Hb and PCV are low. The fall in Hb level or may vary.
- Red cell indices: MCV is high or >100 fl (usually >120 fl, proportionate to the

severity of macrocytosis), elevated MCH or >32 pg (usually 50 pg), and normal or reduced MCHC.

- RDW is high (>14) but not as high as IDA.
- Reticulocyte count: It is usually low to normal in untreated cases.
- RBCs (red cells): RBCs become oval macrocytes (appear fully haemoglobin zed). In addition, PBS demonstrates marked anisocytosis, poikilocytosis and presence of macro-ovalocytes. Basophilic stippling, Howell-Jolly bodies, Cabot rings, circulating megaloblasts (rare) (Fig. 11.2).

Sequence of change: Reduction of vitamin B_{12} and/or folic acid in blood → hypersegmented neutrophils → oval macrocytosis (macro-ovalocytes) → megaloblasts in bone marrow → macrocytic anaemia.

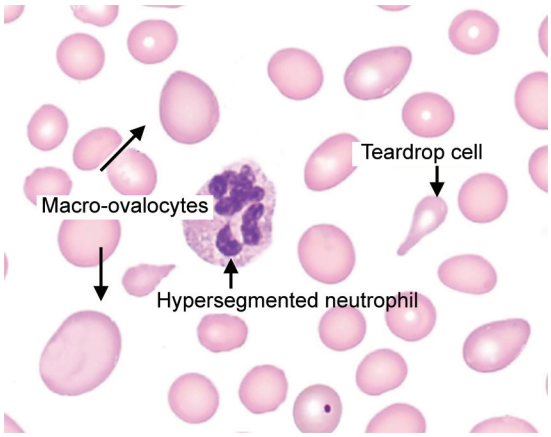


Fig. 11.2: Hypersegmented neutrophil, macro-ovalocytes and teardrop cell. Howell-Jolly body is also seen in an RBC in lower middle area

- **Leucocytes (WBCs):** TLC may be reduced. There may be hypersegmented neutrophils (>5% of neutrophils showing ≥5% lobes, or ≥1% of neutrophils showing ≥6 lobes). An occasional myelocyte or metamyelocyte may also be seen.
- **Bone marrow** (Fig. 11.3)**:** It shows megaloblasts, erythroid hyperplasia, giant metamyelocytes and band neutrophils. Nuclei of megaloblasts lags behind cytoplasm in maturation (nuclear-cytoplasmic asynchronous erythropoiesis), i.e. large nuclei with open chromatin, and relatively haemoglobinized cytoplasm. There is destruction of mature erythroid cells and accumulation of primitive erythroid cells (ineffective erythropoiesis). Megakaryocytes are enlarged and hyperpolypoid.

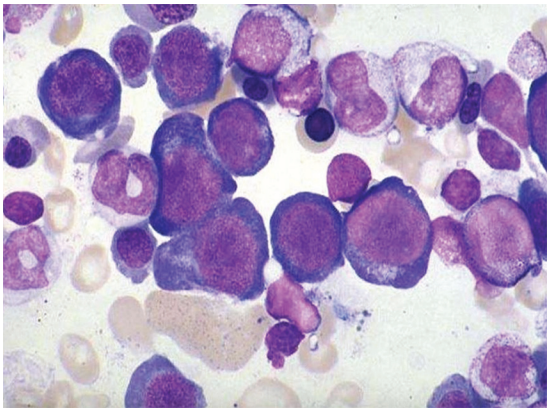


Fig. 11.3: Megaloblasts in bone marrow

- **Platelets:** Platelets count may be moderately reduced in severely anemic patients, bizarre forms of platelets may be seen.
- Blood chemistry: Increased indirect serum bilirubin and lactate dehydrogenase.
- Serum methylmalonic acid: Raised value is the most sensitive indication of vitamin B_{12} deficiency.
- **Causes of oval macrocytes:** Megaloblastic anaemia due to deficiency of folate or vitamin B_{12}, drug therapy (hydroxyurea, anticancer drug like methotrexate, zidovudine).
- **Causes of round macrocytes:** Liver diseases, alcoholism, hypothyroidism.

Table 11.7: Differences between normoblasts and megaloblasts

Parameter	Normoblast	Megaloblast
Cell size	Normal	Larger than normoblast
Nuclear chromatin	Not open type	Open, sieve like or stippled
Nuclear maturation (progressive condensation of nuclear chromatin)	Normal	Lags behind cytoplasmic maturation (haemoglobinization). This is known as **nuclear-cytoplasmic asynchrony or dissociation**.
Maturation in bone marrow	Normal normoblastic maturation (more early normoblasts than late normoblasts which is called **maturation arrest**)	Increased number of early megaloblasts than late megaloblasts (early > intermediate > late megaloblasts)
Granulocytic series (myelopoiesis)	Normal	Displays megaloblastic changes. Giant metamyelocytes are seen with horseshoe-shaped nuclei and finer nuclear chromatin. Band neutrophils are large
Mitosis	Normal	Increased and abnormal
Dyserythropoiesis	Absent	Present (Howell-Jolly bodies, Cabot rings, irregular nuclei)
Megakaryocytes	Normal	Large with multiple nuclear lobes and paucity of cytoplasmic granules
Found in	Normal bone marrow	Bone marrow of megaloblastic anaemia

HEMATOLOGICAL INVESTIGATIONS IN HAEMOLYTIC ANAEMIA/THALASSAEMIA

- Hb level very low (severe anaemia in thalassaemia major, mild anaemia in thalassaemia minor or trait). PCV is also low.
- Red cell indices: MCV, MCH and MCHC are significantly reduced
- RDW: Usually normal in thalassaemia minor/trait.
- Reticulocyte count: It is raised
- Serum bilirubin: Unconjugated bilirubin is raised.
- RBCs: Severe microcytic hypochromic red cells. Marked anisopoikilocytosis, basophilic stippling, many target cells, teardrop cells and normoblasts or nRBCs.
- Leucocytes (WBCs): TLC is raised most of the time. DLC shows some shift to left of the neutrophil series, with presence of some myelocytes and metamyelocytes."
- Platelets: Platelet count is usually normal. But it may be reduced if the patients have massive splenomegaly.
- Bone marrow: It shows normoblastic erythroid hyperplasia with predominance of intermediate and late normoblast granules. These normoblasts are usually normal in size (micronormoblasts). Iron staining demonstrates siderotic granules in the cytoplasm of normoblasts and increased iron in reticuloendothelial cells. Ring sideroblasts are rarely seen.
- Osmotic fragility test: It is done by NESTROF (naked eye single tube rapid osmotic Fragility) test which characteristically reveals increased resistance to saline haemolysis, i.e. decreased osmotic fragility.
- Alkali denaturation test: Increased HBF in thalassaemia major and in hereditary persistent of foetal haemoglobin (HPFH).

- HbA_2 estimation: Done by HPLC (high performance liquid chromatography), column chromatography or HB electrophoresis.
- Haemoglobin electrophoresis: There may be increased amount of HbF; increased amount of HbA_2, and almost complete absence or presence of variable amount of HbA.

LABORATORY INVESTIGATIONS OF DIFFERENT THALASSAEMIAS

Classification: There are two main forms of thalassaemias.

- α-Thalassaemias (deficient/absent synthesis of α-globin chains)
- β-Thalassaemias (deficient/absent synthesis of β-globin chains)

There are three clinical forms of thalassaemias: Thalassaemia major, thalassaemia intermedia and thalassaemia minor.

1. **Thalassaemia major (homogygous thalassaemia, or Cooley's anaemia)**
   - Severe degree of anaemia (Hb <7g/dl).
   - Reticulocytosis (5–15%).
   - Low MCV, MCH and MCHC
   - Blood smear shows marked anisopoikilocytosis (increased RDW or >14), red cell fragments, hypochromic, target cells, basophilic stippling, and nRBCs (nucleated red cells).
   - Markedly elevated HbF on electrophoresis (HbF 10–95%).
   - α/β ratio >3.
2. **β-Thalassaemia intermedia**
   - Moderate degree of anaemia (Hb 7–10 g/dl).
   - Reticulocytosis (5–10%).
   - Hb F variable (5–90%)
   - α/β ratio: >2
   - Blood smear shows less severe abnormalities than thalassaemia major.
3. **β-Thalassaemia minor (thalassaemia trait)**
   - Mild anaemia on normal Hb (>10 g/dl).
   - Low MCV, MCH but normal MCHC
   - Normal serum ferritin
   - HbA_2>3.5 % (3.5–8%), HbF < 5%
   - α/β ratio: 1.2–2.0
   - Blood smear: Microcytic hypochromic RBCs, target cells, and basophilic stippling (Table 11.8).

δβ-Thalassaemia

In the homozygous state, these forms are characterized by thalassaemia intermedia and absence of both HbA and HbA_2 and almost 100% HbF. Heterozygous states have thalassaemia minor with 5–20% HbF and normal HbA_2.

Table 11.8: β-Thalassaemias

Syndrome	Genotype	Clinical features	Haemoglobin pattern
Homozygous state			
• β^+-Thalassaemia	β^+/β^+	Thalassaemia major or intermedia	↓HbA, ↑HbF, variable HbA_2
• β^0-Thalassaemia	β^0/β^0	Thalassaemia major	0 HbA, variable HbA_2, residual HbF
• δβ-Thalassaemia	$\delta\beta^0/\delta\beta^0$	Thalassaemia intermedia	0 HbA, 0 HbA_2, 100% HbF
Heterozygous State			
• β^+-Thalassaemia	β^+/β	Thalassaemia minor	↑HbA_2, slight ↑ HbF
• β^0-Thalassaemia	β^0/β	Thalassaemia minor	↑HbA_2, slight ↑ HbH
• $\delta\beta^0$-Thalassaemia	$\delta\beta^0/\delta\beta$	Thalassaemia minor	5–20% HbF, normal HbA_2

Flowchart 11.8: Diagnosis of different types of α- and β-thalassaemias

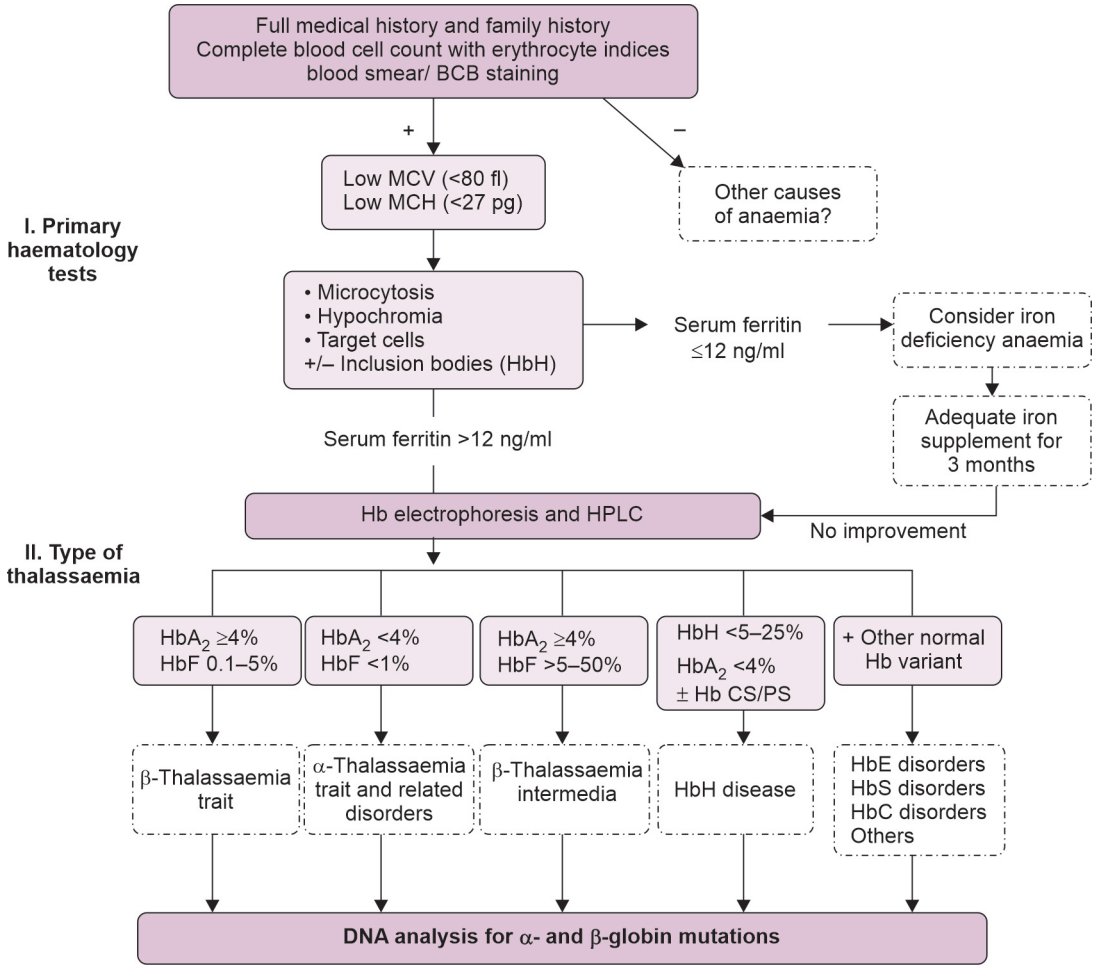


Table 11.9: Different α-thalassaemias and Hb pattern

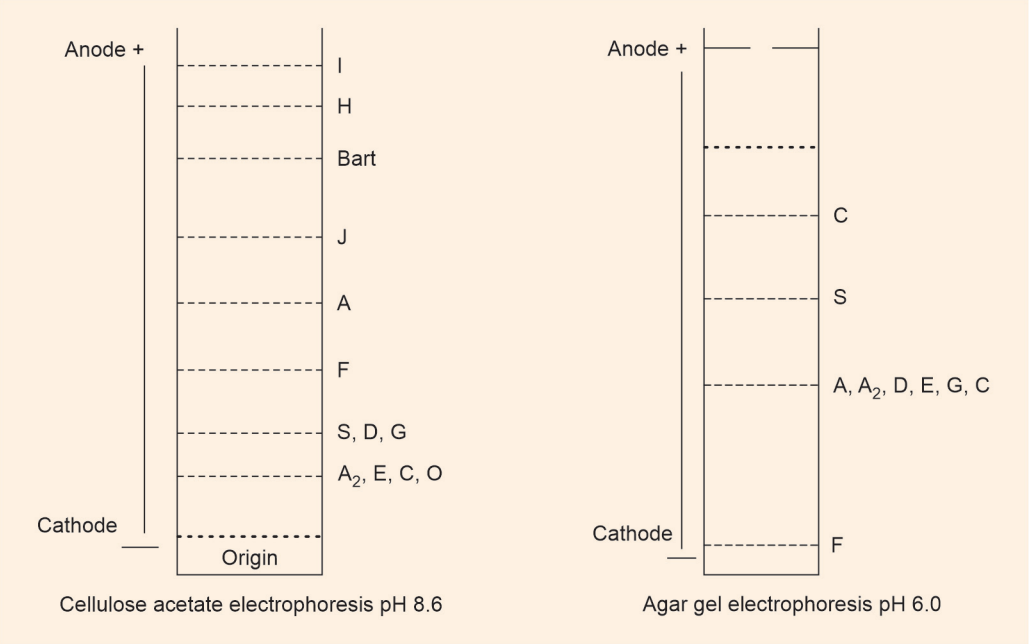
Syndrome	Genotype	Clinical features	Hb in newborn	Hb after first year
Hydrops foetalis	–/–	Foetal or neonatal death with severe anaemia	Hb Bart's (γ_4) >80%	—
HbH disease	–/–α	Chronic haemolytic anaemia	Hb Bart's (γ_4) 20–40%	HbH (excess β or β_4) 5–30%, Hb Bart's trace
Thalassaemia minor	–/αα	Little or no anaemia: Thalassemic RBC	Hb Bart's 2–20%	None
Silent carrier	–α/αα	No clinical or haemato logical abnormality	Hb Bart's 1–2%	None
Normal	αα/αα	No clinical or haemato-logical abnormality	Hb Bart's O-trace	None

HAEMOGLOBIN ELECTROPHORESIS

Haemoglobin molecules in an alkaline solution have a net negative charge and move toward the anode in an electrophoretic system. Those with an electrophoretic mobility greater than that of HbA at pH 8.4–8.6 are known as the fast haemoglobins. These include Hb Bart and the two fastest HbH and HbI. HbC is the slowest of the common haemoglobins.

A practical method for routine haemoglobin electrophoresis is cellulose acetate at alkaline pH (8.4–8.6). It is rapid and reproducible and separate haemoglobins S, F, C, A and A_2.

Citrate agar electrophoresis at an acid pH (6.0–6.2) provides ready separation of haemoglobin that migrate together on cellulose acetate: Haemoglobin S from haemoglobin D and haemoglobin G; and haemoglobin C from haemoglobin E and haemoglobin O (Fig. 11.4).



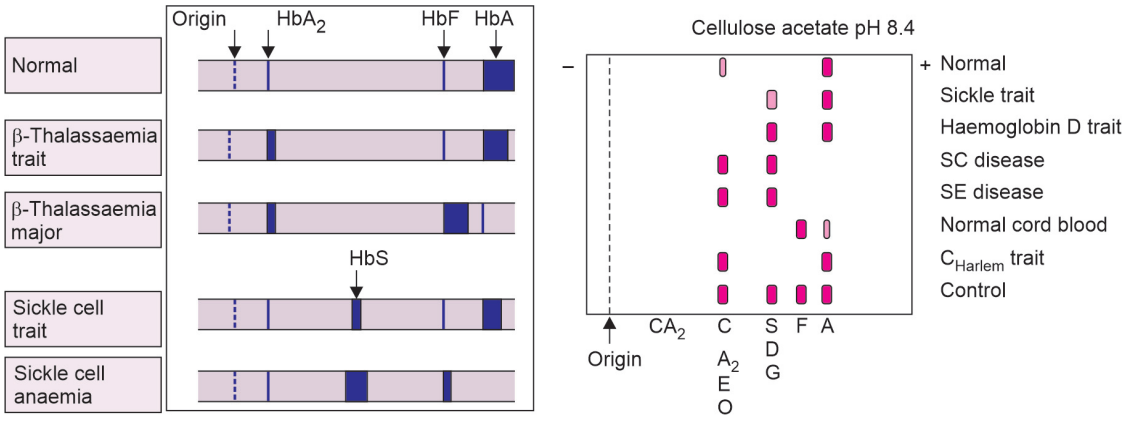


Fig. 11.4: Haemoglobin electrophoresis (cellulose acetate membrane)

Estimation of Foetal Haemoglobin (HbF)

Foetal haemoglobin ($\alpha_2 \gamma_2$) IS THE Predominant form of haemoglobin during foetal life. This foetal haemoglobin or HbF gradually falls and is approximately 25% at one month, 5% at 6 months and <2% at 1 year. During adult life HbF is <1%.

High level of HbF

- Significant increase of HbF occurs in β-thalassaemia major, hereditary persistence of foetal haemoglobin (HPFH), δβ-thalassaemia and sickle cell anaemia.
- Mild increase of HbF occurs during pregnancy and in aplastic anaemia, megaloblastic anaemia, PNH (paroxysmal nocturnal haemoglobinuria, chronic leukaemia and erythroleukaemia.

There are various methods for estimating HbF. If the HbF level is >2%, then it can be identified on serum electrophoresis.

Two commonly used methods for estimation of HbF are

Betke method and **Jonxis and Visser method**. The Betke method is more popular. These methods are done manually in the laboratory. Besides, HPLC (high performance liquid chromatography) method which is done by machine is also available.

MODIFIED BETKE METHOD FOR ESTIMATION OF HBF

Principle of the test: To measure the percentage of HbF in a mixture of haemoglobins, NaOH (sodium hydroxide) is added to a lysate. After a set time the denaturation of haemoglobin protein is stopped by adding saturated ammonium sulphate. Addition of ammonium sulphate lower the pH and precipitates the denatured haemoglobin. The adult haemoglobin (HbA) is denatured but the foetal haemoglobin (HbF) is resistant to this alkali (NaOH) denaturation.

After filtration, the quantity of undenatured or unprecipitated haemoglobin is measured. The proportion of alkali-resistant HbF is then calculated as a percentage of the total amount of haemoglobin present.

Reagents

- Cyanide solution: Potassium cyanide 0.2 g, potassium ferricyanide 0.2 g, water to 1 liter (1000 ml).
- Sodium hydroxide solution: 1.2 mol/L. Sodium hydroxide 48 g, water to 1 litre.
- Saturated ammonium sulphate.

Method

- Before use, all the reagents should be kept at room temperature. Add 0.6 ml of lysate (100 g/L) to 10 ml of cyanide solution, to make a cyanomethaemoglobin (HiCN) solution.
- Add 0.2 ml of sodium hydroxide solution to 2.8 ml of the HiCN solution. Mix thoroughly and leave for exactly 2 mins at room temperature.
- Add 2 ml of saturated ammonium sulphate, mix thoroughly and allow standing for 10 minutes.
- Fitter through a Whatman No. 42 filter paper and collect the filtrate.
- Using the remaining HiCN solution as the standard, read the absorbance of the filtrate and the standard in a spectrophotometer at either 413 nm or at 520 nm.
- Calculate the percentage (%) of HbF as follows:

$$\% \text{ HbF} = \frac{A^{520} \text{ filtrate} \times 100}{A^{520} \text{ standard} \times 100}$$

Where A^{520} is absorbance using 520 nm filter.

Note

The raised HbF control should not contain >10% HbF. This control HbF can be prepared from a mixture of cord blood and adult blood.

Each laboratory must establish its own normal range and this should not differ significantly, for adults the range is 0.2 – 1%.

Results and interpretation of HbF values

HbF level	Interpretation
• HbF 0.2–1%:	Normal result
• HbF 1.0–5.0%:	In 30–50% of β-thalassaemia trait/minor. Some individuals with aplastic anaemia, myelodysplastic syndrome (MDS), pregnant women (third trimester)
• HbF 5.0–20.0 %:	Occasional β-thalassaemia trait/minor. Some types of heterogzyous HPFH. Also seen in 8β-thalassaemia
• HbF 15.0–45.0%:	Heterozygous HPFH (African type). Some cases of β-thalassaemia intermedia.
• HbF > 45%:	β-thalassaemia major. Homo- and zygous African type HPFH and neonates

Flow Cytometric Detection of Fetal Red Blood Cells (Fig. 11.5 and Table 11.10)

Table 11.10: Interpretation of haemoglobin F (HbF) values

HbF range(%)	Interpretation
• 0.2–1.0%	Normal adults
• 1.0–5.0%	In approximately 30% of thalassaemia traits, some pregnant women (2nd trimester). Some hematological disorders (aplastic anaemia, MDS, juvenile myelomonocytic leukaemia).
• 5–20%	Occasional cases of β-thalassaemia trait, some types of HPFH or δβ thalassaemia
• 15–45%	Heterozygous HPFH (African type) (usually 20%), some cases of β-thalassaemia intermedia
• >45%	β-Thalassaemia major, some cases of β-thalassaemia intermedia
• >95%	Homozygous HPFH (African type), some neonates (particularly if premature)

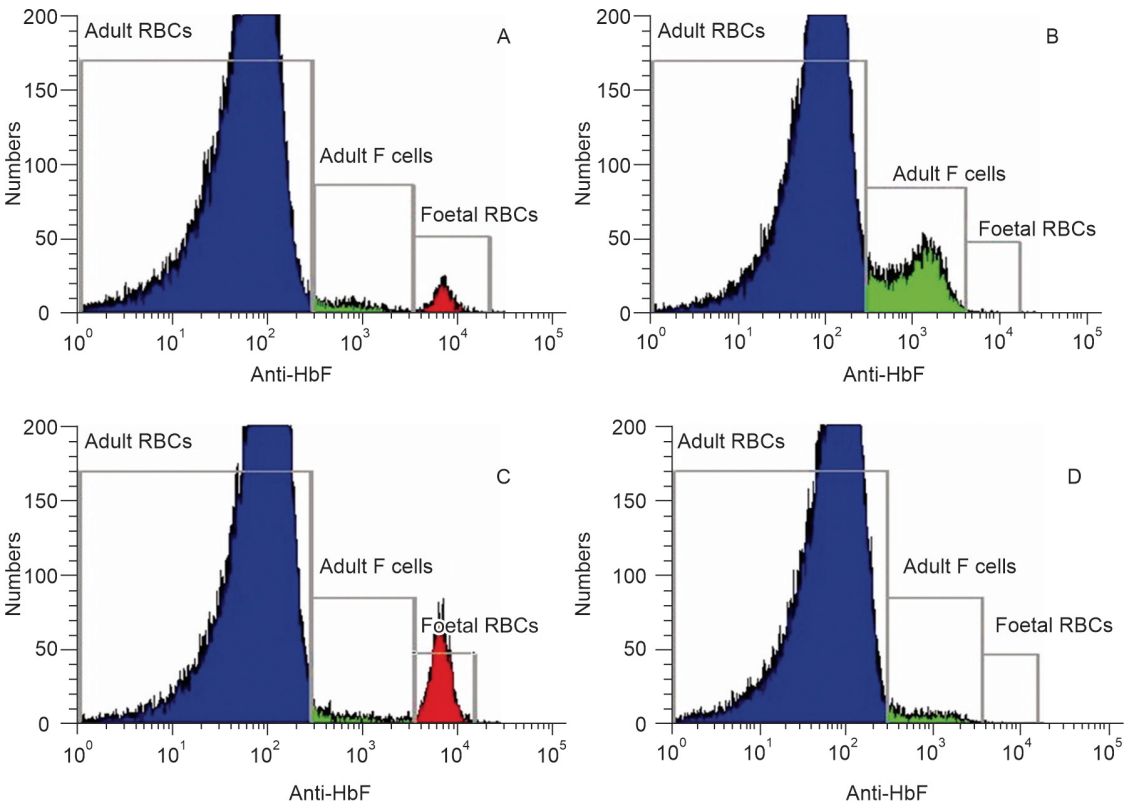


Fig.11.5: Flow cytometric detection of foetal red blood cells/RBCs

Cellulose Acetate

Principle of the test: HbA_2 is separated from HbA on cellulose acetate electrophoresis at pH 8.9, eluted into buffer and the percentage calculated by measuring and comparing the absorbance of the HbA_2 eluate and the eluate prepared from the remaining haemoglobin.

Equipment

- Electrophoresis tank and power pack
- Wicks of double filter paper
- 10 µl micropapillary pipette
- Cellulose acetate membranes (10 × 5 cm size)
- pH meter

Reagents

- *Electrophoresis buffer:* Tris/EDTA/borate (TEB,) pH 8.9. Tris 14.4 g, EDTA 1.5 g, boric acid 0.9 g, water to make 1 litre. The buffer should be stored at 4°C and the pH checked before use.
- *Working buffer:* KCN 200 mg, stock buffer 100 ml; water to 2 liters. Adjust the pH to make it 8.5 with concentrated HCl.

Preparation of Haemolysate

Blood is taken in EDTA anticoagulant. Packed red cells are prepared by centrifugation. The cells are washed three times in isotonic saline (0.15 mol/L).

Preparation of Lysate for Immediate use

Lyse 1 volume of washed packed cells in 4 volumes of lysing reagent prepared as follows:

- EDTA (tetrasodium salt): 3.8 g
- Potassium cyanide (KCN): 0.7 g
- Water to 1 litre

This type of lysate can be used only for 1–2 days, at 4°C as it tends to gel. If required it can be frozen at –20°C for up to 1 month. But try to avoid repeated freezing and thawing (bring to room temperature).

Preparation of Lysate for Long-term Storage

Lyse 2 volumes of washed packed cells in 1 volume of distilled water. To it, add 1 volume of CCl4 (carbon tetrachloride). After vigorous shaking, preferably using a mechanical agitator, centrifuge the mixture at 3000 rpm (1200 g) for 30 minutes. Transfer the clear solution to a tube and adjust the haemoglobin concentration to 100 g/L with water.

These prepared samples can be stored frozen at –20°C for up to 3 months.

Method

- With the power supply disconnected, fill the electrophoresis tank with TEB buffer. Soak and position the wicks.
- In a separate disk soak the cellulose acetate membrane in TEB buffer for at least 5 min. Remember the membrane should be immersed slowly and avoid trapping air bubbles. Ensure even saturation of the membrane.
- Blot the cellulose acetate membrane between two pieces of absorbent paper. But do not let it dry out before sample application.
- Stretch the strips across the bridges of the tank so that they are connected to the buffer chambers by a double-layered wick of filters paper.
- Apply 10 µl of haemolysate (100 g/L) to the cathode end of the membrane by micropipette. The application should stretch to within 0.5 on of the edge of the strip.
- Connect the power supply and run at 3 mA for 30–40 min or until a good separations is achieved.
- Disconnect the power supply, remove the membrane. Cut the HbA_2 and HbA zones into small pieces and elute into 1.5 ml and 15 ml of buffer respectively.
- Allow to elute with occasional mixing for 20–30 minutes.
- Remove the eluted pieces and centrifuge the elutes at 1200 g for 5 min to sediment the debris.

- Measure the absorbance of each eluate at 413 nm using a spectrophotometer and using TEB buffer as the blank.
- Calculation of HbA_2 using this formula:

$$\% \, HbA_2 = \frac{A^{413} \, HbA_2}{(10 \times A^{413} \, HbA) + A^{413} \, HbA_2} \times 100$$

Notes

- If a haemoglobin variant is present, the eluate used to calculate the major Hb fraction must include this variant.
- Particular care must be taken when cutting strips on which HbS is present, as the separation between HbS and HbA_2 is diminished.
- This method is unsuitable in the presence of HbsC, HbC_{Harlem}, HbE and HbO_{Arab}. Because they all have electrophoretic mobilities similar to that of HbA_2.

Table 11.11: Interpretation of haemoglobin A_2 (HbA_2)

HbA_2 range (%)	Interpretation
• < 2%	δβ-Thalassaemia (if HbF is increased), α-thalassaemia trait, haemoglobin H disease, δ-thalassaemia, iron deficiency anaemia
• 2–3.3%	Normal, δβ-thalassaemia (if HbF is increased, α-thalassaemia trait, α- or δ-chain variant)
• 3.4–3.7%	Severe iron deficiency anaemia in β-thalassaemia trait, some β-thalassaemia trait.
• 3.8–7.0%	β-Thalassaemia trait, unstable haemoglobin
• >7%	Rare; exclude a structural variant. Repeat HbA_2 estimation. Rare β-thalassaemia mutations

Laboratory Tests for Sickle Cell Disease

Blood: The anaemia is normochromic and normocytic: polychromatia is increased; and

Flowchart 11.9: Evaluation of haemolytic anaemia

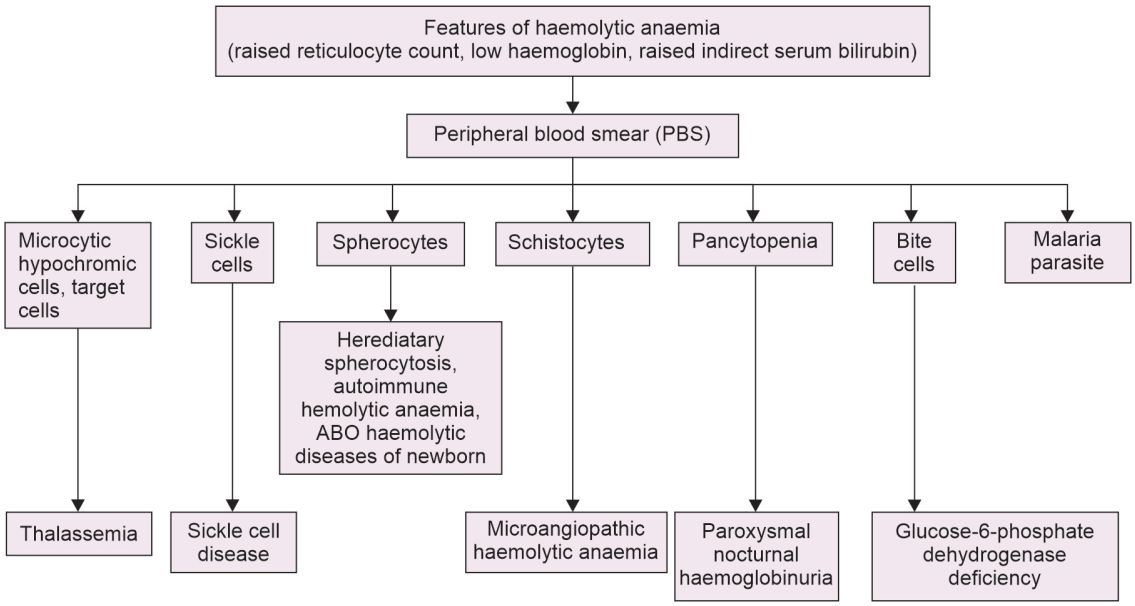


Table 11.12: Different haemoglobin in normal and in haemoglobinopathy

Person/Patient	HbA	HbF	HbA_2	HbS
Normal newborn	25%	75%	<1%	0
Normal adults	97%	<1%	1–3%	0
β-Thalassaemia minor	90–75%	0–5%	3.5–7%	0
β-Thalassaemia major	0–5%	95–98%	2–5%	0
Sickle cell trait	56–60%	0	1–3%	40%
Sickle cell anaemia	0	5–10%	1–3%	90–95%

normoblasts are present. Target cells are numerous (sickle cell trait) and Howell-Jolly bodies are regularly seen in older children and adults as a result of asplenia.

Sickle cells are often found in the stained smears. ESR is decreased as sickling prevents rouleaux formation. But the micro haematocrit (PCV) as an estimate of degree of anaemia is unreliable because of excessive plasma trapping. Osmotic fragility is decreased and mechanical fragility is increased. Neutrophilia and thrombocytosis are usual. The marrow shows normoblastic hyperplasia and increased storage iron (Fig. 11.6).

Sickle Cell Slide Test (Sickling Test)

Principle of the test: Sickling is induced by adding a reducing (oxygen consuming) agent like 2% sodium metabisulphite or sodium dithionite to blood sample.

Method

- Take a clear glass slide.
- Place 1 drops of 2% sodium metabisulphite solution.
- Add 1 drop of anticoagulated venous or capillary blood
- Mix it properly and place a cover slip on the slide.
- Seal the edges of coverslip with petroleum jelly paraffin wax or nail polish.
- Wait for 30 minutes and examine under microscope.
- If sickle cells are not seen, examine the slide gain after 2 hours, and 24 hours.

Result

Positive: If red cells become sickle-shaped (crescent-shaped with pointed ends) or holly-leaf shaped (Fig. 11.7).

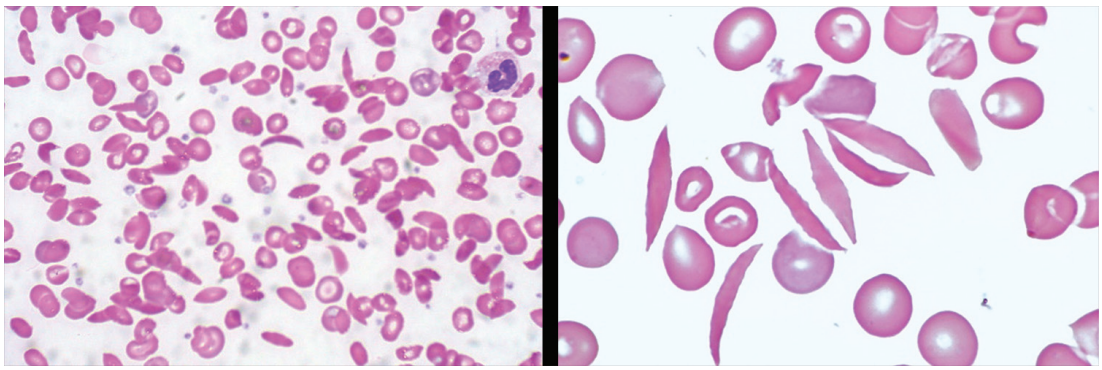
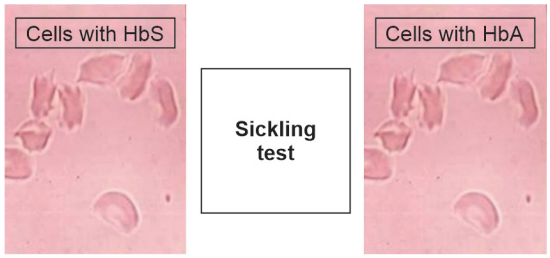


Fig.11.6: Peripheral blood smear shows many sickle cells (RBCs)

Negative: If the red cells remain normal looking round shaped.



Method	Use
• 2 drops of 2% sodium metabisulphite + • 1 drop of blood observed at 15 and 30 minutes	• It is a qualitative test • This test is used to identified HbS erythrocytes

Fig.11.7: Sickling test by sodium metabisulphite

Note

- A positive test indicates presence of HbS.
- The test cannot differentiate between sickle cell disease (homozygous) and sickle cell trait (heterozygous).
- However, in sickle cell disease both sickle cells and target cells are seen on PBS while in sickle cell trait only target cells are seen.
- Hb electrophoresis is useful for confirmation of HbS and to differentiate between various sickle cell disorders (sickle cell trait, sickle cell anaemia, sickle cell-β thalassaemia, sickle cell D-Punjab disease, etc.)
- Concentration of HbS may be low in infants below 6 months, in severe anaemia or following recent blood transfusion.
- False positive test can occur if the concentration of sodium metabisulphite is excessive or if there is drying of the wet smear.

Solubility Test for Haemoglobin S (Fig. 11.8)

In this test, anticoagulated venous blood is added to the reagent solution (consisting of phosphate buffer, saponin and sodium dithionite). RBCs are hemolyzed and if HbS is present, it will be reduced by dithionite. HbS forms tactoids and refract light. The solution appears turbid, whereas normal Hb gives a clear solution.

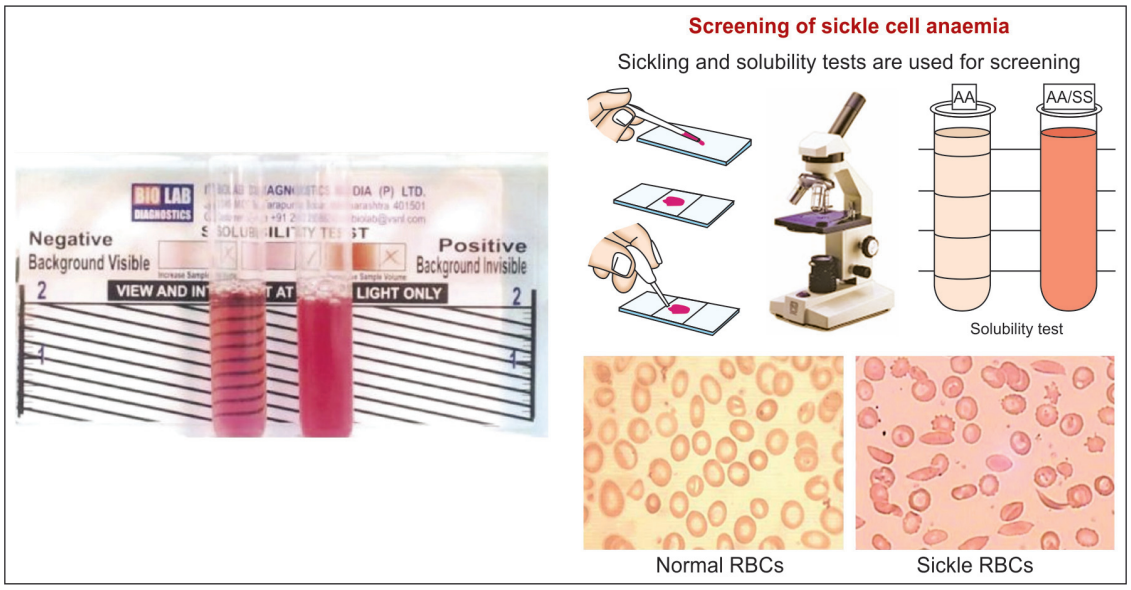


Fig.11.8: Sickling and solubility test

Note

- The test cannot differentiate between various sickle cell disorders.
- False positive cases; polycythaemia, paraproteinemias, hyperlipidemia and marked leukocytosis.
- False negative cases: Outdated or old reagent, recent blood transfusion and low concentration of HbS (e.g. infants <6 months, severe anaemia).

NESTROFT (Naked Eye Single Tube Red Cell Osmotic Fragility Test)

Method

- 0.02 ml of patient's blood + 5 ml of 0.35% saline in a test tube.
- Similarly, a control tube contains 0.2 ml of blood of normal person + 5 ml of 0.35% saline.
- After 30 minutes, white paper with a dark black line is held behind the tube.
- The microcytic hypochromic RBCs of thalassaemia minor/trait are resistant to lysis (hence not clear) compared to normocytic normochromic RBCs of control (normal person).
- Hence, the black line on the paper is not clearly visible through the test tube containing patient's blood (of thalassaemia minor/trait) compared to test tube containing blood of normal person.

Result

Positive: Thalassaemia minor/trait
Negative: Normal person

Note

- Naked eye single tube red cell osmotic fragility test (NESTROFT) is a screening test and not a diagnostic test for thalassaemia minor on trait.
- All antenatal females with Hb <11 g/dl should undergo NESTROFT (Fig. 11.9).

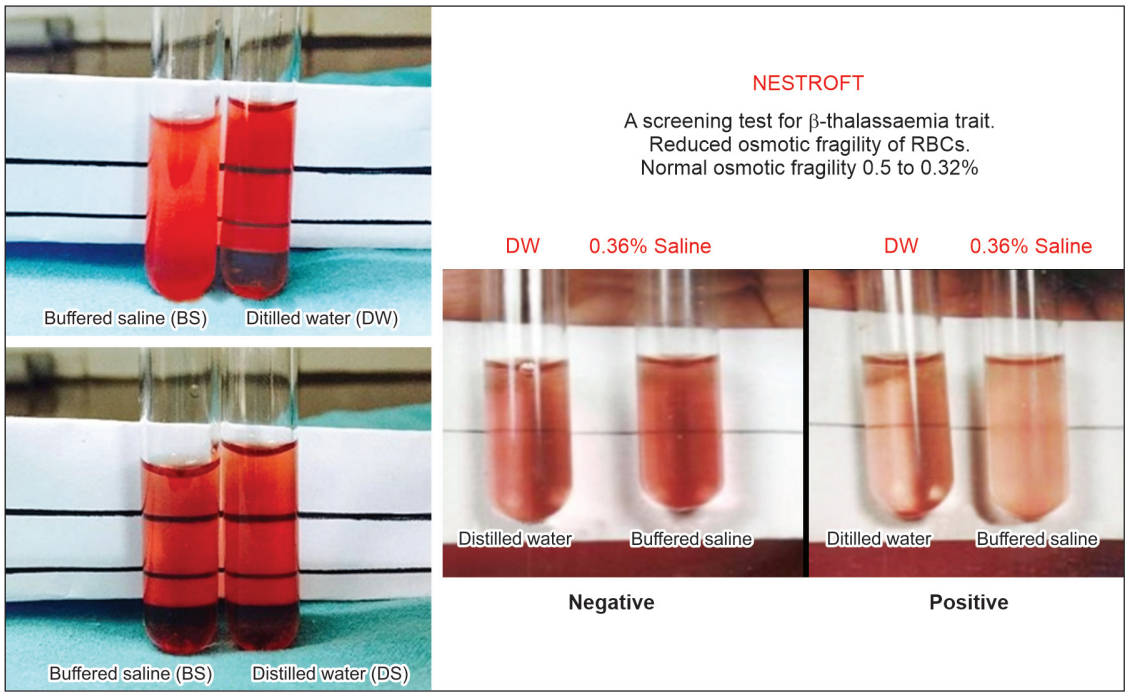


Fig. 11.9: Osmotic fragility test for β-thalassaemia minor/trait

LABORATORY TESTS FOR HEREDITARY SPHEROCYTOSIS

Osmotic Fragility Test (Fig. 11.10)

Principle of the test: In this test, red cells are suspended in decreasing concentrations of hypotonic saline solutions to determine the ability of the red cells to withstand osmotic stress.

In hypotonic solutions, water enters into RBCs causing cellular swelling, and at one point, cell lysis occurs. Normal RBCs are biconcave and disc-shaped and have high surface area to volume ratio. So, they permit entry of water and increase their volume up to 70% before they are lysed. But spherocytes have decreased surface area to volume ratio. Hence, they cannot take up as much water as normal RBCs and they lyse earlier (i.e. at relatively higher saline concentration than normal red cells).

With normal RBCs, haemolysis usually starts at saline concentration of 0.5 g/dl and haemolysis is complete at 0.30 g/dl.

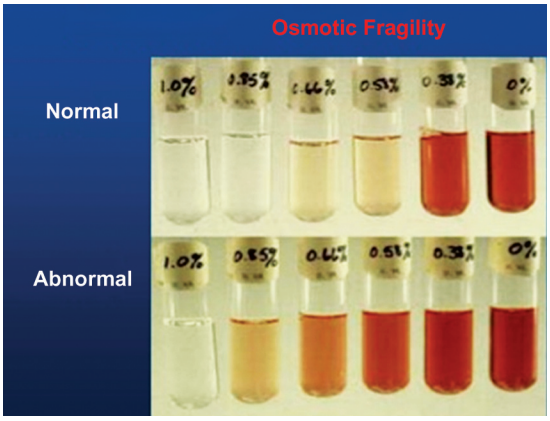


Fig.11.10: Osmotic fragility test for hereditary spherocytosis

Method

- A series of test tube, containing varying concentration of NaCl (sodium chloride) ranging from 0.9–0.10% (0.9, 0.75, 0.65, 0.6, 0.55, 0.5, 0.4, 0.35, 0.3, 0.2, and 0.10%) are taken. Add 5 ml of water to tube 12 for (positive control).
- Red cells are suspended in each test tube and incubated at room temperature for 30 minutes. For this 30 µl of well mixed blood (heparinized venous blood or defibrinated blood) may be used. But remember oxalated or citrated blood is unsuitable for this test as it contains additional salt. Mix well the blood in all 12 tubes.
- After incubation of 30 minutes room temperature, mix it again. Then centrifuge for 30 mins at 1200 g (3000 rpm).
- Remove the supernatants and estimate the amount of lysis in each using spectrophotometer at a wavelength setting of 540 nm on a photoelectric colorimeter (yellow-green filter). Use as a blank the supernatant from tube 1 (0.9% NaCl).
- Then calculate the osmotic fragility of each tube (tube 1 to tube 11) comparing the tube 12 (100% lysis).
- Make a osmotic-fragility curve using data of haemolysis of all 12 tubes (0% in tube 1 and 100% in tube 12).

Note

- The amount of blood delivered in each tube should be same (50 µl). Special care is to be taken during blood delivery in tube 12 which is used as control. Use automatic pipette for accuracy.
- In case of availability of limited blood (e.g. form babies) take 1 ml of normal saline (instead of 50 µl). Because, spectrophotometer allows 1ml cuvette (not less than that). But delivery of 10 µl of blood (so little amount) is difficult. For this a Pasteur pipette or capillary pipette is to used.
- When transferring the supernatant from a tube to spectrophotometer cuvette, care must be taken so that pellet is not disturbed.
- With the above method (50 µl blood) the reading for 100% lysis (tube 12) will be 0.7 in non-anemic blood. Reading of 0.5 to 1.5 is acceptable using a modern spectrophotometer.
- If the value is <0.5, then test should be repeated using more blood (>50 µl) or less saline (<5 ml). The reverse should be done (less blood and more saline) of the reading is >1.5.

Factors Affecting Osmotic Fragility

- A proportion of 1 volume of blood to 100 volumes of saline is chosen so that effect of plasma on the final tonicity the supernatant is minimized. For the most accurate result, the blood be mixed with saline until bright red (complete haemolysis).
- Effect of pH: A shift of 0.1 of a pH unit is equivalent to altering the saline concentrating by 0.1 g/L. Fragility of RBCs is increased by a fall in pH.
- Effect of temperature: A rise in temperature will decrease the fragility. A rise of 5°C being equivalent to an increase an saline concentration of about 0.1 g/L.

Test Result

- Highest concentration of saline at which haemolysis starts in normal non-anemic persons is 0.5 g/dl NaCl. Highest concentration of saline at which haemolysis is complete in 0.3 g/dl and onwards in normal persons.
- 50% haemolysis (median corpuscular fragility) is seen 0.40–0.40–0.45 gm/dl NaCl. Higher value denotes increased fragility.

Increased Osmotic Fragility (OF)

- **Hereditary spherocytosis:** Red cells show beginning of haemolysis at 0.6–0.8 g/dl (i.e. increased OF). As spherocytes have decreased surface/volume ratio, they have a limited capacity to expand in hypotonic solutions. There will be a shift to right of normal range in the graph.
- **Hereditary elliptocytosis:** As in hereditary spherocytosis, but haemolysis is less marked.
- **Other causes:** Autoimmune haemolytic anaemia, ABO haemolytic disease of newborn, and burns.

Decreased Osmotic Fragility

- **Thalassaemia**: Median corpuscular fragility (MCF) is decreased, i.e. OF is decreased in all forms of thalassaemia, except in some α-thalassaemia (heterozygotes). Target cells of thalassaemia have increase surface/volume ratio (opposite to hereditary spherocytosis). The osmoticfragility curve is shifted to left.
- **Others:** Enzyme abnormalities of RBC, iron deficiency anaemia, sickle cell disease and liver disease.

Remember OF may be negative if only a few spherocytes are present in PBS. Sensitivity of the test can be increased by incubating the red cells at 37°C for 24 hours before performing the test (OF test after incubation).

AUTOHAEMOLYSIS TEST

In this test, sterile defibrinated blood is taken (1 ml) in a tube. The test can be performed with or without the addition of glucose (50 µl of sterile 100 g/L glucose solution). Then blood is incubated at 37°C for 48 hours and degree of spontaneous haemolysis is noted. Amount of haemolysis is measured in a colorimeter and the result is expressed as a percent lysis (Normal: <4%; with addition of glucose: <0.5%).

Significance: In membrane disorders such as HS (hereditary spherocytosis) the rate of glucose consumption is increased to compensate for an increased cation leak through the membrane. During 48 hours of incubation, glucose is therefore used up relatively rapidly so that energy production, fails more quickly than normal unless glucose is added. This contributes to increased autohaemolysis in HS (type I autohaemolysis).

Other disorders which show increased autohaemolysis are: Pyruvate kinase deficiency, G6PD deficiency, and disorders of the pentose phosphate pathway.

ACIDIFIED SERUM TEST (HAM TEST)

Principle of the test: The patient's red cells are exposed to the action of normal or patient's own serum suitably acidified to the optimum pH for lysis (pH 6.5–7) at 37°C.

Patient's red cells: It can be obtained from defibrinated, heparinized, oxalated, and citrated or EDTA blood. If necessary, the blood can be stored at 4°C in ACD (acid citrate dextrose) solution for 2–3 weeks.

Patient's serum: It is best obtained by defibrination.

Normal serum: obtained by defibrination also. But serum obtained from blood allowed to clot spontaneously at room temperature or at 37°C can also be used.

Method: Six pairs of test tubes are used: Three for tests and three for controls. The test tubes are prepared in the following manner (Fig. 11.11):

Standard and blank: Add 0.05 ml of cell suspension of RBCs to 0.55 ml of water for preparation of standard for subsequent quantitative measurement of lysis.

Retain 0.5 ml of serum for use as a blank.

Measurement of lysis: Deliver 0.3 ml volumes of the supernatants of the test and

Reagent	Test (ml)			Control (ml)		
	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6
Fresh normal serum	0.5	0.5	0	0.5	0.5	0
Heat inactivated normal serum	0	0	0.5	0	0	0.5
0.2 mol/L HCl	0	0.05	0.05	0	0.05	0.05
50% patient's RBC	0.05	0.05	0.05	0	0	0
50% normal RBC	0	0	0	0.05	0.05	0.05
Magnesium chloride (250 mmol/L or 23.7 g/L)	0.01	0.01	0.01	0.01	0.01	0.01
Lysis (in a positive test)	Trace (2%)	+++ (30%)	–	–	–	–

Unacidified fresh serum: Tube 1 and 4
Acidified fresh serum: Tube 2 and 5
Acidified inactivated serum: Tube 3 and 6 (inactivation done by keeping the tube at 56° C for 10–30 minutes)
After that mix the contents carefully and leave the tube at 37°C. Centrifuge them after about 1 hour

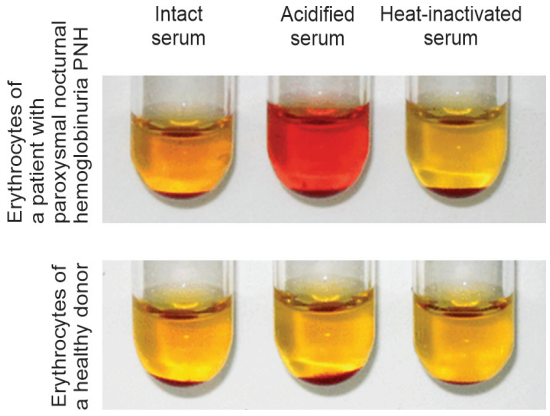


Fig. 11.11: Ham test (acidified serum test) in upper row (PNH patient). Tube 2 or middle tube which is acidified with HCl, shows maximum haemolysis

control series of cell-serum suspensions, and of the blank serum and lysed cell suspension equivalent to 0% and 100% lysis, respectively, into 5 ml of 0.4 ml/L Drabkin reagent or ammonia.

Measure the lysis in a spectrophotometer at a wavelength of 540 nm or in a photoelectric colorimeter using a yellow-green filter.

Significance of the Test

In PNH (paroxysmal nocturnal haemoglobinuria) 10–50% lysis is usually obtained. Exceptionallly, there may be 80% lysis (very high) or 5% lysis (very low).

In normal person (control), there will be no lysis.

ANTIGLOBULIN TEST (COOMBS' TEST)

Principle of the test: The test is used to detect antibodies or complements attached to RBC or present freely in serum. Patient's blood is taken and 2–5% cell suspension of RBCs is prepared. Anti-human globulin (AHG) is added to the cell suspension. AHG is commercially available which is prepared by immunizing mouse or rabbit with whole human serum or with specific fraction of human globulin. The Fab portion of AHG molecule (anti-IgG) reacts with the Fc portion of two adjacent antibodies (IgG molecule on RBC). Thus, it bridges the gap between RBCs causing agglutination.

Direct Antiglobulin Test (Direct Coombs' Test)

This test is used to detect incomplete antibody (IgG) or components of complement coating patients' RBCs.

Method

- One drop of 2–5% of patient's cell suspension is placed in a glass tube and washed three times with saline.
- Add 1–2 drops of anti-human globulin (AHG) to it.
- The content is mixed and centrifuged at 1000 rpm for 1 minute.
- The tube is gently shaken to dissolve the precipitate and agglutination is observed (naked eye as well as under microscope).
- If there is no agglutination, the sensitivity of the test may be increased by adding 1 drop og IgG coated red cells. The content is mixed and centrifused at 1000 rpm for 1 minute and checked for agglutination. If no agglutination occurs, the whole test becomes invalid and should be repeated again.

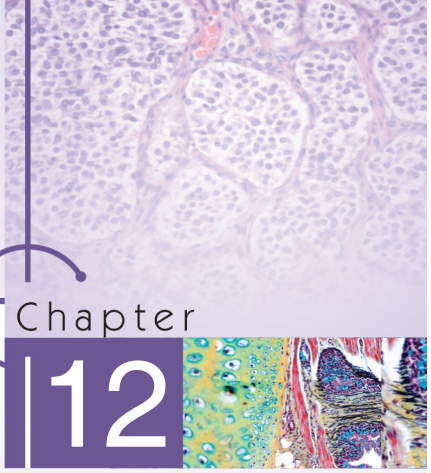
Result

Result is positive when there is agglutination, i.e. presence of immune antibody (IgG) or complement (C3d) on the surface of patient's RBC.

Use

Direct antiglobulin test is of use in the diagnosis of:

- Autoimmune haemolytic anaemia
- Haemolytic disease of newborn
- Haemolytic transfusion reaction
- Drug-induced red cell sensitization



Chapter 12

Leukaemia: Common Types and their Investigations

Leukaemia is the cancer of the marrow and blood, European physicians in the 19th century were the earliest observers of patients who had markedly increased white cell counts. The term **Weisses Blut** or "white blood" emerged as a designation for the disorder. Later, the term "leukaemia", which is derived from the Greek word *leukos*, means "white" and *haima*, means "blood", was used to indicate the disease.

One confusing aspect of the lymphoid neoplasms concern the use of terms lymphocytic leukaemia and lymphoma. Leukaemia is used for neoplasms that present with widespread involvement of the bone marrow and (usually, but not always) the peripheral blood. Lymphoma is used for proliferations that arise as discrete tissue masses. Originally, these terms were attached to what were considered distinct entities, but with time and increased understanding these divisions have blurred. Many entities called "lymphoma" occasionally have leukaemic presentations, and evolution to "leukaemia" is not unusual during the progression of incurable "lymphomas". Conversely, tumors identical to "leukaemias" sometimes arise as soft tissue masses unaccompanied by bone marrow disease. Hence, when applied to particular neoplasms, the terms leukaemia and lymphoma merely reflect the usual tissue distortion of each disease at presentation.

The major form of leukaemia is divided into four categories:

1. Acute lymphoblastic leukaemia (ALL)
2. Acute myeloblastic leukaemia (AML)
3. Chronic lymphocytic leukaemia (CLL)
4. Chronic myelocytic or myeloid leukaemia (CML)
   - **ALL:** Presence of ≥20% lymphoblasts in the blood and/or bone marrow.
   - **AML:** Presence of ≥20% myeloblasts in the blood and/or bone marrow.
   - **CLL:** Lymphocytosis (>90% small mature lymphocytes) and TLC >5000/mm^3. In bone marrow lymphocytes >30%.
   - **CML:** Proliferation of myeloid cells (neutrophils, myelocytes, metamyelocytes, band form, etc.) in blood and bone marrow.

Classification of ALL (Acute Lymphoblastic Leukaemia)

➢ Criteria that are not met (or intermediate result) are considered result with no score. The possible total score for a case ranges from –4 to +2. A **score of 0 to +2** results in a diagnosis of **L1** and a **score of –1 to –4** in a diagnosis of **L2** (Table 12.1). N/C ratio means nuclear cytoplasmic ratio.

Note

- Approximately 71% of cases of childhood ALL are L1, 27% are L2 and 2% are L3 variants. In adult patients with ALL, however, L2 is the most commonly observed cytologic variant (Table 12.2).

Table 12.1: FAB scoring system for L1 and L2 variants of ALL

Criteria	Score
• High N/C ratio ≥75% of cells	+
• Low N/C ratio ≥25% of cells	–
• Nucleoli: 0–1(small) ≥75% of cells	+
• Nucleoli: 1 or more (prominent) ≥25% of cells	–
• Irregular nuclear membrane ≥25% of cells	–
• Large cells >50% of cells	–

Table 12.2: French American British (FAB) classification

Cytology	L1	L2	L3
• Size	Small	Large	Large and homogeneous
• Chromatin	Homo-geneous	Variable	Finely stippled
Nuclear			
• Shape	Regular	Irregular	Oval to round
• Nucleoli	Rare	Present	1–3
• Cytoplasm	Scant	Moderate	Intense, basophilic, vacuoles

Laboratory Diagnosis of ALL

1. **Blood:** Anaemia is present if clinical manifestations are fully developed. It is usually normocytic. Frequently, nucleated red cells on normoblasts are present. Thrombocytopenia of moderate to marked degree is the rule. The leukocyte count occasionally is very high (>100 × 10^9/L or 100,000/mm^3). But many times, TLC is slightly elevated. Sometimes it may be normal or even may be decreased (aleukemic leukaemia). The predominant cell type is the lymphoblast or immature lymphocyte (at least 20% by definition) (Fig. 12.1).
2. **Bone marrow:** By the time the patient is symptomatic, the haematopoietic cells and fat usually are replaced by diffuse infiltration of lymphoblast.
   In the L1 type, according to the FAB classification, the lymphoblast has a high nuclear/cytoplasmic ratio. The nuclei are regular and not indented on twisted. The chromatin pattern is fine and uniform. The cytoplasm is scanty in amount, pale blue, and homogeneous, usually without granules, this type is common in children.
   In L2 type ALL, a larger type prevails and usually there is more variation in cytoplasmic features within and between cases. It is less common in children and is the usual adult type of ALL (Fig. 12.2).
   L3 represents the Burkitt type of ALL. The cells are larger and uniform, they have a round or oval nucleus with prominent nucleoli and deeply basophilic cytoplasm that usually contains vacuoles (Fig. 12.1B). The lymphoblast should be differentiated from haematogones. Haematogones may be increased in the bone marrow in **iron deficiency anaemia (IDA), neuroblastoma, and idiopathic thrombocytopenic purpura (ITP)** as well as **following cytotoxic therapy**. These cells have a very high nuclear cytoplasmic ratio and a homogeneous nuclear chromatin. The nuclei may show indentations or clefts. Nucleoli are not usually identified, if present they are indistinct. Haematogones are not

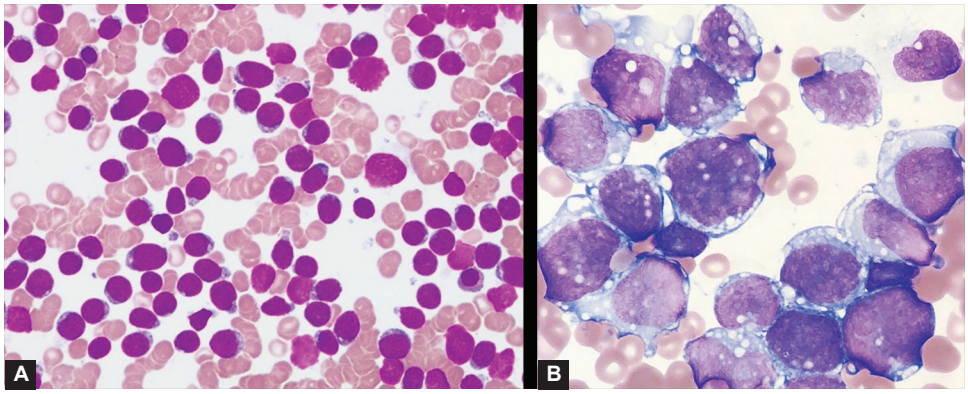


Fig. 12.1: (A) PBS showing ALL; (B) L3 type of ALL showing multiple cytoplasmic vacuoles

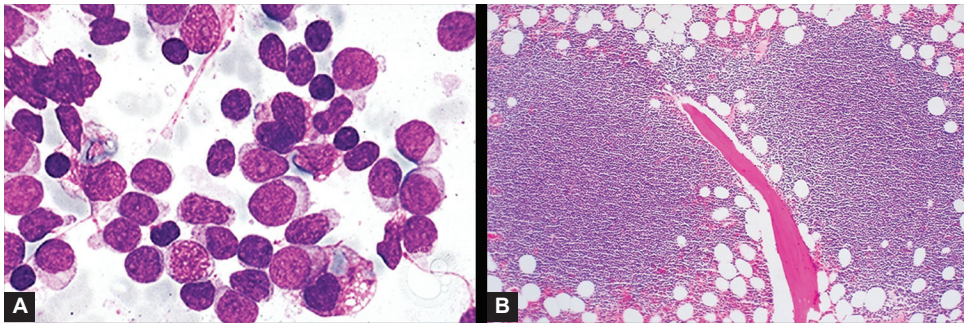


Fig. 12.2: (A) Bone marrow smear showing lymphoblasts in ALL; (B) Trephine biopsy in ALL showing hypercellular marrow occupied by lymphoblasts

generally found in the peripheral blood. In marrow biopsies, the haematogones distributed uniformly in the interstitium. The nuclear chromatin is very clumped. Nucleoli and mitotic figures are rarely identified (Fig. 12.3).

Both haematogones and lymphoblasts are TdT positive and may express CD10. Haematogones lack aberrant antigenic expression and show a reproducible pattern of coexpression of markers associated with B cell lymphoid differentiation including CD10, CD19, CD20, CD34, and CD45. There is a continuum of expression of these antigens in haematogones, indicating maturation. There is a predominance of intermediate (CD10+, CD19+, TdT negative, SIg) and late immunophenotypic stage (CD19+, CD20+, SIg+). In contrast, the lymphoblast of early pre-B cell ALL differ from normal B cells with a predominance of immature cells (TdT+, CD19+, SIg–, CD20–) and a paucity of mature cells.

3. **Cytochemistry:** The lymphoblasts are negative for Sudan Black B, myeloperoxidase and naphthol ASD chloroacetic esterase. The acid phosphatase reaction is moderately or strongly positive in the blasts in about 20% of cases of ALL. Most of these appear to be T cell leukaemias. The PAS stain usually shows coarse blocks of material (block positivity) in at least some lymphoblasts.
4. **Immunophenotyping/flow cytometry:** Already discussed in Tables 12.3 and 12.4.
5. **CNS leukaemia (ALL):** 5 WBC/µl with the presence of lymphoblasts on cytospin preparations or the presence of cranial nerve palsy.

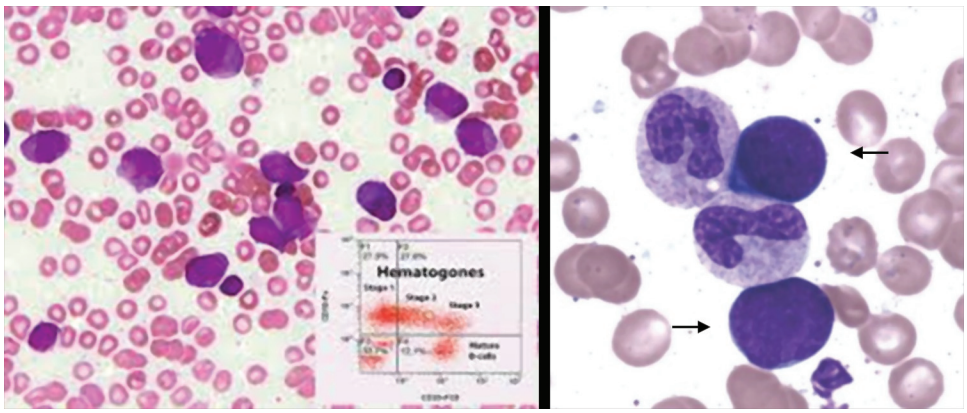


Fig. 12.3: Haematogones in peripheral blood smear and in bone marrow

- B-ALL: CD19, CD20, CD21, CD22, CD 79a, CD10 (common ALL antigen or CALLA), cytoplasmic μ-chain, surface immunoglobulin.
- T-ALL: CD2, CD3, CD5, CD7, cytoplasmic CD3 (Table 12.3).

FAB Classification of AML

1. Acute myeloid leukaemia, minimally differentiated: M_0 (AML–M_0)
2. Acute myeloid leukaemia, without maturation: M1 (AML–M1)
3. Acute Myeloid Leukaemia, with maturation: M2 (AML–M2)
4. Acute promyelocytic leukaemia: M3 (AML–M3)
   a. Hypergranular variant acute promyelocytic leukaemia, APL.
   b. Microgranular (hypogranular) acute promyelocytic leukaemia M3V (APL–V)
5. Acute myelomonocytic leukaemia: M4 (AML–M4)
   – Acute myelomonocytic leukaemia with increased eosinophils (AML–M4Eo)
6. Acute monocytic leukaemia: M5 (AML–M5)
   – Acute monoblastic leukaemia, M5A (AML–M5A)

Table 12.3: Immunophenotypic classification (WHO) of ALL

Subsets of ALL	Immunophenotype									
	Tdt	DR	CD2	CD5	CD10	CD19	CD20	SIg	CIg	Percentage (%)
• T cell	+	–	+	+	+/–	–	–	–	–	10–20%
• Early pre-B	+	+	–	–	+	+	–	–	–	60–70%
• Pre-B	+	+	–	–	+	+	+	–	+	15–20%
• B cell	–	+	–	–	–	+	+	+	–	1–3%

Pre: Precursor, TdT: Terminal deoxynucleotidyl transferase, DR: HLA–DR histocompatibility antigen, CD10: Common acute lymphoblastic leukaemia antigen (CALLA), SIg: Surface immunoglobulin, CIg: Cytoplasmic immunoglobulin (heavy chain, IgM).

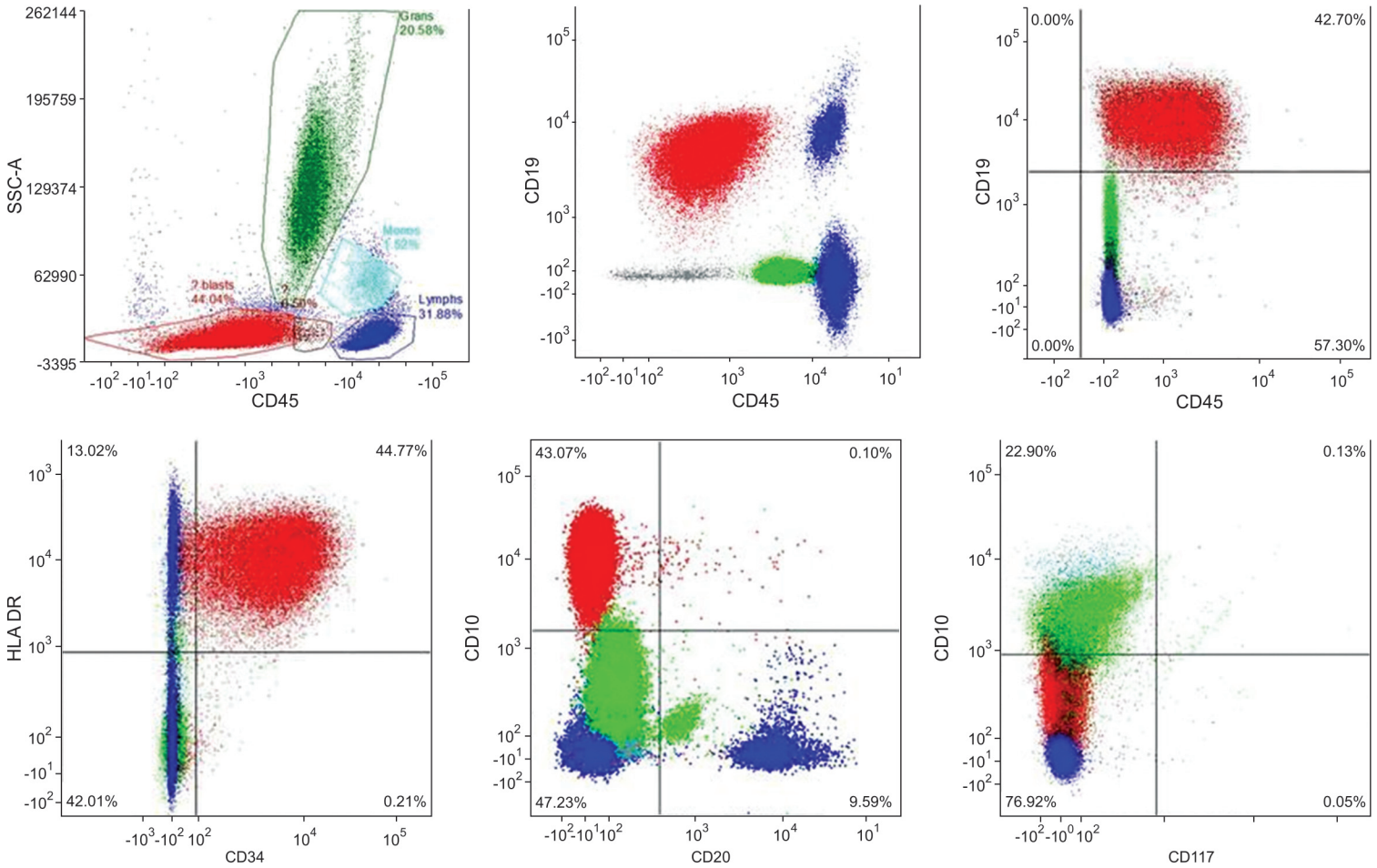


Fig. 12.4: Flow cytometry in a case of B-ALL

Flow cytometry analysis of peripheral blood. The blasts (red) represent approximately 43% of the WBC and are dim to negative for CD45; positive for CD19, CD34, CD10 (bright), CD22, HLA-DR and CD58; and negative for CD20, CD117, CD13, CD33, and surface light chains. This immunophenotype is typical for B lymphoblastic leukemia (B-ALL).

Table 12.4: Major subtypes of AML in the WHO classification 2017

Subtype of AML	Prognosis	FAB Subtype	Morphology/Comments
I. AML with recurrent genetic aberrations			
a. AML with balanced translocations	Favourable	M2	Auer rods++, abnormal cytoplasmic granules
• AML with t (8;21) $RUNX_1$ $RUNX_1T_1$			
• AML with inv (16) CBFB–MYH II	Favourable	M4	Abnormal eosinophilic granules
• AML with t (15;17) PML–RARA	Intermediate	M3	Auer rods++, high incidence of DIC
• AML with (9;11) KMT_2A–MLL	Poor	Variable	Responds to treatment
• AML with BCR–ABL_1 mutation			
b. AML with genetic mutations			
• AML with mutated NPM_1	Favourable		
• AML with biallelic mutation of CEBPA	Favourable		
II. AML with MDS like features			
• AML with MDS like cytogenetic abnormalities	Poor	Variable	Associated with $5q^-$, $7q^-$, Monosomy 5 and 7
III. AML, therapy related			
• Post-alkylating agents • Post-DNA topoisomerase II	Very poor	Variable	• Seen after 5–10 years • Balanced chromosomal translocations • Unbalanced loss of chromosome 5 and 7; loss of P53
IV. AML, not otherwise specified			
• AML of FAB from M0 to M7 • Acute basophilic leukaemia • Acute panmyelosis with myelofibrosis			

Table 12.5: Cytochemical features in acute leukaemia

Type of leukaemia	MPO	PAS	NSE	AP
• B-ALL	–ve	+ve (blocks)	–ve	–ve
• T-ALL	–ve	–ve	–ve	+ve (focal)
• AML M0	–ve (+ve on EM)	–ve	–ve	–ve
• AML M1–M3	+ve	–ve	–ve	–ve
• AML M4:	+ve	–ve	+ve	–ve
• AML M5:	±	–ve	+ve	–ve
• AML M6	±	+ve (fine granular)	–ve	–ve
• AML M7	–ve	–ve	–ve	–ve

+ve: Positive, –ve: Negative ±: May or may not be positive, MPO: Myeloperoxidase, NSE: Non-specific esterase, PAS: Periodic acid–Schiff, AP: Acid phosphatase

Table 12.6: Laboratory features of AML (as per FAB classification)

Type	Morphology	Cytochemistry	Other features
1. AML M0 (minimal differentiation)	Large, agranular blasts, blasts >90%, medium size, round to slightly indented nuclei with 1–2 nucleoli, scant, agranular cytoplasm	• MPO, SBB, CAE: Negative (<3% blasts are positive) • α-Naphthyl acetate and butyrate esterase negative	CD13+, CD33+, CD34+, HLADR+, CD117+
2. AML M1 (without maturation)	• Blasts 90% • Mature granulocyte <10% • Auer rods+ • Scant cytoplasm with granules • 1–3 prominent nucleoli	• MPO and SBB+ in at least 3% of blasts (3% blasts) • CAE+ • NSE–	• CD13+, CD33+, CD117, CD34+, HLADR+ • t (9;22)
3. AML M2 (with maturation)	• Blasts 20% • Mature granulocytes (promyelocytes, myelocytes, and mature neutrophils) 10% • Auer rods++ (Faggot cells) • Cytoplasm may or may not have azurophilic granules	• MPO, SBB, and CAE+	• CD13, CD33, CD11b, CD15, CD34+ • CD14 and CD64 are negative • t (8;21)
4. AML M3 (acute promyelocytic leukaemia)	• Promyelocytes are predominant (>50%) • Hypogranular and microgranular variant. Auer rods++	• MPO strong+	• CD13, CD33+ • t (15,17) • DIC
5. AML M4 (acute myelomonocytic leukaemia)	• Monocytic differentiation (20–80%) shows indented and lobulated nucleus • Both myeloblasts and monoblasts are seen • Auer rods+	• MPO+ (>3% blasts) • Monoblasts, promonocytes and monocytes are positive for NSE • Double staining for NSE, CAE and MPO may show dual +ve cells	• CD13, CD33, CD14, CD11b, CD11C, CD64, CD68 positive cells
6. AML M5 (acute monocytic/monoblastic leukaemia)	• Acute monocytic leukaemia: Monocytic cells 80% with majority being promonocytes	• Monoblasts and promonocytes are NSE+ • MPO is negative	• CD13, CD33, CD15, CD65+ • CD117, HLADR+

Contd.

Table 12.6: Laboratory features of AML (as per FAB classification) (Contd.)

Type	Morphology	Cytochemistry	Other features
	• Acute monoblastic leukaemia: >80% monoblasts • Both myeloblasts and monoblasts 20% of nonerythroid cells in acute monoblastic leukaemia	• Lysozyme +	• Inversion 16 in M4 with eosinophilia • ↑ Lysozyme in blood and urine
7. AML M6 (acute erythroid leukaemia)	• Proerythroblasts ≥80% of bone marrow cells • ≥20% of nonerythroid cells are myeloblasts, 50% of all nucleated cells are erythroblasts, marked dyserythro-poiesis	• MPO and SBB negative • Acid phosphatase+ • PAS+ • α-naphthyl acetate esterase+	• Immature cells express carbonic anhydrase 1, CD36 • Mature cells show glycophorin A+
8. AML M7 (acute mega-karyoblastic leukaemia)	• Most of the blasts are megakaryoblasts. They are medium to large cells with a round nucleus with fine reticular chromatin and 1–3 nucleoli	• MPO, SBB and CAE negative and CD36 positive • Maybe PAS positive	• t (1,22), (p13, q13) specific for infantile M7 Trisomy 21 • CD41, CD61 and • CD36 Positive

CAE: Chloroacetate esterase, NSE: Non-specific esterase

– Acute monocytic leukaemia, M5B (AML – M5B)

7. Erythroleukaemia (DiGuglielmo syndrome): M6
8. Acute megakaryoblastic leukaemia: M7

CYTOCHEMISTRY AND SPECIAL STAINS IN ACUTE LEUKAEMIA

1. **Myeloperoxidase (MPO):** Activity is specific for myeloid differentiation. Myeloperoxidase activity is stable for approximately four weeks in unstained smears kept at cool room temperature and protected from light. Myeloperoxidase activity in myeloblasts is granular and often concentrated in the Golgi zone. Monoblasts may be negative or positive with scattered fine granules. Lymphoblasts and megakaryoblasts are MPO (myeloperoxidase) negative.
2. **Sudan Black B (SBB):** SBB reactivity is similar to MPO in myeloblasts and monoblasts, and is stable for months in unstained slides. Positive cells usually stain more intensely with SBB than MPO. Lymphoblasts are usually negative. In rare case, lymphoblasts contain granules which may stain light gray, contracting to the black granules in neutrophils and myeloblasts.
3. **Non-specific esterase (NSE)**
   i. **Alpha-naphthyl butyrate esterase (ANBE):** Reactivity in monoblasts with

most methodologies is diffuse cytoplasmic. Neutrophils are negative or only weakly positive. Lymphoblast, especially granular lymphoblasts, may show multifocal punctate or Golgi zone cytoplasmic positivity.

ii. **Alpha-naphthyl acetate esterase (ANAE):** Reactivity in monoblasts and lymphoblasts is similar to ANBE, megakaryoblasts and erythroblasts may contain multifocal punctate cytoplasmic positivity that is partially resistant to NaF (sodium fluoride) inhibition. The ANA positivity in monoblasts is totally inhibited by NaF. Lymphoblast positivity is variably inhibited.

4. **PAS stain:** Coarse blocks of material in lymphoblasts (at least in some) while in myeloblasts there are fine granular positivity.
5. **Serum lysozyme (muramidase):** To diagnose myelomonocytic (M4) and monocytic (M5) leukaemia in which level can be high.
6. **Acid phosphatase (AP):** Strong focal acid phosphatase activity is seen in T cell ALL. However, focal activity is also seen in AML M6 and M7. Monoblasts show a strong and diffuse reaction.

   If tartrate is used during reaction, then it inhibits AP in most cells except in hairy cell leukaemia which is resistant to tartrate inhibition. The **tartrate-resistant acid phosphatase (TRAP)** activity is a characteristic feature of **hairy cell leukaemia**.

LABORATORY DIAGNOSIS OF AML

1. **Blood:** The number of leukemic cells (myeloblasts, or promyelocytes, promonocytes) in the blood is highly variable. Blasts may be >100,000/mm^3 but are under 10,000/mm^3 in about 50% of patients. Occasionally, blasts are entirely absent from the blood (**aleukaemic leukaemia**). For this reason, a bone marrow examination is essential to exclude acute leukaemia in pancytopenia patients.
2. **Bone marrow aspiration (BMA):** At least 500 cells should be counted. First, it is necessary to calculate the percentage of erythroid precursor cells. With the exception of erythron-leukaemia (M6), the diagnosis of AML is established when 20% of the nucleated cells of the bone marrow are blasts and/or leukemic cells (abnormal promyelocytes and promonocytes). The diagnosis of erythron-leukaemia is established when >50% of the bone marrow cells are erythroid precursor cells and when myeloblast represent >20% of remaining non-erythroid cells (NECs).

   The FAB group recognizes three types of myeloblasts. All have central nuclei with fine, uncondensed chromatin and prominent nucleoli (usually 3–5). **Type I blasts** lack cytoplasmic granules, but **type II blasts** have a small number (<20) of primary (azurophilic) granules. **Type III blasts** are similar to type II blasts with the exception of more abundant azurophilic granules.

   A helpful finding in the diagnosis of AML is the presence of **Auer rods**. With Romanowsky stains, Auer rods are linear on spindle shaped, red-purple inclusions in the cytoplasm of myeloblasts or promyelocytes. Less commonly, they may be seen in more mature neutrophils. **Auer rods are derivatives of azurophil granules** and stain positively for Sudan Black B, myeloperoxidase, ASD chloroacetate esterase, and acid phosphatase. Auer rods can be found in any of the subtypes of AML, but they are especially associated with M1 to M3. Their presence ensures the diagnosis of AML or at least, refractory anaemia with excess blasts in transformation. Numerous Auer rods in the cytoplasm of promyelocytes (not myeloblasts) are known as **Faggot cells**. They have appearance of a bundle of sticks, from which the cells are given their name. They are usually seen in **acute promyelocytic leukaemia (M3-AML)** (Fig. 12.5).

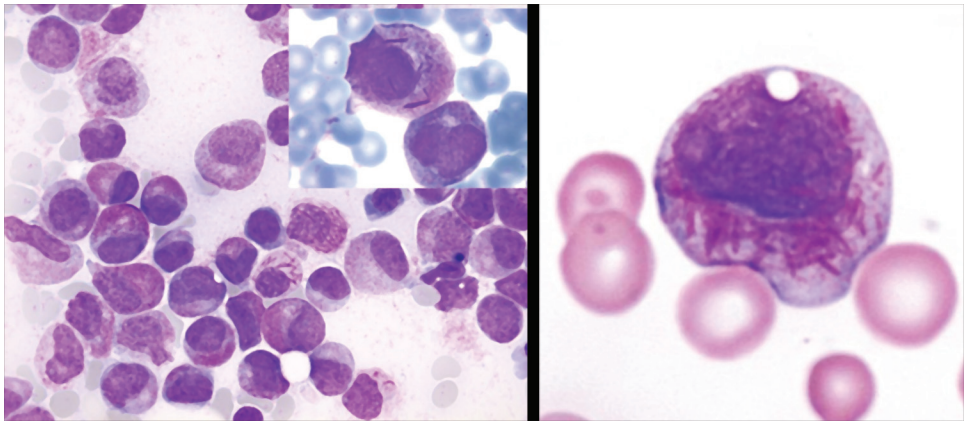


Fig.12.5: Auer rods in myeloblasts. Faggot cells on right side

Monoblasts have folded or lobulated nuclei, lack Auer rods, and are nonspecific esterase positive. In some AMLs, blasts show megakaryocytic differentiation, which is often accompanied by marrow fibrosis caused by the release of fibrogenic cytokines.

Phi bodies (Fig. 12.6)**:** Unique fusiform-shape thin rods in cytoplasm of myeloblasts. Phi bodies are observed in AML blasts but are seen more frequently than Auer rods. **Phi bodies derive from catalase-containing granules (microperoxisomes)** and are distinct from Auer rods, which derive from peroxidase-containing (primary or azurophilic) granules. Phi bodies are better seen with the peroxidase cytochemical reaction using 3, 3′-diaminobenzidine (DAB) and a pH of 9.7. But the number is reduced significantly in the presence of 3-amino-1,2,4-triazole.

3. **Bone marrow biopsy:** It is necessary for evaluation of cellularity and recognition of marrow fibrosis. In addition, in cases in which inadequate smear specimens are obtained because of fibrosis or other factors. Immunohistochemistry (IHC) with a variety of antibodies may be the primary methodology for determining cell lineage.
4. **Hypocellular AML** (Fig. 12.7)**:** In some case of AML, the marrow cellularity is <20%, so called "hypocellular" or "hypoplastic" AML. But the blasts count is 20%. Immunohistochemical reaction with antibody to CD34 will usually show a significant positive population in hypoplastic AML. In aplastic anaemia, the reaction is negative or only a rare cell may be positive. Moreover, in aplastic anaemia, the interstitial cells are well differentiated lymphocytes, plasma cells, mast cells and scattered maturing haematopoietic cells.

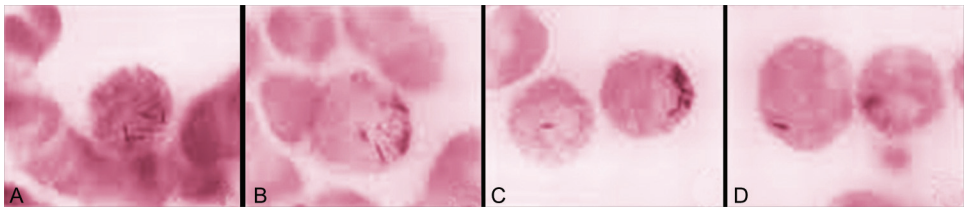


Fig. 12.6: Blast cells of AML cases showing Phi bodies (A,B,C,D). Phi bodies were seen as thin, sometimes multiple (A), rods

Panel of Monoclonal Antibodies for the Classification of Acute Leukaemia

- Haematopoietic precursors: CD34, HLA–DR, TdT, CD45[2]
- B-lineage: CD19, CD20, cyto 79a[1,3], cyto CD22[1]
- T-lineage: CD2, cyto CD3[1], CD5, CD7
- Myeloid: CD13, CD33, CD15, MPO[1], CD117 or c-Kit
- Erythroid: Haemoglobin A, glycophorin A

Note

- Antigens identified with an asterisk are considered lineage specific when detected in the cytoplasm by flow cytometry.
- [1]Cytoplasmic expression
- [2]CD45 is generally more dimly expressed than on normal lymphocytes and it may be negative in some cases of ALL or megakaryoblastic leukaemia.
- [3]CD79a has been reported in some cases of precursor T-ALL.
- CD41 glycoprotein IIb/IIIa, CD61 glycoprotein IIIa

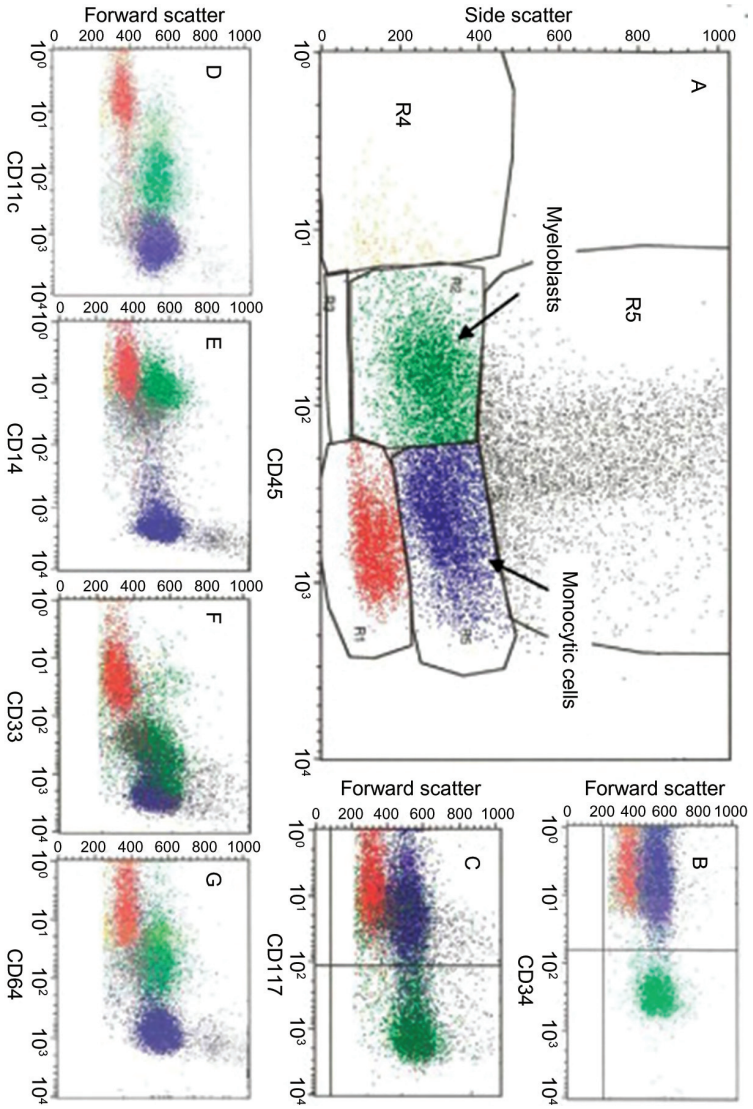


Fig. 12.7: Flow cytometry in AML

Table 12.7: Difference between lymphoblasts and myeloblasts

Parameter	Lymphoblasts	Myeloblasts
1. Nuclear character		
• Nuclear chromatin	Uniform, coarse or clumped, irregularly distributed	Uniform, fine, delicate or stippled or lacy, uniformly distributed
• Nucleoli	Number 0–2, may be indistinct or inconspicuous. Nucleoli are folded or convoluted	Number 3–5, prominent or conspicuous. No fold in nucleoli
• N:C ratio	High	High
2. Cytoplasm		
• Amount	Scanty	More amount than lymphoblast
• Colour	Blue	Gray
• Granules	Absent	Fine granules
• Auer rods, Phi bodies and Faggot	Absent	May be present
3. Cytochemistry		
• MPO, SBB	Negative	Positive
• PAS	Block positive	Negative or fine granular positivity
• Non-specific esterase	Negative	Positive in M4 and M5
4. Accompanying cells	Lymphocytes	Promyelocytes, myelocytes, metamyelocytes, band form and neutrophils

Table 12.8: Normal bone marrow and AML bone marrow in adults

Normal bone marrow	AML bone marrow
• Cellularity:Fat space = 1:1	Cellularity highly increased and fat space markedly decreased. Many blast cells are present (20%). Normal blast cell in marrow 1%
• Myeloid:Erythroid 1.2:1 to 5:1 (average 3:1)	• Myeloid: Erythroid ratio is severely altered due to proliferation of myeloid cells
• Granulocytic cells 40–60% of surface area (trephine biopsy)	• Normal granulocytic precursors decreased markedly
• Megakaryocytes 1–3 under low power or 100X magnification	• Megakaryocytes decreased
• Lymphocytes (5–20%), not >20%	• Lymphocytes normal or decreased
• Erythroid cells 10–20% of surface area	• Decreased erythroid cells

Chronic Myeloid Leukaemia (CML)

There are three phases of CML:

1. **Chronic phase (CP):** In chronic phase, leukemic cells retain the capacity for differentiation and maturation and are largely able to function normally. The disease is amenable or responsive to chemotherapy. Patient remains stable for variable period. The duration of this stage is 3–5 years.

2. **Accelerated phase (AP):** In many cases (70% of patients), chronic phase gradually evolves into accelerated phase. In this phase, leukemic cells show increasing loss of differentiation and maturation, increased proliferation and resistance to chemotherapy. This phase lasts for 6–12 months and eventually progress to blast crisis.
3. **Blast crisis phase (BP)** (Table 12.9)**:** In this phase, the disease transforms into acute leukaemia and patient becomes extremely resistant to chemotherapy. Median survival is 2–6 months without successful treatment. About 30% of patients progress to blastic phase without intervening AP (accelerated phase).

Chronic Phase (CP) of CML

1. **Peripheral blood smear (PBS)**
   - Leukocytosis (12–1000 × 10^9/L) with increased myeloid cells
   - Less than <10% blasts (myeloblasts), usually <2% of WBC.
   - There may be basophilia or eosinophilia.
   - Platelet count normal or increased (Fig. 12.8).
2. **Bone marrow**
   - Myeloblasts <10%, usually <5% of marrow cells
   - Megakaryocytes are smaller and hypolobated "dwarf megakaryocytes".

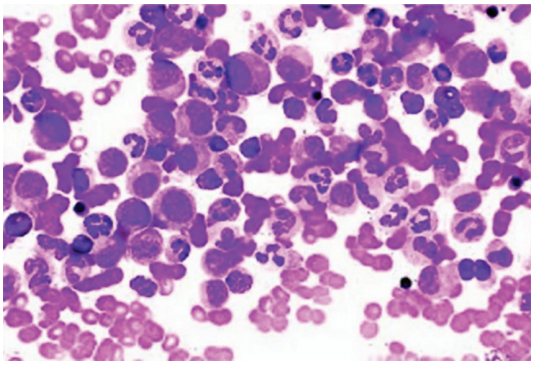


Fig. 12.8: Peripheral blood smear in CML

- Pseudo-Gaucher cells and sea blue histiocytes may be seen.

Table 12.9: WHO diagnostic criteria for accelerated and blast crisis phase (WHO 2017)

Accelerated phase	Blast phase/blast crisis phase
• Blast 10–19% in bone marrow • Peripheral blood basophilia 20% • Persistent thrombocytopenia (<100 ×$10^{9/2}$) • Persistent thrombocytosis (>1000 × 10^9), unresponsive to therapy • Increasingly splenomegaly • Provisional response to TKI (tyrosine kinase inhibitors) • Criteria added: i. Provisional response to TKI ii. Occurrence of ≥2 mutations or resistance to therapy	• Blasts 20% • Clusters of blast cell in trephine biopsy • Extramedullary myeloid tumors (granulocytic sarcomas, chloromas) • Two-thirds of the blasts are of myeloid lineage, whereas the reaming one-third is of lymphoid lineage. • Most of the cases resemble AML • Lymphoblasts in any number should be reported as they signify poor prognosis
Cytogenetic abnormalities like trisomy 8, chromosome 17q, duplication of philadelphia chromosome may develop	90–95% cases show t(9;22) (q34; q 11.2) that results in Philadelphia (Ph) chromosome

Table 12.10: Typical PBS findings in CP of CML

Differential white blood cells	Percentage (%)
• Myeloblasts	1–10%
• Myelocytes	20–30%
• Promyelocytes	2–8%
• Metamyelocytes	15–25%
• Neutrophils	40–60%
• Basophils	1–8%
• Eosinophils	2–10%
• Band form	5–15%
• Monocytes	1–3%
• Lymphocytes	10–15%

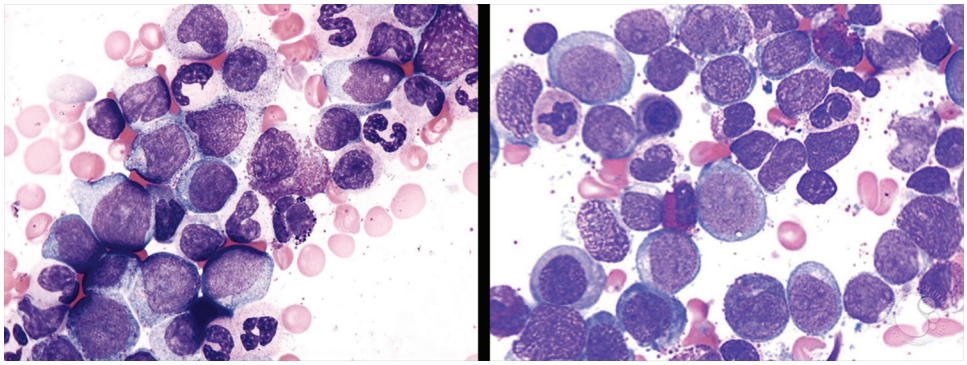


Fig. 12.9: Accelerated phase (left picture) and blast crisis (right picture) of CML

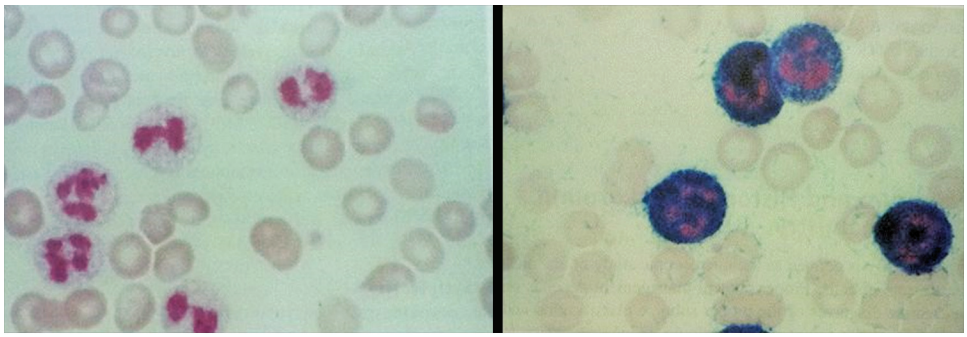
Leukocyte Alkaline Phosphatase (LAP) Score (Table 12.11)

This cytochemical stain is used to demonstrate the presence as well as the amount of the enzyme alkaline phosphatase within neutrophils. Blood smear is prepared from finger stick and air dried, fixed and stained. Enzyme activity is indicated by the presence of bright blue granules in neutrophils. The nuclei are stained red. LAP score is also known as neutrophil alkaline phosphatase (NAP) score.

In CML, LAP is either absent or low. In leukaemoid reaction and in other myeloproliferative disorders (polycythaemia vera or PV, essential thrombocythaemia and myelofibrosis), LAP score is increased. LAP score is graded in this way:

LAP or NAP score	Individual cell (neutrophil) findings
Score 0	: Negative, no granule in cytoplasm
Score 1	: Positive, diffuse pale cytoplasm with occasional granules
Score 2	: Positive, few to moderate number of granules
Score 3	: Strongly positive with numerous granules
Score 4	: Very strongly positive with cytoplasm crowded with granules

LAP score is done after examining 100 consecutive neutrophils. It is calculated as follows: (Number of 1+ cells × 1) + (Number of 2+ cells × 2) + (Number of 3+ cells × 3) + (Number of 4+ cells × 4). Normal score is 40–100 (Fig. 12.10).



Negetive LAP reaction Positive LAP reaction

Fig. 12.10: Alkaline phosphatase is located in the specific granules of neutrophils

Table 12.11: Causes of high and low LAP score

High LAP score (>130)	Low LAP score (<15)
• Infections and leukaemoid reactions • Myeloproliferative disorders other than CML (myelofibrosis, polycythaemia rubra vera, essential thrombocytosis) • AML • Hodgkin lymphoma • Pregnancy, oral contraceptives • Growth factor therapy • Stress, inflammatory disorders • Drugs (corticosteroids, lithium, estrogen)	• CML • PNH (paroxysmal nocturnal haemoglobinuria) • MDS (myelodysplastic syndrome) • Atypical CML (elderly adults) • Juvenile CML (infantile variant <5 years and adult variant >5 years) • Hereditary hypophosphatasia

Laboratory Diagnosis of CML

- Haemoglobin: Usually <11 g/dl
- RBCs in PBS: show moderate degree of normocytic normochromic anaemia. Severity of anaemia is proportional to the increase in leukocytes. There may be mild anisocytosis.
- WBCs in PBS:
  i. There is marked leukocytosis (12–600 × 10^9/L or 12–600 × 10^3/mm^3). The total leukocyte count (TLC) usually exceeds 100 × 10^9/L (100,000/mm^3). If left untreated, TLC progressively increases.
  ii. There is shift to left (shift to immaturity) with granulocytes at all stages of development (neutrophils, myelocytes, metamyelocytes, promyelocytes and occasional myeloblasts) are seen in PBS. Predominant cells in PBS are neutrophils and myelocytes. Blast count 1–10% (always <10% in chronic phase).
  iii. Basophilia and eosinophilia are usually observed.
  iv. Platelets number is 15–450 × 10^9/L but may be >1000 × 10^9/L. Up to half of the patients of CML have thrombocytosis. Thrombocytopenia in CML usually indicate transformation into accelerated or blast phase.
  v. Decreased LAP or NAP score.
- **Bone marrow**
  i. Cellularity: Highly cellular due to marked myeloid hyperplasia
  ii. M:E ratio: Often exceeds 20:1.
  iii. Myelopoiesis: It shows marked hyperplasia of myeloid cells (promyelocytes, myelocytes, metamyelocytes, band form and neutrophils). Blast cells are usually <10%. Basophils and eosinophils and their precursors are usually found.
  iv. Erythropoiesis: Diminished erythroid cells as they are replaced by myeloid hyperplasia.
  v. Megakaryopoiesis: Megakaryocytes may be either increased or normal. These megakaryocytes are small and dysplastic (hypolobated and fewer granules in cytoplasm), known as dwarf megakaryocytes. There is megakaryocyte clustering.
  vi. Sea blue histiocytes (Gaucher like or pseudo-Gaucher cells): Bone marrow often reveals the presence of scattered large storage histiocytes with wrinkled, green blue cytoplasm.
  vii. Increased fibrosis in the bone marrow. Reticulin stain reveals increased deposition of reticulin fibres.
- **Biochemical findings**
  i. Serum LDH level is increased
  ii. Serum alkaline phosphatase is increased
  iii. Serum uric acid is increased due to high turnover of leukemic white cells.
  iv. Serum vitamin B_{12} is also elevated due to production of binding protein by the granulocytic cells.

Laboratory Findings in CLL

- Haemoglobin: Usually <13 g/dl and as the disease progresses, it may decrease < 10 g/dl. This low Hb, is due to marrow failure and associated autoimmune hemolysis.
- RBCs in PBS: It shows normochromic normocytic anaemia
- WBCs in PBS:
  i. TLC is usually 20–50 × 10^9/L or 20,000–50,000/mm^3.
  ii. There is lymphocytosis and absolute lymphocyte count >5 × 10^9/L or 5000/mm^3. Lymphocytes are usually >50% of the WBCs and may reach up to 90–98%, with neutropenia.
  iii. Morphology of lymphocytes: They are small, mature lymphocytes. These lymphocytes have scant or a very thin rim of blue cytoplasm (Fig. 12.11). The nuclei are usually round with regularly clumped coarse chromatin ("soccer ball" or ball type chromatin). Occasional cells may have indentation or notching in the nuclei. Nucleoli are inconspicuous or absent.
  iv. Smudge cells: 'Smudge' cells or 'basket' cells or 'parachute' cells are seen in PBS. They are disintegrated lymphocytes and represent the spread out of nuclear chromatin. They are produced due to rupture of the neoplastic lymphoid cells while making PBS.
- **Platelets:** In initial phase, platelet count is usually normal. The bone marrow proliferation of lymphocytes as well as autoimmune destruction of platelets associated with hypersplenism may result in decreased platelet count.
- **Bone marrow**
  i. Cellularity: Bone marrow is infiltrated by mature lymphocytes which cause hypercellular marrow.
  ii. Percentage of lymphocytes: Lymphocytes constitute >30% of nucleated to in the marrow and is diagnostic of CLL. The infiltration in the trephine biopsy may be in the form of non-paratrabecular aggregates or interstitial infiltrates of small lymphocytes.
  iii. Erythropoiesis: Usually erythropoiesis is normal. If the patient develops autoimmune disease then there may be haemolytic anaemia with normoblastic erythroid hyperplasia.
  iv. Myelopoiesis: Myelopoiesis is normal in initial phase.
  v. Megakaryopoiesis: It is within normal limits (Tables 12.12 and 12.13).

Myeloma defining events (Fig. 12.12) (any One is sufficient for the diagnosis of multiple myeloma)

a. ≥60% clonal plasma cells
b. Involved/uninvolved free light chain ratio (>100)
c. ≥2 focal bone lesions of ≥5 mm in size on radiology (MRI).

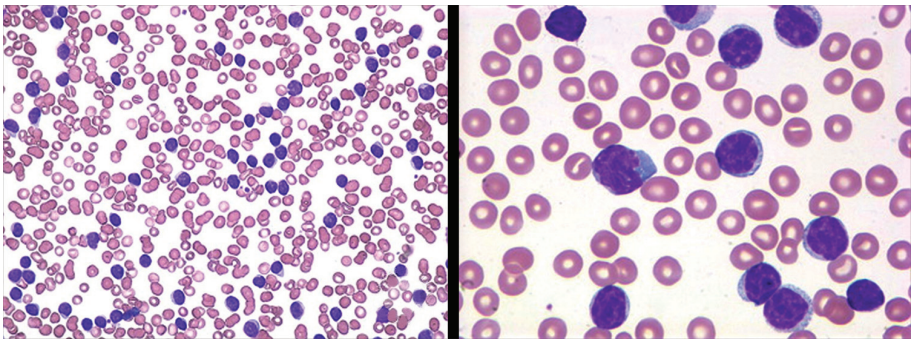


Fig. 12.11: Low power and high power view of peripheral blood smear in CLL

Table 12.12: Criteria for diagnosis of CLL

Parameter	Criteria
Absolute lymphocyte count PBS	≥5000/mm^3 (5 × 10^9/L) persistent for 3 months
Bone marrow lymphocytes	≥30%
Prolymphocytes	≥55%
Lymphocyte morphology	Small, mature lymphocyte with high N/C ratio and scanty cytoplasm. Dense and clumped nuclear chromatin. No visible nuclei. Presence of basket or smudge cells.
Immune phenotype (IHC markers)	B cell markers 1 positive of CD19, CD20, CD23 but CD20 is dim. T cell marker CD5 positive (aberrantly) Negative : CD79b, FMC7, CD10

Table 12.13: Staging system for CLL

Rai stage	Binet stage
0: Lymphocytosis only I : Lymphadenopathy II: Hepatomegaly and/or splenomegaly ± lymphadenopathy III: Haemoglobin <11g/dl IV: Platelet count <1,00,000/mm^3	A. <3 enlarged lymphoid areas* B. ≥3 enlarged lymphoid areas C. Anaemia (haemoglobin <10 g/dl) and/or thrombocytopenia (platelet count <1 lac/mm^3). Lymphoid areas mean lymph nodes (unilateral or bilateral cervical, axillary and inguinal), liver, spleen * Lymphoid areas mean lymph nodes (unilateral or bilateral cervical, axillary and inguinal)

Table 12.14: Classification of plasma cell neoplasms (WHO 2017)

MGUS	Smoldering myeloma	Multiple myeloma
IgG/A/M level: • Serum monoclonal antibody IgG or IgA or IgM <3 g/dl, and • Clonal BM plasma cells <10%, and • No myeloma defining events (see below)	• Serum monoclonal antibody (IgG or IgA) 3 g/dl • Urinary monoclonal antibody 500 mg/24 hours and/or • Clonal BM plasma cells 10–60%, and • No myeloma defining events or amyloidosis (no CRAB and no SLiM) as detailed below	• Clonal BM plasma cells 10%, or • Biopsy proven bony or extra medullary plasmacytoma, and • 1 or more myeloma defining events as detailed below 1 CRAB feature(s) and 1 SLiM Feature(s)

C : Calcium elevation (>11 mg/dl or >1 mg/dl higher than upper normal)

R : Renal insufficiency (creatinine clearance <40 ml/min or serum creatinine >2 mg/dl)

A : Anaemia (Hb <10 g/dl or 2 g/dl or 2 g/dl lower than normal value/limit)

B : Bony lesion (1 lytic lesion on skeletal radiography (CT or PET–CT)), or

In the absence of CRAB, any one or more of the following biomarkers or malignancy, referred to here as the SLiM criteria.

S: Sixty-percent (60%) clonal BM plasma cells

Li: Serum free light chain ratio involved: uninvolved ≥100

M: >1 focal lesions (5 mm each) detected by MRI studies.

Myeloperoxidase Reaction

Myeloperoxidase is a lysosomal enzyme localized in the azurophil granules of neutrophils and monocytes. Azurophil granules in neutrophils correspond to the large electron dense (primary) granules visualized

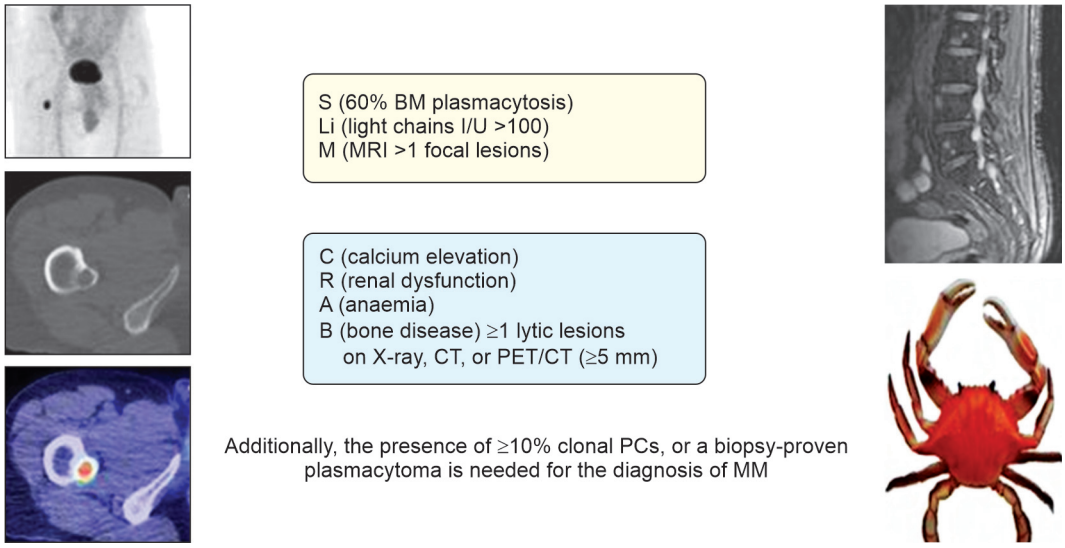


Fig.12.12: Myeloma defining events

under electron microscope. The secondary or specific granules are less electron dense and appear at the myelocyte stage. In the monocytic series the azurophil granules are smaller and should not be called primary as they do not appear first.

In the monoblasts, the lysosomal granules are very small and have acid phosphatase activity but lack myeloperoxidase activity.

Myeloperoxidase activity may be found in the specific granules of eosinophils and basophils.

There methods are commonly used to stain and demonstrate myeloperoxidase activity. These are:

i. 3-amino-9-ethyl carbazole
ii. 2,7-fluorenediamine (FDA)
iii. 3,3′-diaminobenzidine (DAB) tetrahydrochloride

Among those methods, DAB is the substrate of choice for ultrastructural studies because its oxidized product is electron-dense and can be intensified by post-fixation with osmium tetroxide. DAB is also frequently used to visualize the myeloperoxidase reaction and to diagnose AML.

Myeloperoxidase Stain using DAB

- **Fixative:** A mixture of 1.25% glutaraldehyde and 1% formaldehyde in 0.1 mol/L phosphate buffer (pH 7.3).
  Make 50 ml of a 25% solution of gluteraldehyde, 27.8 ml of a 36% solution of formaldehyde and add the buffer up to 1 litre.
- **Incubation mixture:** DAB, 5 mg; tris-HCl buffer, 50 mmol/L, pH 7.6, 10 ml; H_2O_2, 30% (W/V), 0.1 ml. Add the reagents in this order and mix well after each addition. This medium should be prepared just before use.
- **Enhancer:** Dissolve $CuSO_4$ (copper sulphate) 0.5 g or copper nitrate $\{Cu(NO_3)_2 .3H_2O\}$, 0.5 g in 100 ml of tris-HCl buffer, 50 mmol/L, pH 7.6.
- **Counter stain:** Dissolve 10 g of Giemsa's stain in 100 ml of 66 mmol/L phosphate buffer, pH 6.4.

Staining Method

- Fix peripheral blood or bone marrow films in fixative described above for 1 minute.
- Rinse in normal saline (9 g/L NaCl)

- Immerse the slides in the incubation mixture for 1 minute in a Coplin jar at room temperature (20–25°C).
- Rinse briefly in tris-HCl buffer (three changes)
- Then immerse the slides in the reaction enhancer.
- Rinse in saline and keep in the saline until counterstained. Counterstain for 10 minutes.
- Mount in DPX.

Myeloperoxidase Stain Using 2, 7-FDA

- Fixative: 10% formal-ethanol solution (9 volumes of 95% ethanol and 1 volume of 40% formaldehyde).
- Incubation mixture: Dissolve 40 mg of 2, 7 -FDA in 40 ml of tris-HCl buffer (pH 8.6) in order to obtain a saturated solution. Shake the mixture vigorously for 5 minutes at room temperature and then filter to remove excess of precipitated substrate. The solution (without H_2O_2) is stable for >6 weeks at room temperature. Add 2 drops of 30% H_2O_2 just before use to clear filtrate.
- Giemsa counterstain: 10 g of Giemsa powder in 66 mmol/L phosphate buffer.

Staining Method

- Fix the smear in fixative for 1 minute
- Rinse in water.
- Transfer the slides to the incubation mixture in a Coplin jar.
- Incubate for 5 minutes at room temperature.
- Wash for a few seconds and counterstain with Giemsa for 15 minutes.
- Dry the smears and mount in DPX mountant.

Results

- **Positive**
  i. Myeloblasts, and precursors of granulocytes (strong + vein promyelocytes and myeloeytes)
  ii. All mature neutrophils
  iii. Eosinophils and basophils
- **Negative:** Monoblasts, lymphocytes, lymphoblasts.

Note

- Freshly withdrawn (uncoagulated blood or bone marrow is used. However, EDTA, heparin, or oxalate blood may be used as myeloperoxidase is not inhibited by them. But stain should be done within 6 hours.
- Before staining, the smears should be dry and then fixed.
- Auer rods is better identified than in MGG stain.
- Phi bodies are visualized when DAB is allowed. But it is not when 2, 7-FDA is used.
- The method using DAB demonstrates a significantly high percentage of positive rods than techniques with other substrates.

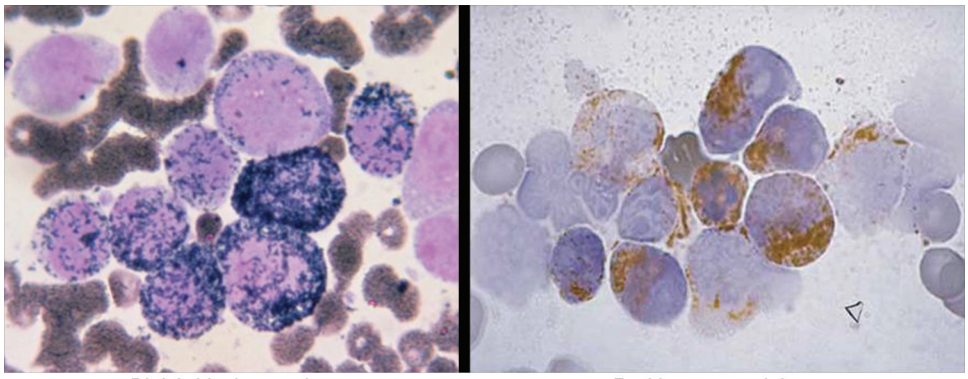


Fig. 12.13: Myeloperoxidase positivity in myeloblasts

Sudan Black B Staining

Both Sudan Black B and myeloperoxidase are positive in the azurophil granules of neutrophils and monocytes and in the specific granules of eosinophils. The biochemical basis for Sudan Black B staining or Sudanophilia is poorly understood. One possible view is that Sudan Black B stains the lipid membrane of the granules which contain myeloperoxidase enzyme. Another view is that it stains through an enzymatic mechanism, perhaps lined to myeloperoxidase and not by physical solution of lipids only.

The simplicity of Sudan Black B stain, makes it very popular among the histological laboratory.

Reagents

- Fixative: 40% formaldehyde (formalin)
- Staining solution. This is a mixture of two solutions, A and. B.
  - A. Sudan Black B: 0.3 g in 100 ml of absolute ethyl alcohol or ethanol. Shake well to dissolve the stain and filter to remove particles.
  - B. Buffer: 16 g of crystalline phenol in 30 ml of absolute ethanol. Add the phenol-ethanol mixture to 100 ml water in which 0.3 g of disodium hydrogen phosphate (Na_2HPO_4; 12 H_2O) has been dissolved. Shake well (vigorously) until all phenol was dissolved and then filter.

  Add 30 ml of solution A (Sudan Black B) to 20 ml of solution B (buffer). The mixture can be kept for 2–3 months at 4°C.
- Counterstain: MGG stain (preferably) or safranin.

Staining Method

- Fix air-dried smear of blood or bone marrow for 10 minutes in formalin vapour. It can be done by immersing the smears in a solution of 9 volumes of absolute alcohol (ethanol), and 1 volume of 40% formaldehyde. Alternatively, it can be done by soaking filter paper in formalin and placing the slides inside a 37°C incubator.
- Wash the smears gently in water for 5–10 minutes. But longer period of washing (30–60 minutes) will result in stronger staining. Place the slides in working staining solution in Coplin jar for 60 minutes.
- Wash in 70% ethanol by waving the slides in the alcohol in a Coplin jar for 3–5 minutes (to remove excess dye).
- Wash in water for 2 minutes and then dry.
- Counterstain the slides in MGG stain for 5 minutes.
- Dry and mount it in DPX.

Result

Positive: Cytoplasmic granules stain faintly in neutrophil precursors and strongly in mature neutrophils with a brown black color. Some myoblasts also show positive staining. Eosinophilia granules have scattered fine brown black granules (Fig. 12.14).

Negative: Lymphoblasts and lymphocytes.

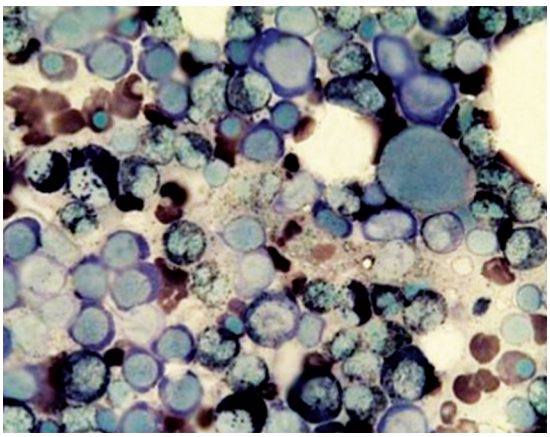


Fig. 12.14: Sudan Black B stain in AML

Nonspecific Esterase Staining

Reagents

- **Fixative:** Phosphate buffered acetone-formaldehyde. Acetone 40 ml; 35% formaldehyde 25 ml; Na_2HPO_4 20 mg, and K HPO_4 100 mg, in 30 ml of water. Filter the

solution before use and must be clear. It can be kept at room temperature for one month.

- **Buffer:** Sorensen's phosphate buffer (M/15, pH = 7.6).
- **Incubation mixture:** Add in the following manner:
  i. Buffer: 44.5 ml
  ii. Hexazotized pararosanilin: 3 ml
  This can be prepared by mixing equal volumes of pararosanilin solution or pararosanilin chloride (sigma) and fresh 4% aqueous $NaNO_2$ for 1 min just before use.
  Pararosanilin solution (pararosanilin chlorde) (sigma): 2 g in 50 ml of 2 mol/L HCl. Heat gently, without boiling cool down to room temperature and filter.
  iii. Alpha-naphthol acetate 50 g dissolved in 2.5 ml ethylene glycol monomethyl ether.
- Mix well all the above reagents. Filler mixture through Seitz filter.
- Counterstain: Harris haematoxylin (alternatively 1% methyl green in veronal acetate buffer, pH 4.0 may be used).
- Mounting medium: Glycerol/gelatin. Add 15 g of gelatin to 100 ml of glycerol and 100 ml of water.

Staining Method

- Place air dried blood or bone marrow films/smears in fixative for 30 seconds at 4°C.
- Rinse the slides well in running tap water.
- Incubate the slides in incubation mixture for 45–60 minutes.
- Wash the slides in running tap water.
- Counterstain with Harris haematoxylin for 10 minutes (or with methyl green for 1 minute).
- Mount in glycerol/gelatin while the slides are still wet.

Result

Positive: Dark red granules in cytoplasm. The nuclei blue or blue-black (pale green if methyl green is used as counterstain).

Alpha-naphthol acetate esterase activity is found in monocytes but not in neutrophils or neutrophil precursors, other granulocytes or lymphocytes. However, it may be found in activated or atypical lymphocytes (Fig. 12.15).

Inhibition with Sodium Fluoride (NaF)

Add 75 mg of NaF to 50 ml of the incubation mixture (concentrations 1.5 mg/ml = 37° mmol/L).

Carry out the test simultaneously with alpha-naphthol acetate esterase (ANAE)

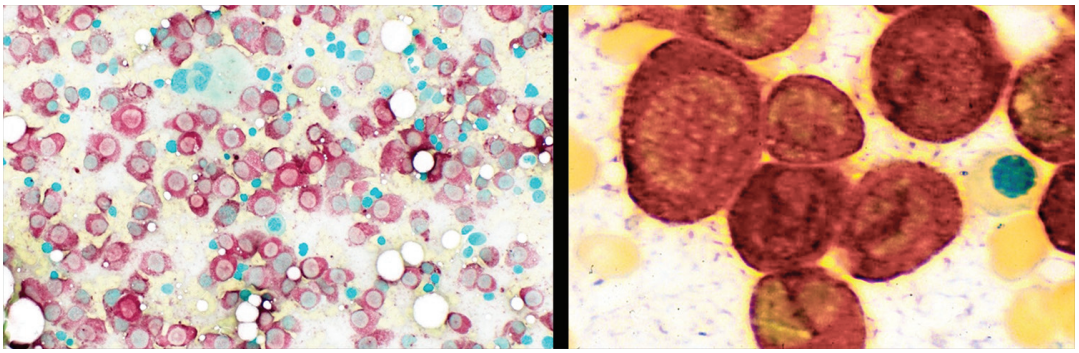


Fig. 12.15: Non-specific esterase positivity in case of acute monocytic leukaemia and in acute monoblastic leukaemia

reaction to investigate the NaF sensitivity or inhibition of a particular cell population.

Significance

- In monocytic leukaemia of AML (FAB, M5 subtype), the lukemic cells give a strong diffuse reaction sensitive to NaF. In erythroleukemia (M6) and megakaryoblastic leukaemia (M7), the blast cells give a positive ANAE reaction localized to the Golgi zone and sensitive to NaF.
- In the chronic B and T lymphoid leukaemia, it helps to distinguish T-PLL (prolymphocytic leukaemia) which gives positive reaction from B-PLL (negative reactions). The typical dot-like pattern of normal T lymphocytes is not observed in large granular lymphocyte (LGL) leukaemias.

Periodic Acid–Schiff (PAS) Staining

Reagents

- Fixative: Methanol
- Periodic acid ($HIO_4.2H_2O$): 5 gm, is dissolved in 500 ml of distilled water. Stored in dark bottle and is good for 3 months.
- Schiff's reagent (leukobasic fuchsin): Basic fuchsin, 1 g dissolved in 400 ml of boiling water.

  Cool the solution to 50°C and then filter. To the filtrate, add 1 ml of thionyl chloride ($SOCl_2$) and allow the solution to stand in the dark for 12 hours. Then add 2 g of activated charcoal and after shaking for 1 min filter the preparation. Store in the dark at 0–4°C.
- Rinsing solution: Sodium metabisulphite (100 g/L), 6 ml; HCl (1 mol/l), 5 ml; water to 100 ml.
- Counterstain: Harris haematoxylin, 2 g; water to 100 ml.

Staining Method

- Fix the smears (films) in methanol for 5 – 15 minutes.
- Then wash in running tap-water for 15 minutes.
- Expose some of the smears to digestion in diastase (1 g in in 1 litre of 9 g/L NaCl) for 1 hour at room temperature.
- Thereafter, allow both treated and untreated slides to stand in the periodic acid solution for 10 minutes.
- Wash in tap water and immerse the slides in Schiff's reagent for 30 minutes at room temperature in the dark.
- Rinse the slides three times in the rinsing solution.
- Wash in distilled water for 5 minutes
- Counterstain with haematoxylin for 10 minutes.
- Blue in tap-water for 5–10 minutes.
- Air dried the smears and mount.

Results

Positive: Block positive in lymphoblast, and fine-granular positivity (or negative reaction) in myeloblasts. Mature neutrophils react most strongly. Monocytes have a faint staining reactions. Positivity means pink/magenta colored granules in cytoplasm due to presence of glycogen.

Negative: Normoblasts (but in erythroleukaemia and in thalassemia, some of the erythroid precursors are PAS positive)

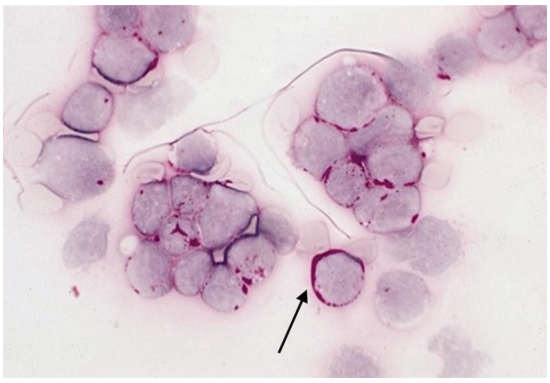
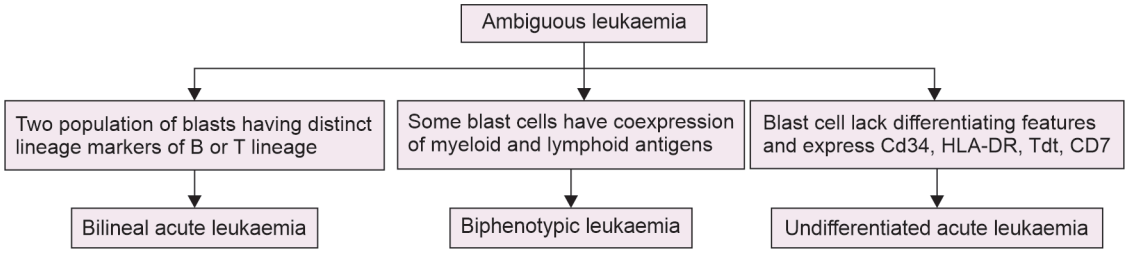


Fig. 12.16: A PAS stain shows block and granular positivity in lymphoblasts of ALL

Flowchart 12.1: Ambiguous leukaemia



Flowchart 12.2: Diagnostic approach to leukaemia

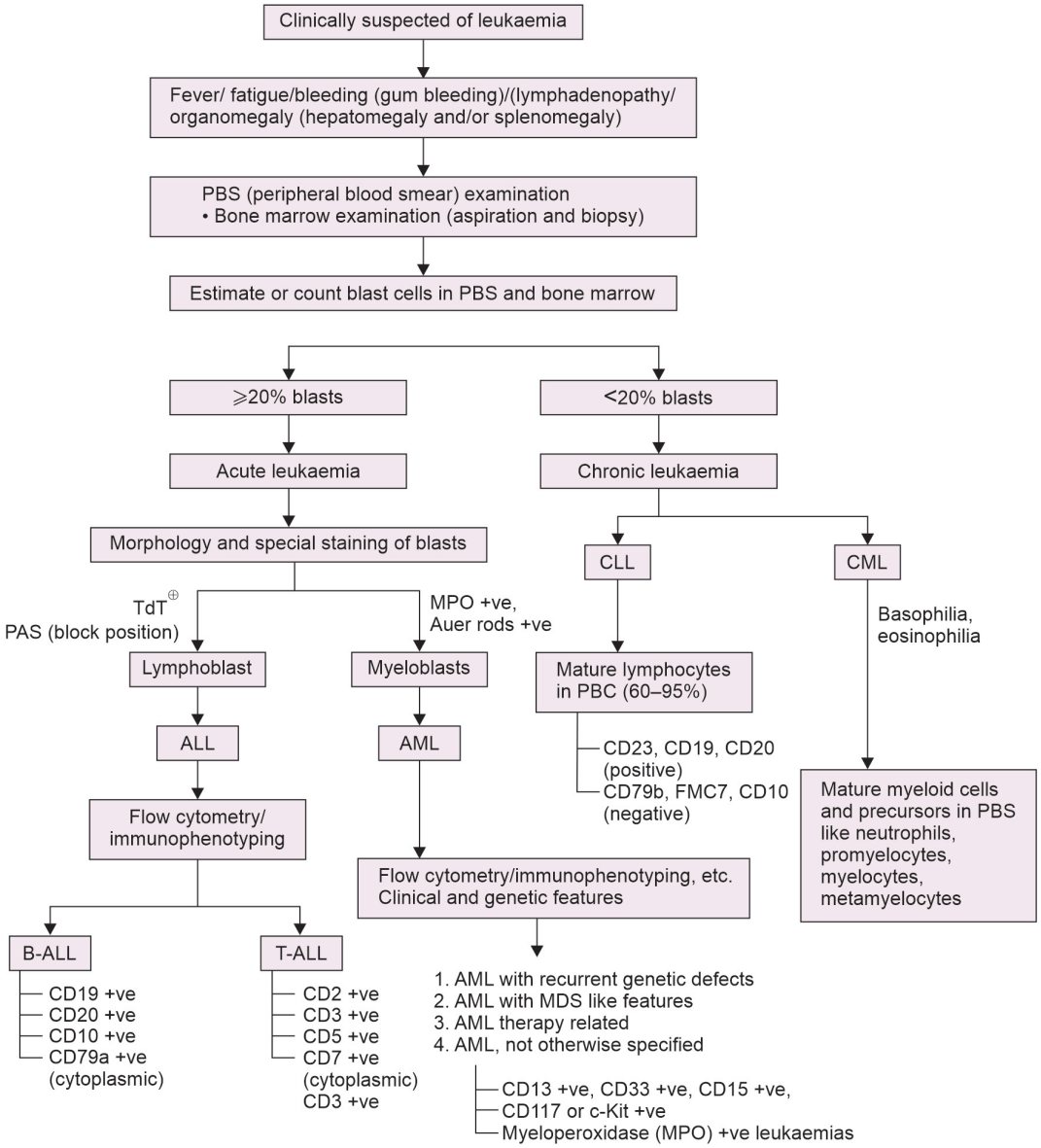


Table 12.15: Differences between chronic phase (CP), accelerated phase (AP) or blast phase (BP) of CML

Parameter	Chronic phase (CP)	Accelerated phase (AP)	Blast phase
• No. of blast %	<10%	10–19%	20%
• Basophil %	<20%	20%	Variable
• LAP score	Low	Increased	Increased
• Clonal evaluation	_	Yes	–
• Extramedullary blast proliferation	Absent	Absent	May be present
• Whether leukocytosis, thrombocytosis and splenomegaly responsive to therapy	Yes	No	_
• First line treatment	Imatinib	TKI therapy followed by bone marrow transplant	TKI therapy followed by bone marrow transplant

TKI: Tyrosine kinase inhibitor

Table 12.16: Differences between leukaemoid reaction and CML

Parameter	Leukaemoid reaction	CML
Clinical features	As per underlying cause or disease	Splenomegaly, lymphadenopathy and bone pain
Peripheral blood smear (WBC)		
• TLC (Total leukocyte count)	Usually <50,000/mm^3, but rarely exceeds >50,000/mm^3	>50,000/mm^3, usually >100,000/mm^3
• DLC (differential leukocyte count)	Shifted to left with few immature myeloid cells (promyelocytes, myelocytes and metamyelocytes)	Shifted to left with numerous immature myeloid cells seen Predominant cells are myelocytes and neutrophils
• Blast cells	Usually not seen	May be seen but less than <10%
• Basophilia and eosinophilia	Variable	Present
• Toxic granules and Döhle bodies	Present	Absent
LAP score	Normal or increased	Low
Anaemia (RBC)	Absent or minimal	Severe and progressive
Platelet number	Variable	Normal or increased
Extramedullary myeloid	Absent	Present
Bone marrow examination	Trilineage hyperplasia	Myeloid hyperplasia
Philadelphia chromosome	Absent	Present

Chapter 13

Platelet Disorders: Investigation of Haemostasis and Bleeding Tendency

PLATELET DISORDERS AND THEIR INVESTIGATION

Platelet disorders include:

- Thrombocytopenia
- Thrombocytosis
- Platelet dysfunction

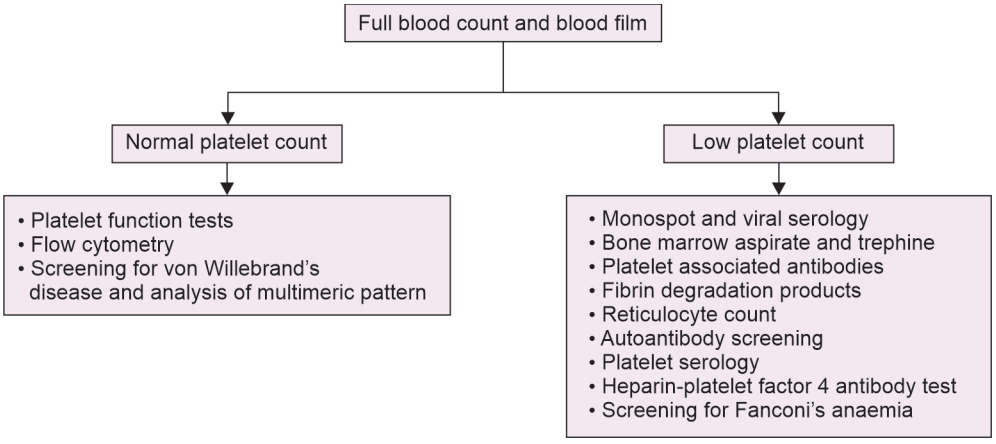
Thrombocytopenia: It refers to decrease in the number of platelets in PBS below normal, i.e. <1.5 lacs/mm^3. It may result from four main mechanisms:

i. Increased destruction of platelets in peripheral blood.
ii. Decreased production of platelets by megakaryocytes in bone marrow.
iii. Dilutional thrombocytopenia
iv. Sequestration in enlarged spleen

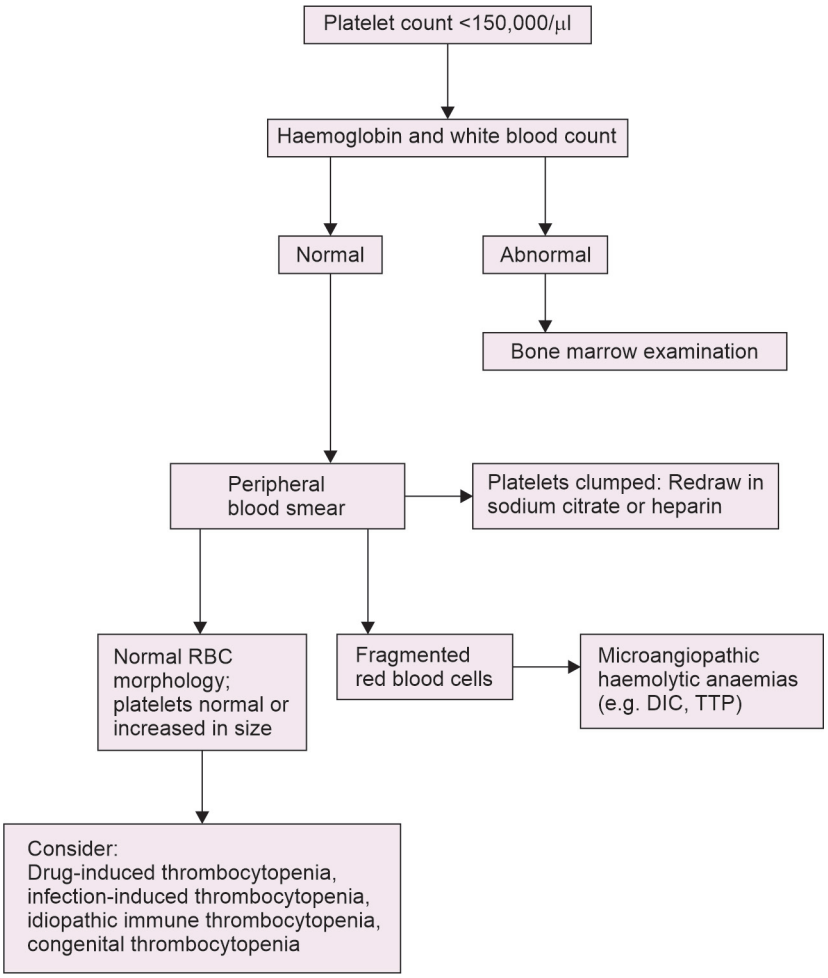
Platelet count and clinical significance

- Normal platelet count: 1.5–4 lacs/mm^3
- Platelet count 50,000–1.5 lacs/mm^3: Thrombocytopenia but no clinical significance or bleeding.
- Platelet count 20,000–50,000/mm^3: Bleeding usually starts immediately after trauma (post-traumatic bleeding).
- Platelet count <10000/mm^3: It can cause life-threatening intracranial bleeding.

Flowchart 13.1: Full blood count and blood film



Flowchart 13.2: Algorithm for thrombocytopenia evaluation

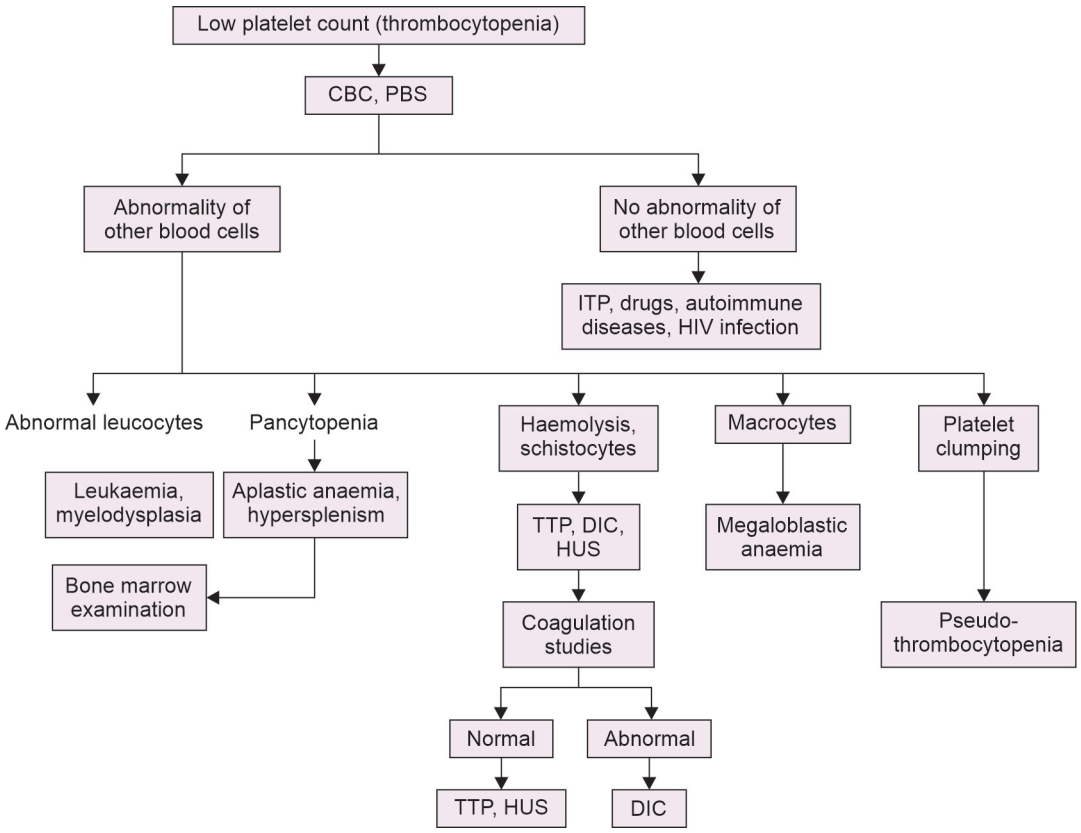


Pseudo-thrombocytopenia

When blood samples are collected in EDTA and platelet counts are determined by electronic cell counters, then sometimes a falsely low result may be obtained. Peripheral blood smear made from this EDTA-mixed blood may reveal large clumps of platelets and platelets rosetting around neutrophils. This clumping of platelets results from presence of EDTA-dependent antiplatelet antibody. EDTA anticoagulant alters the conformation of GpII/IIIa complex and exposes neoantigen. The antibody reacts with this cryptic antigen and causes platelet clumping only *in vitro*. Clinically, these antibodies do not have significance. Incorrect diagnosis of thrombocytopenia by electronic cell counter can avoided by simultaneous examination PBS (Flowchart. 13.3).

Thrombocytosis: This refers to platelet count >4 lacs/mm^3.

Flowchart 13.3: Evaluation of thrombocytopenia



CBC: Complete blood count, PBS: Peripheral blood smear, ITP: Idiopathic thrombocytopenic purpura, TTP: Thrombotic thrombocytopenic purpura, DIC: Disseminated intravascular coagulation, HUS: Haemolytic uraemic syndrome

Platelet clumps seen in EDTA anti-coagulated blood sample in a patient with EDTA, dependent platelet agglutinins (Fig. 13.1).

No platelet clumps seen and platelet count normal in the blood sample from the same patient anti-coagulated with sodium citrate.

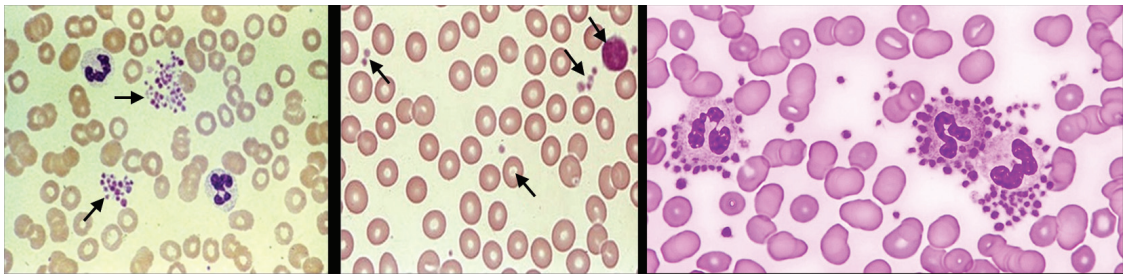


Fig. 13.1: Platelet clumps in EDTA blood, no platelet clumps in sodium citrate blood and platelet rosetting around neutrophils due to EDTA excess in blood

PLATELET FUNCTIONAL DISORDER

A. Hereditary

- Bernard-Soulier syndrome: Disorders of platelet adhesion
- Glanzmann thrombasthenia: Disorders of platelet aggregation
- Storage pool deficiency: Disorders of platelet secretion

B. Acquired

- Drugs: Aspirin, NSAID, sulfinpyrazone
- Haematologial malignancies: Myelodysplastic syndrome and myeloproliferative neoplasms
- Renal failure: Uraemia

Table 13.1: First line investigation for acute haemostatic failure

Coagulation Test			Platelet count	Clinical significance
PT	APTT	TT		
1. N	N	N	N	Disorders of platelet function. Factor XIII deficiency. Disorder of vascular homeostasis. Normal homeostasis
2. Long	N	N	N	Factor VII deficiency, early oral anticoagulant
3. N	Long	N	N	Factor VIII : C, IX, XI, XII, prekallikrein, HMWK deficiency, von Willebrand's disease, heparin administration, circulating anticoagulant
4. Long	Long	N	N	Vitamin K deficiency, oral anticoagulants, Factor V,VII,X and II deficiency.
5. Long	Long	Long	N	Heparin administration, fibrinogen deficiency. Hyperfibrinolysis, liver disease.
6. N	N	N	Low	Thrombocytopenia
7. Long	Long	N	Low	Massive blood transfusion, liver disease
8. Long	Long	Long	Low	DIC, liver disease

Table 13.2: Contents of platelet granules

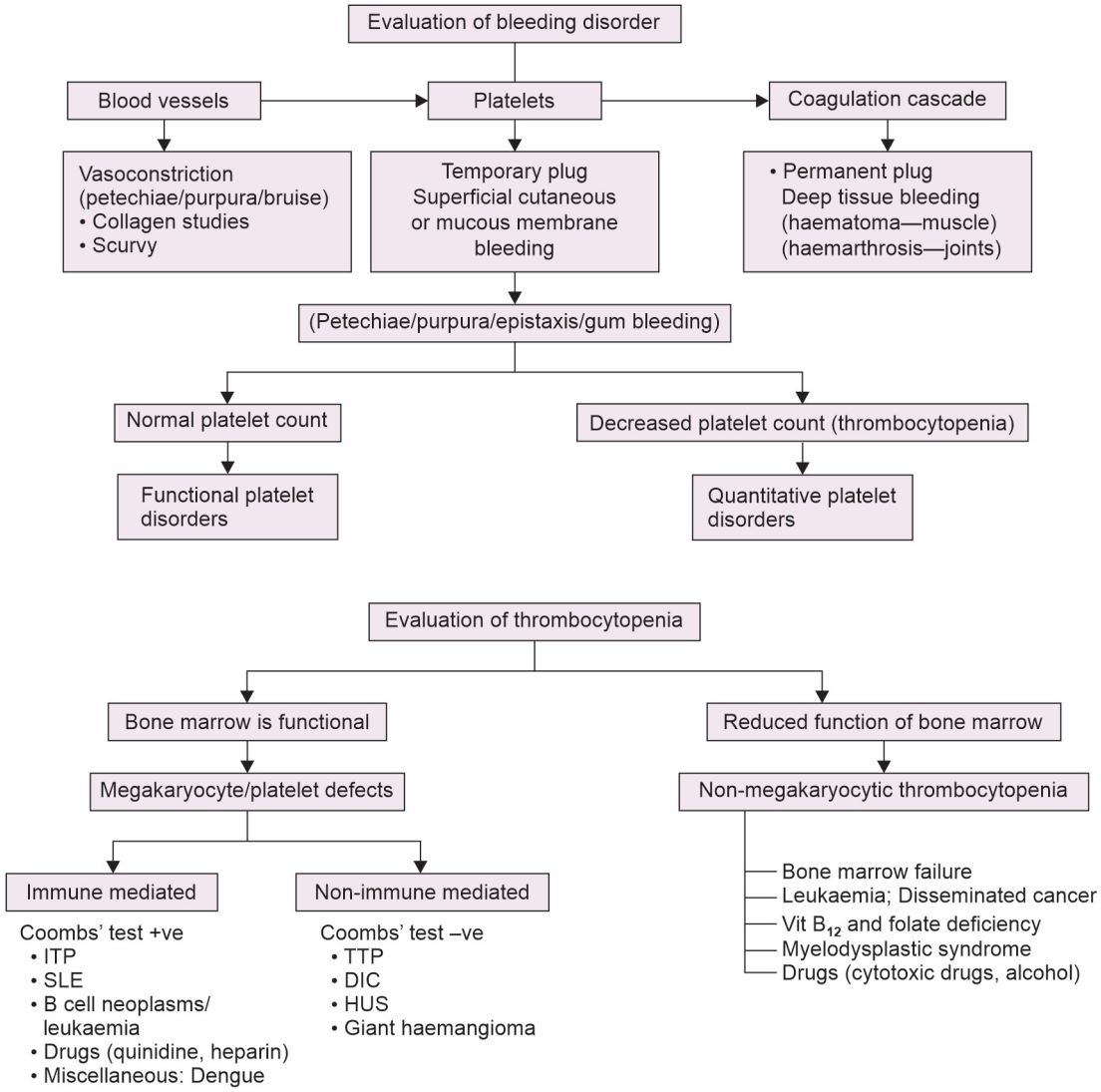
Granules	Dense (Delta or δ) granules	Lysosomal vesicles
Factor VIII	ATP	Galactosidases
vWF	ADP	Glucuronidases
Fibrinogen	GDP	Hexosaminidase
PF4	GTP	Fucosidase
PDGF	Calcium	Microperoxisomes
PAI-1	Serotonin	+ Others
β-thromboglobulin	Pyrophosphate	
Thrombospondin		

Platelet Function Tests

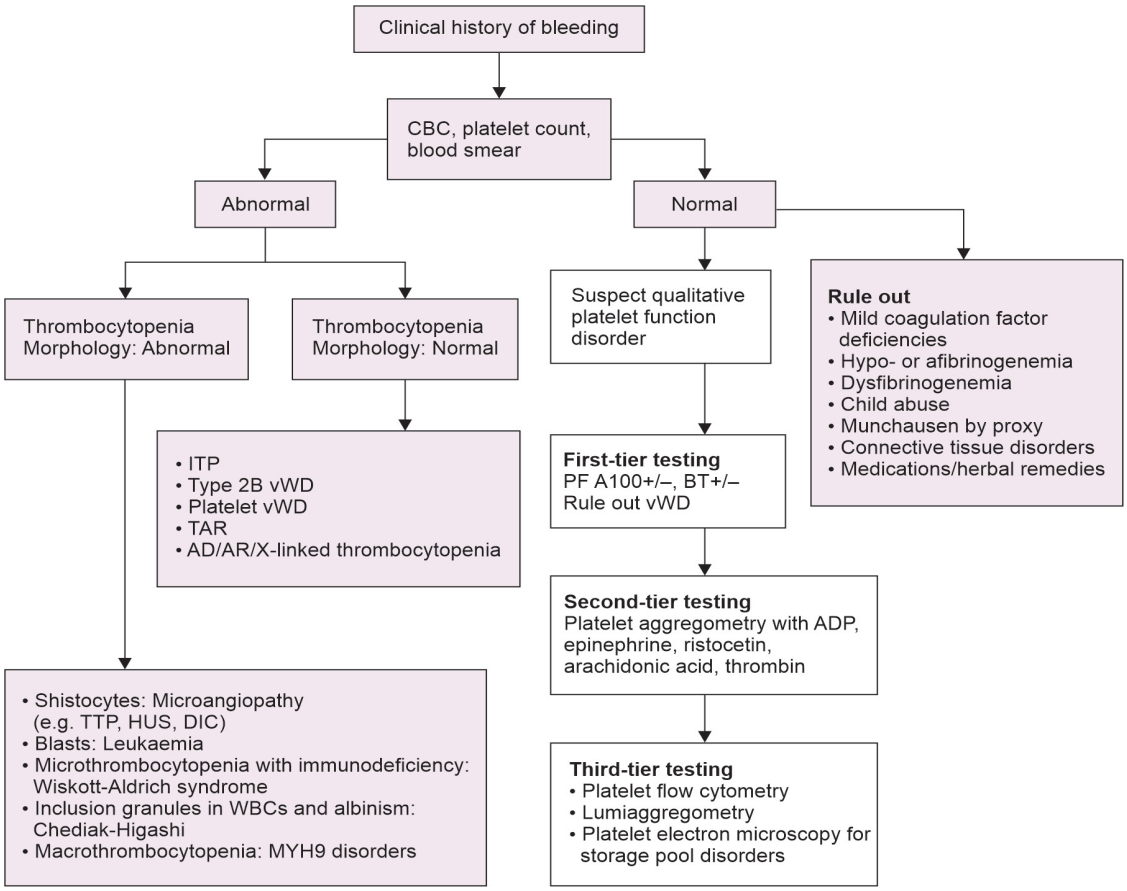
1. Adhesion tests:
   i. Retention in a glass-bead coloumn,
   ii. Baumgartner's technique
2. Aggregation tests: Turbidometric technique using ADP, ristocetin, collagen, arachidonic acid, adrenalin, thrombin.
3. Investigation of granular content and release:

i. Dense bodies: ADP and ATP content (bioluminescence), serotonin release, electron microscopy.
ii. α-Granules: β-Thromboglobulin, PF4 (platelet factor 4), vWF
4. Platelet coagulation activity: Prothrombin consumption index (Flowcharts 13.4 and 13.6).

Flowchart 13.4: Evaluation of bleeding disorder and thrombocytopenia

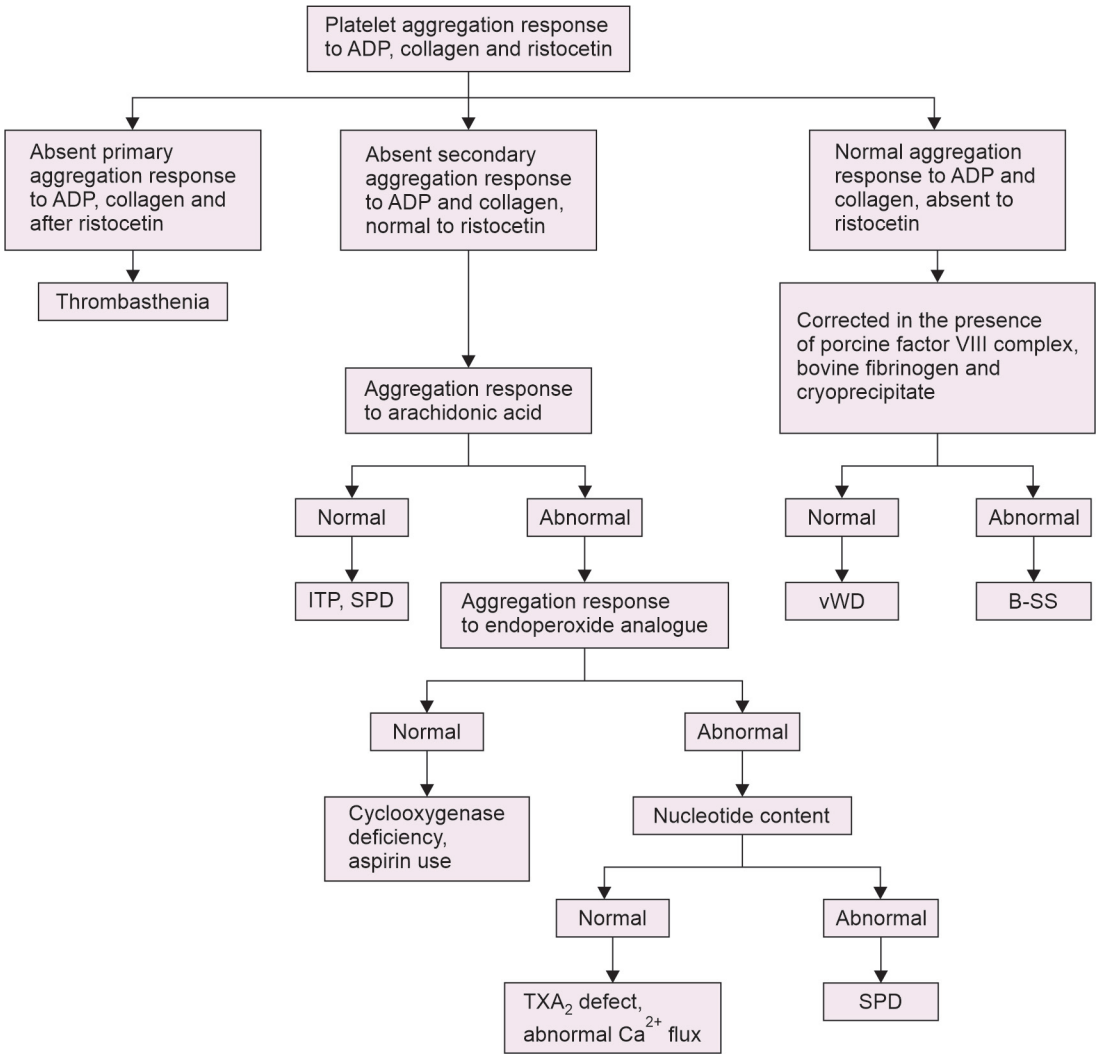


Flowchart 13.5: Algorithm for evaluation of a patient with suspected platelet disorders



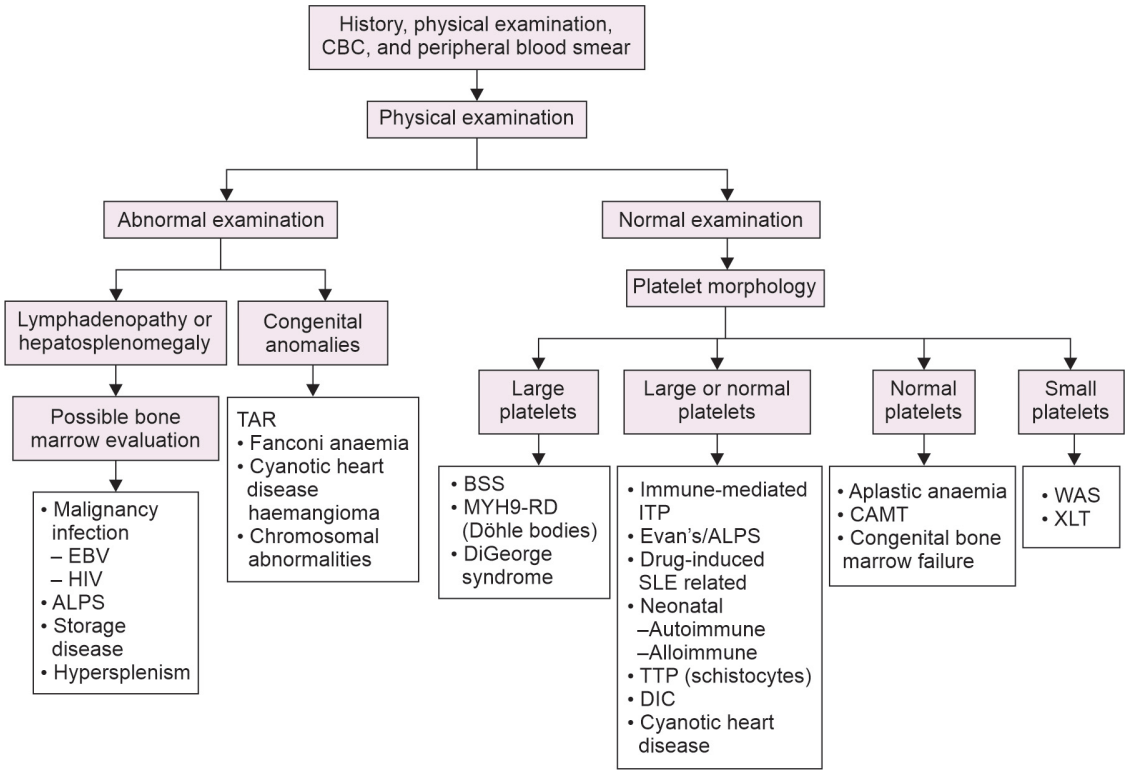
Abbreviations: BT: Bleeding time; PFA: Platelet function abnormalities; N: Normal; TTP: Thrombotic thrombocytopenic purpura; HUS Haemolytic uraemic syndrome; DIC disseminated intravascular coagulation; WBCs: White blood cells; MYH9: Myosine heavy chain gene disorders: ITP: Immune thrombocytopenic purpura; vWD: von Willebrand's disease; TAR: Thrombocytopenia and absent radi; AD: Autosomal dominant; AR: Autosomal recessive; ADP: Adenosine diphosphate.

Flowchart 13.6: Flowchart for suspected platelet dysfunction.



ITP: Idiopathic thrombocytopenic purpura; SPD: Storage pool defect, vWD: von Willebrand disease, B-SS: Bernard-Soulier syndrome

Flowchart 13.7: A diagnostic approach to the patient with thrombocytopenia



ALPS: Autoimmune lymphoproliferative disorder; BSS: Bernard-Soulier syndrome; CAMT: Congenital amegakaryocytic anemia; CBC: Complete blood count; DIC: Disseminated intravascular coagulation; EBV: Epstein-Barr virus; HIV: Human immunodeficiency virus; ITP: Immune thrombocytopenic purpura; MYH9-RD: MYH9-related disorders; SLE: Systemic lupus erythematosus; TAR: Thrombocytopenia absent radius; TTP: Thrombotic thrombocytopenic purpura; WAS: Wiskott-Aldrich syndrome; XLT: X-linked thrombocytopenia

Table 13.3: Planet count, size and aggregation tests in different planet function disorders

Condition	Platelet		Aggregation with				
	Count	Size	ADP	Collagen	Ri	AA	Factor VIII complex (porcine)
Thrombasthenia	N	N	0	0	Ab	0	Ab
Bernard-Soulier syndrome	Large	Large	N	N	0	N	0
von Willebrand disease	N	N	N	N	0	N	N
Storage pool defect	N	N	1	Ab	1/0	1/0	1/0
Aspirin ingestion	N	N	1	Ab	N/Ab	Ab	N/Ab
Ehlers-Danlos syndrome	N	N	N	Ab	N	N	--
Cyclooxygenase deficiency	N	N	1/N	Ab	N	Ab	--
Thromboxane synthetase deficiency	N	N	1/N	Ab	n	Ab	

N: Normal, 0: Absent,1: Primary wave only, Ab: Abnormal, Col: Collagen, Ri: Ristocetin, AA: Arachidonic acid

Chapter 14

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 hosts 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 malaria* and *Plasmodium ovale* (Fig. 14.1). Among these four species *P. vivax* is the most widely distributed species in the world. In India, incidence is *P. vivax* (70%), *P. falciparurm* (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 occuring 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.

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 glycophorin 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 falciparun 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.

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).
- 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

Table 14.1: Different features of *P. vivax* and *P. falciparum*

Features	*P. vivax*	*P. falciparum*
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 14.1: Different features of *P. vivax* and *P. falciparum* (Contd.)

Microscopic examination	*P. vivax*	*P. falciparum*
Gametocyte	Spherical or globular much larger than RBC	Crescentic or sickle-shaped larger than a RBC but host cell (RBC) hardly recognisable
Haemozoin	Yellowish-brown, fine granules	Dark brown or blackish
Schüffner's dots or Maurer's dots in cytoplasm	Schüffner's dots	Maurer's dots

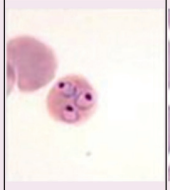
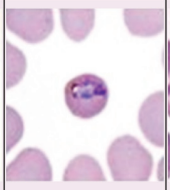
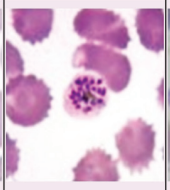
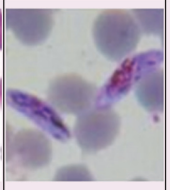
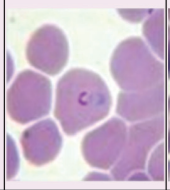
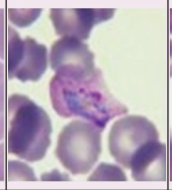
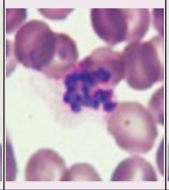
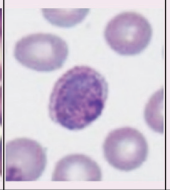
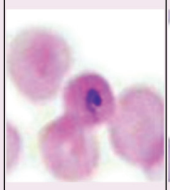
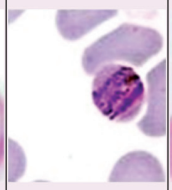
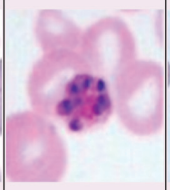
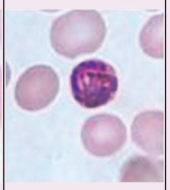
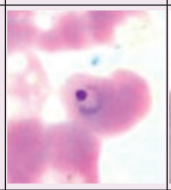
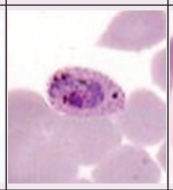
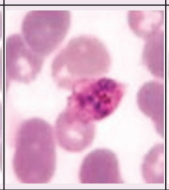
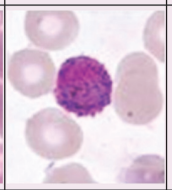
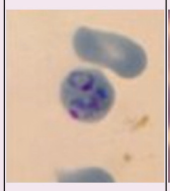
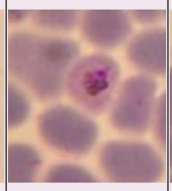
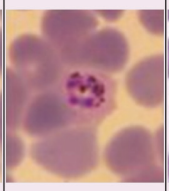
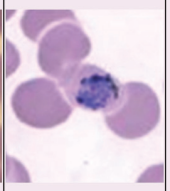
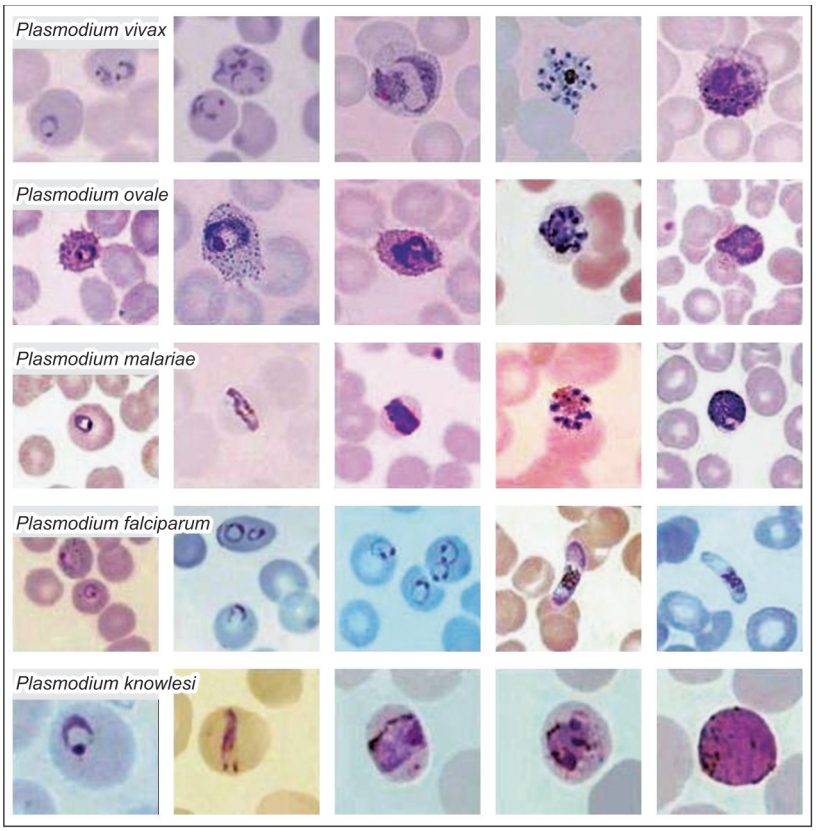
Stages / Species	Ring	Trophozoite	Schizont	Gametocyte	
P. falciparum					• Parasitised red cells (pRBCs) not enlarged. • RBCs containing mature trophozoites sequestered in deep vessels. • Total parasite biomass = circulating parasites + sequestered parasites.
P. vivax					• Parasitised prefer young red cells • pRBCs enlarged. • Trophozoites are amoeboid in shape. • All stages present in peripheral blood.
P. malariae					• Parasitised prefer old red cells. • pRBCs not enlarged. • Trophozoites tend to have a band shape. • All stages present in peripheral blood
P. ovale					• pRBCs slightly enlarged and have an oval shape with tufted ends. • All stages present in peripheral blood
P. knowlesi					• pRBCs not enlarged. • Trophozoites, pigment spreads inside cytoplasm, like *P. malariae*, band form may be seen • Multiple invasion and high parasitaemia can be seen like *P. falciparum* • All stages present in peripheral blood.

Fig. 14.1: Human malaria



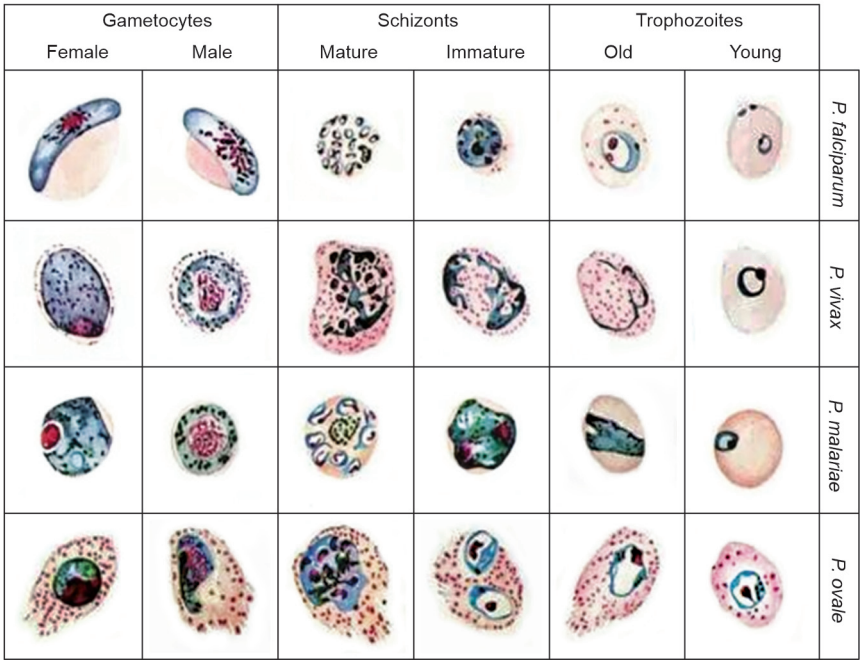


Fig. 14.2: Different species of human malaria

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 on Leishman stain. Thick smear also can be stained by Field's stain. For preparation of thin and trick 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 (Fig. 14.2).

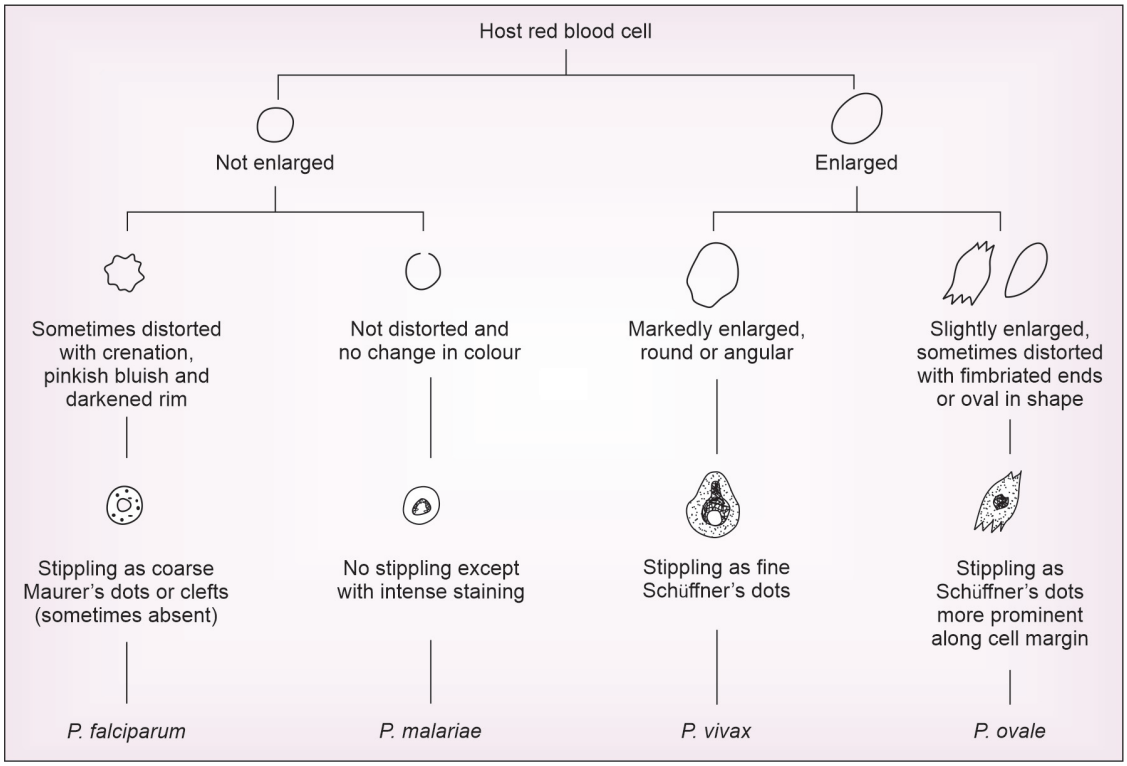


Fig. 14.3: Schematic diagram to diagnose four different species of malaria

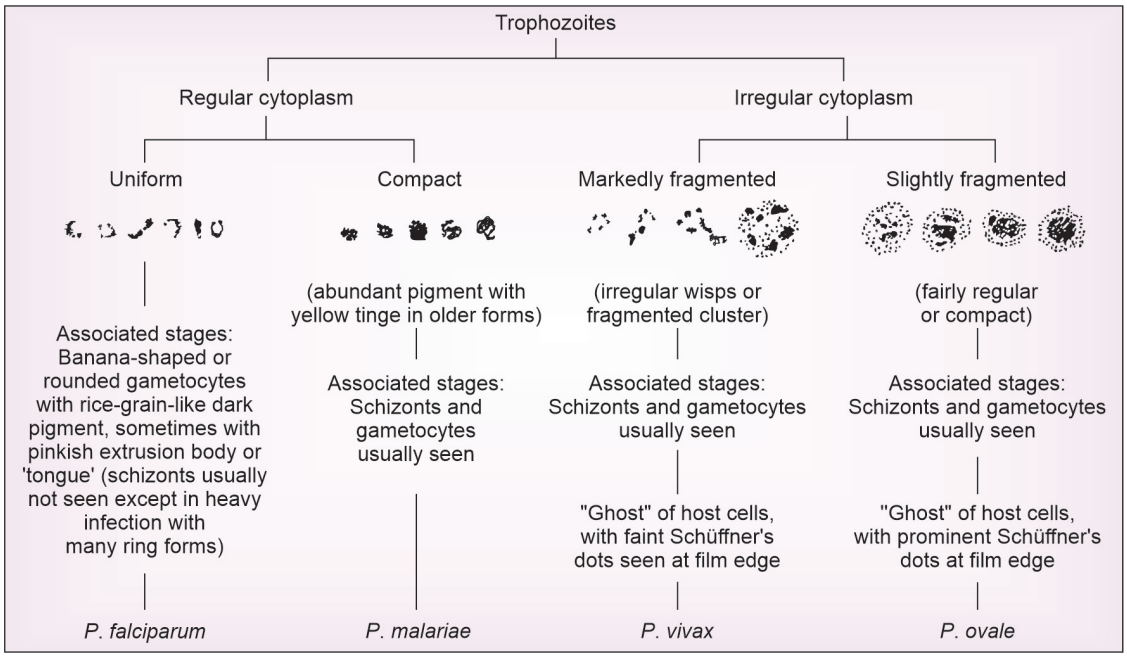


Fig. 14.4: Species differentiation of malarial parasites by cytoplasmic pattern of trophozoites in Giemsa-stained thick blood films

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 (accole 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 14.5 and 14.6).

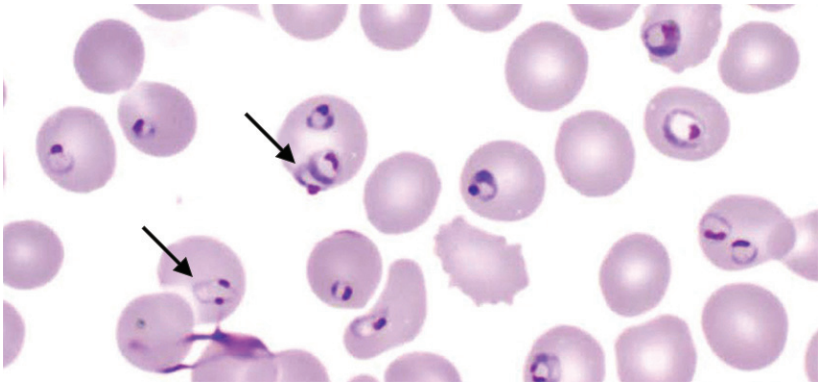


Fig. 14.5: Trophozoites of *P. falciparum*

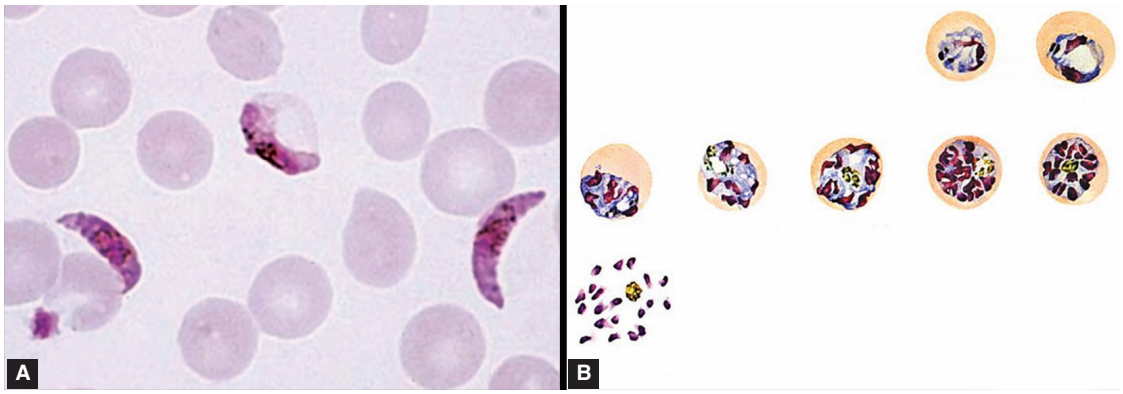


Fig. 14.6: (A) Gametocytes of *P. falciparum;* (B) Schizonts of *P. falciparum*

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. 14.7)

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. 14.5).

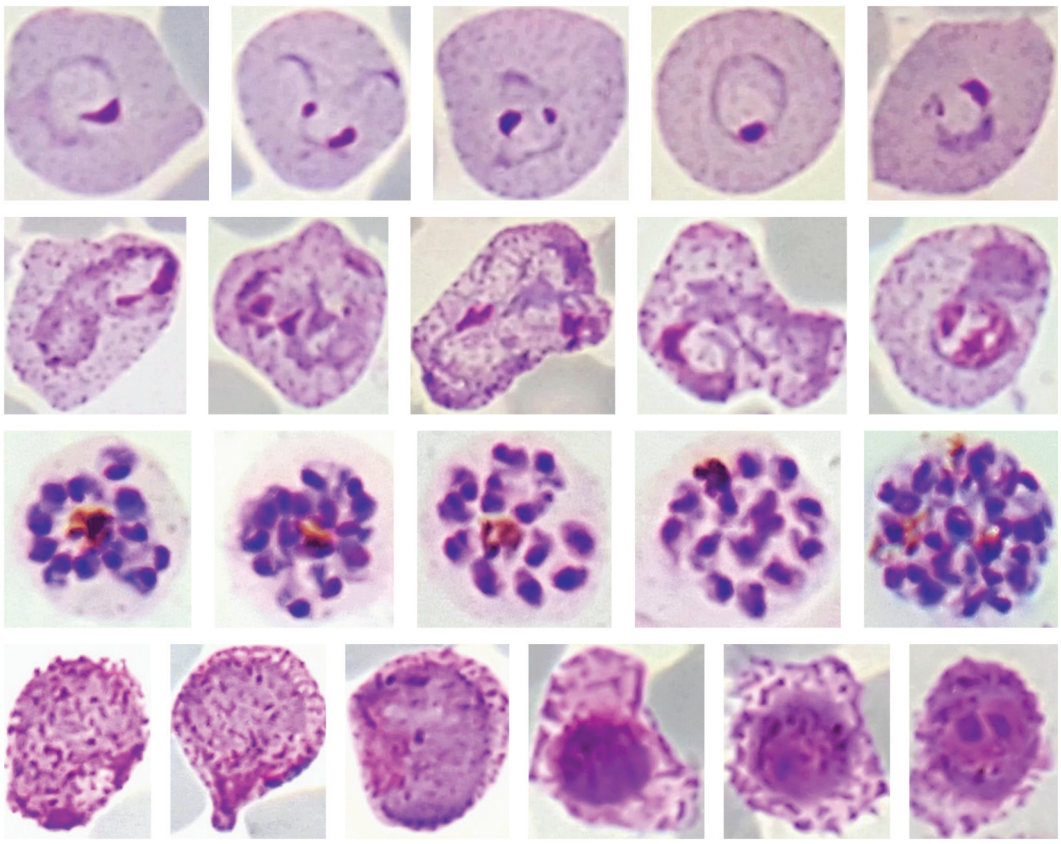


Fig. 14.7: *Plasmodium vivax*. First row: Early trophozoites, Second row: Late trophozoites, Third row: Schizonts, Fourth row: Gametocytes

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. 14.8).

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

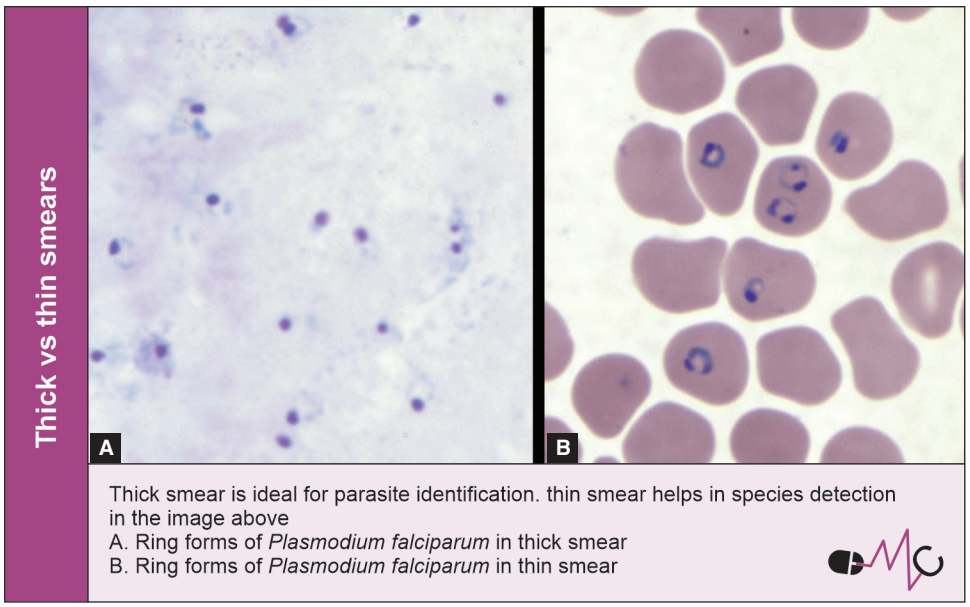


Fig. 14.8: *Plasmodium falciparum;* (A) Thick smear; (B) Thin smear

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. 14.9).

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:

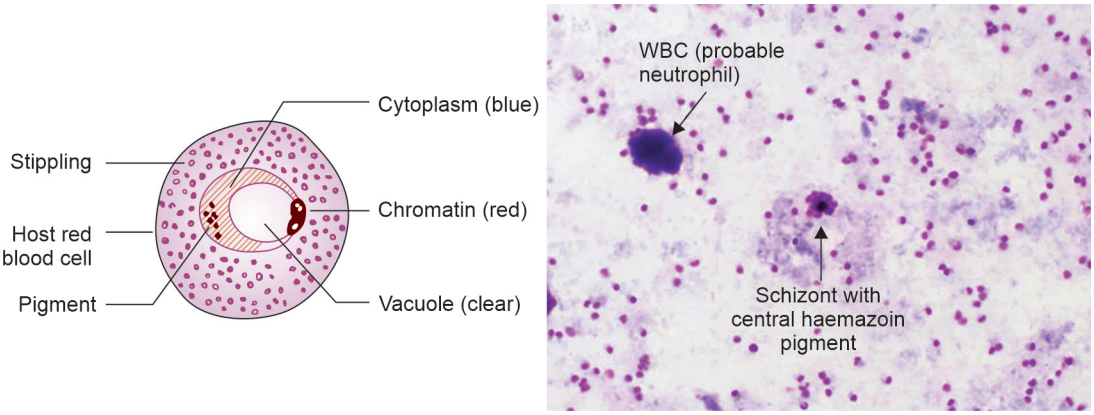


Fig. 14.9: Malarial pigments

Parasite density (PD) on thick smear
= Total WBC count/µl

$$\times \frac{\textit{Number of parasites}}{200}$$

that is (total WBC count/µl × Number of parasites) ÷ 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 of blood can be calculated if RBC count in millions/µl is known.

PD on thin smear = Red cell count in millions/µl × 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) =

$$\frac{\textit{Number of parasitised RBCs}}{1000\ \textit{RBCs}} \times 100\%$$

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, accole 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 immunochromatographic 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. 14.10).

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.

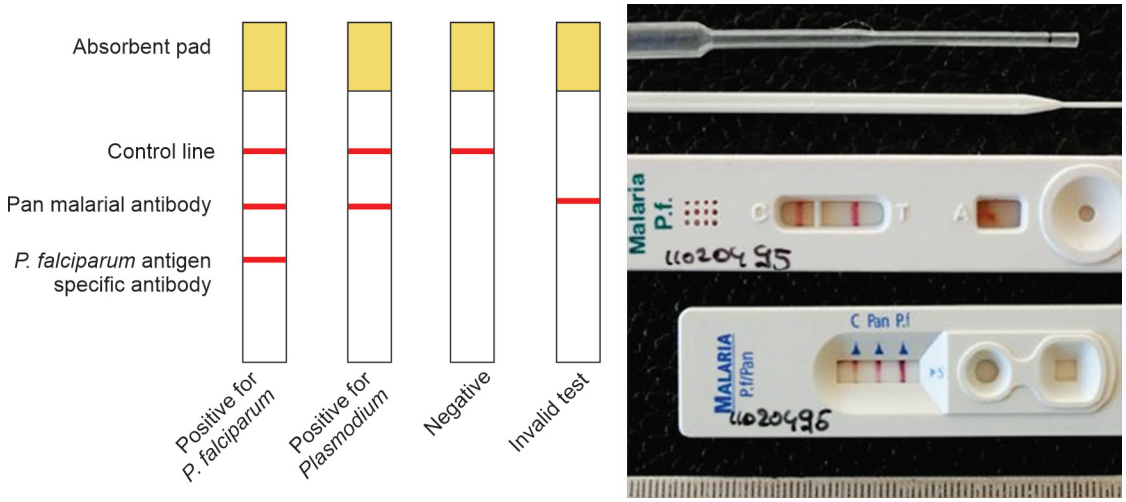


Fig. 14.10: Rapid diagnostic kits for malaria

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. 14.11).

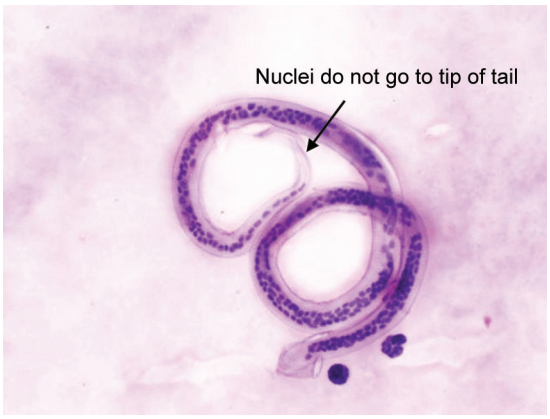


Fig. 14.11: Filaria (*Wuchereria bancrofti*) in blood

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. 14.12).

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. 14.13).

The diagnosis can be made by demonstrating **trypomastigote forms (amastigates of trypanosoma)** in PBS or bone marrow.

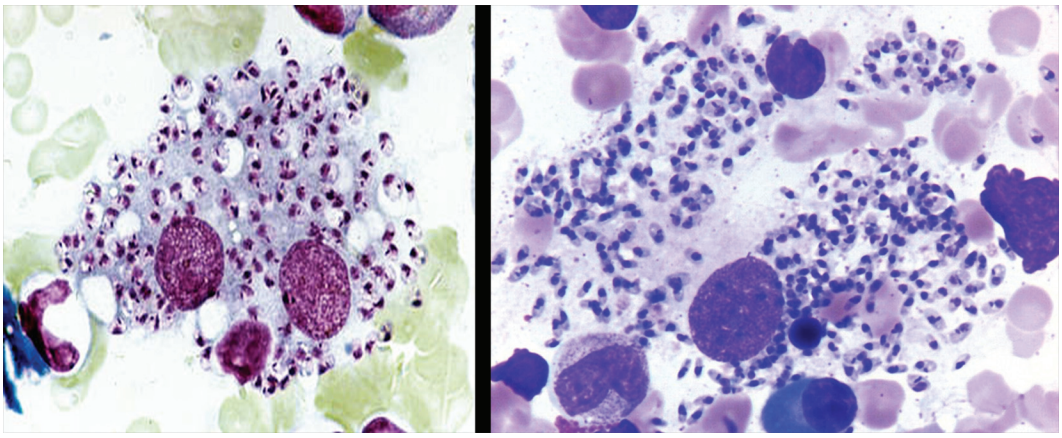


Fig. 14.12: LD bodies in bone marrow

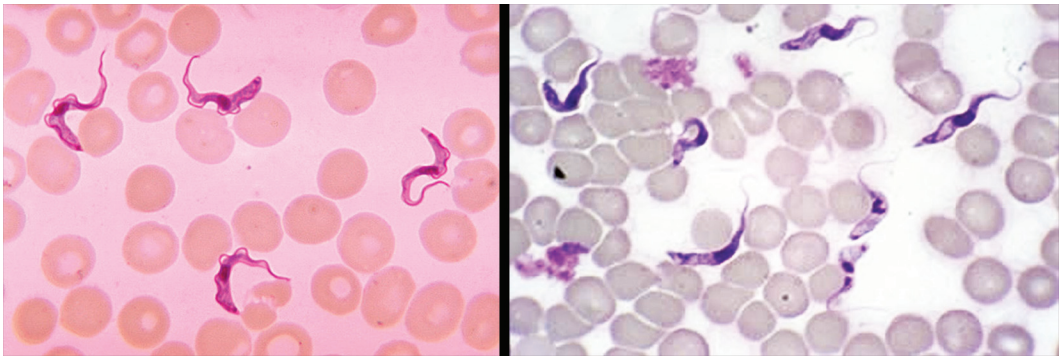


Fig.14.13: *Trypanosoma cruzi* in peripheral blood smear

Babesiosis (Fig. 14.14)

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. 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. 14.15).

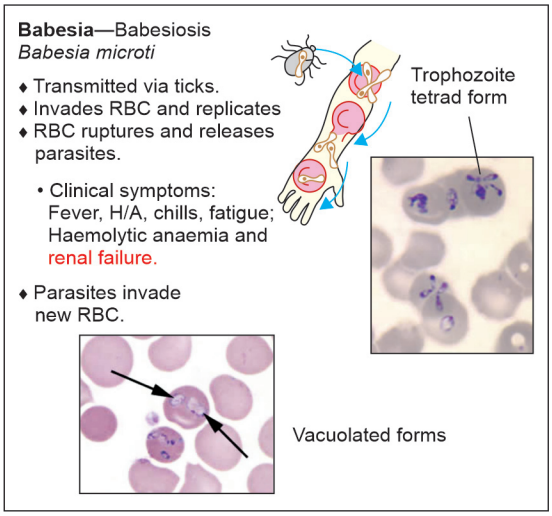


Fig.14.14: Schematic diagram of babesiosis

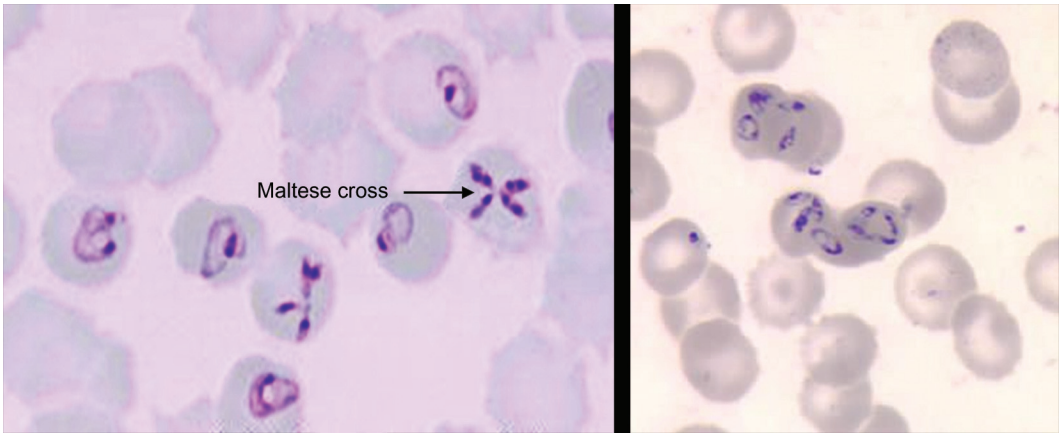
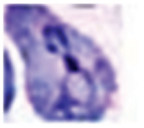
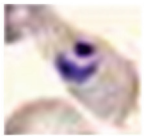
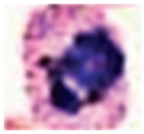
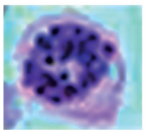
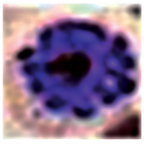
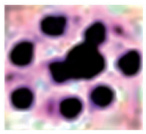
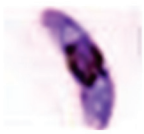
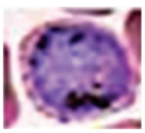


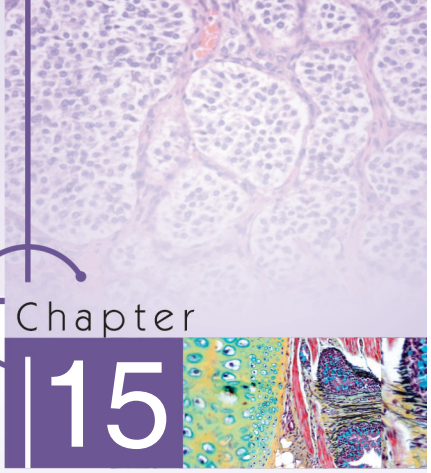
Fig.14.15: Babesiosis on peripheral blood

Table 14.2: Morphological differentiation of malaria parasites

	P. vivax	*P. falciparum*	*P. ovale*	*P. malariae*
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 accole, small chromatin dot	Thick, compact rings	Very small, compact rings
Late trophozoites	Amoeboid, central vacuole, light blue cytoplasm	Compact,vacuolated, somatimes 2 chromatin dots	Smaller than *P. vivax*, 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 *P. vivax* but similar	Round, fills one-half to two-thirds of cell, similar to *P. vivax* 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; Accole forms: Parasite that is marginalized to edge of cell

Species / Stages	P. *falciparum*	P. *vivax*	P. *malariae*	P. *ovale*
Ring stages				
Trophozoite				
Schizont				
Gametocyte				



Chapter 15

Automation in Haematology

Automation in haematology includes

- Cell counter (automated haematology analysers)
- Diagnosis of haemoglobinopathy by HPLC
- Flow cytometry
- Automated coagulometers
- Automated ESR

CELL COUNTER (AUTOMATED HAEMATOLOGY ANALYSERS)

Evolution of the Analyser

The first automated cell counters came out in the 1950s based on **Coulter's electrical impedance principle** in which cells pulled through an aperture break an electric circuit, indicating both the presence of a cell and the size of the cell. Those were the "prehistoric" analysers which just did counts and indices.

In the 1970s, automated platelet counters, 7 parameter complete blood count (CBC) analysers, and 3-part differential leucocyte counters (for lymphocytes, monocytes and granulocytes) entered the market. In the 1980s, a single instrument could produce a 10-parameter CBC. The 1990s brought further advancement in leucocyte differentials with the use of **flow-cell techniques** based on either electrical impedance or light scatter properties.

Now, some analysers are primarily based on impedance principles, some use flow principles to perform laser light scatter, and others use **fluorescent flow cytometer**. For instance, one analyser may determine leucocyte differentials by inserting a fluorescent dye into the cell nucleus and measuring how strongly it fluoresces. One may alter the permeability of a cell and see how quickly it absorbs a dye. Another may measure enzyme activity in a cell placed in a particular substrate. Then there is the **volume conductivity and scatter (VCS) method** that analyzes cell in their "near-native" state.

Some of the popular automated haematology analysers are supplied by Sysmex, Horiba, Mindray, Erba (Transsasia), etc.

Reticulocyte counts can be performed by many analysers, giving an alternative to time-consuming manual counts. Many automated reticulocyte counts, like their manual counterparts, employ, the use of a supravital dye/stain such as new methylene blue to stain the red cells containing reticulin prior to counting (Fig. 15.1).

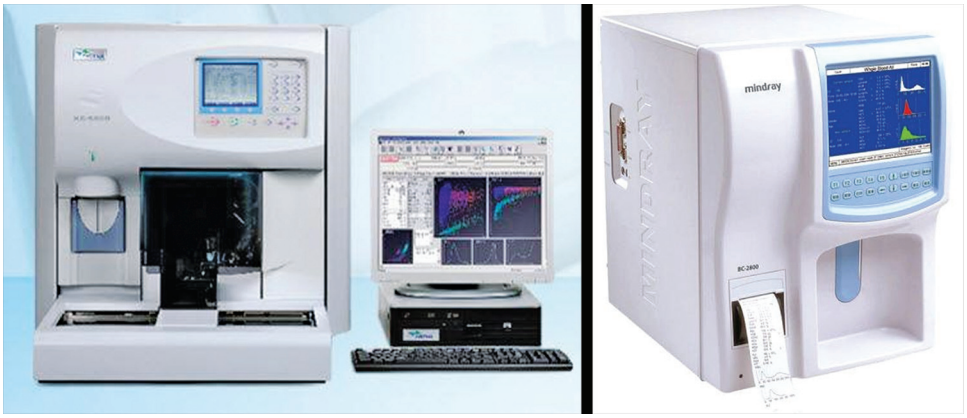


Fig. 15.1: Haematology autoanalyser

Table 15.1: Advantages and disadvantages of cell counter

Advantages	Disadvantages
• Speed with efficient handling of large number of samples	• Flagging of a laboratory test (or flags) result demands labour intensive examination of a blood smear
• Accuracy and precision in quantitative blood tests	• Comments on red cell morphology cannot be generated
• Ability to perform multiple tests on a single platform	• Platelet clumps are counted as single, so give erroneous low platelet count
• Significant reduction of labour requirements	• Due to interfering factors falsely increased or decreased results
• Invaluable for accurate determination of red cell indices (MCV, MCH, MCHC)	• Machines are expensive with high running costs
	• Giant platelets are counted as RBCs
	• Nucleated RBCs and platelet clumps are counted as leucocytes

Accuracy: A test method is said to be accurate when it measures what is supposed to measure. This means it is able to measure the true amount or concentration or percentage of a substance in a sample.

For accuracy of particular test(s), laboratory is required to participate in **External Quality Assurance Scheme (EQAS)**. This is called **proficiency testing (or PT)**. Participating in PT or EQAS determines the accuracy of laboratory results. In India, EQAS/PT providers are AIIMS, New Delhi (haematology), CMC, Vellore (haematology), Bio rad (haematology), etc.

Precision: A test method is said to be precise when repeated determinations (analyses) on the same sample give similar results. When a test method is precise, the amount of random variation is small.

For precision of results, laboratory is required to participate in **Internal Quality Assurance (IQA).** Laboratory's performances of comparison of two consecutive measurement values within the laboratory to test the precision of laboratory's autoanalyser.

So, in short, EQA: Test results acceptable
IQA: Precision acceptable.

Types of Cell Counters

(Table 15.2 and Fig. 15.2)

- **Semi-automated:** Some steps are carried out manually like dilution of blood. It measurers only a few parameters
- **Fully automated:** It requires only anti-coagulated blood samples. Measures multiple parameters.

Table 15.2: Types of cell counters

3-part differential	5-part differential	7-part differential
• Granulocytes (large cells) • Lymphocytes (small cells) • Monocytes (mid)	• Neutrophils • Eosinophils • Basophils • Lymphocytes • Monocytes • Also, there are large unstained cells (atypical, abnormal cells)	5-part differentials (neutrophils, eosinophils, basophils, lymphocytes, monocytes) + Large immature cells (blasts, immature granulocytes) + Atypical lymphocytes (including blast)

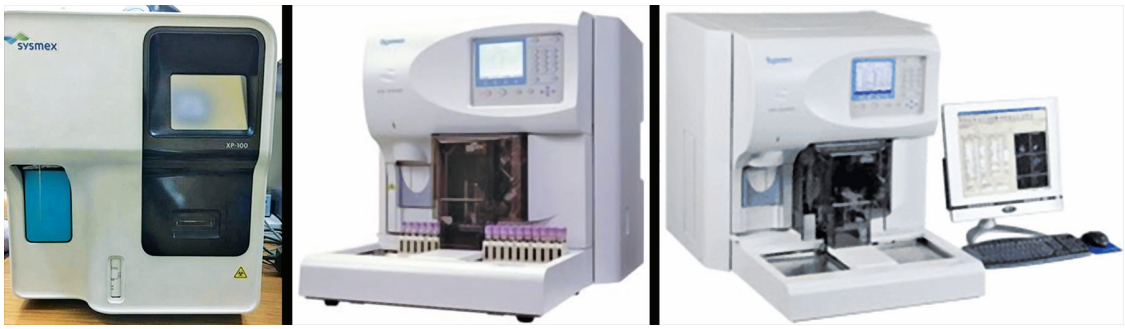


Fig. 15.2: 3-part, 5-part and 7-part haematology analysers

COMPONENTS OF A CELL COUNTER

Three basic components

- **Hydraulics:** It includes aspirating unit, dispensers, diluters, mixing chambers, aperture baths and haemoglobinometer.
- **Pneumatics:** Vacuums and pressure for operating valves.
- **Electronics:** Analyser and computing circuit.

18 Parameter electronic cell counter

1. WBC (while blood cells);
2. RBC (red blood cells);
3. Hgb (haemoglobin);
4. HCt (hematocrit);
5. MCV (mean corpuscular/cell volume);
6. MCH (mean corpuscular haemoglobin);
7. MCHC (mean corpuscular haemoglobin concentration);
8. RDW (red blood cell distribution with);
9. LYM # (lymphocytes total count);
10. MID # (mid cells total count);
11. GRN # (granulocyte total count);
12. LYM % (lymphocyte percentage),
13. MID % (mid cells percentage);
14. GRN % (granulocytes percentage);

15. Pct (plateletcrit);
16. PDW (platelet distribution width);
17. WBC histogram; and
18. Platelet histogram

PRINCIPLES OF AUTOMATED HAEMATOLOGY ANALYSERS

Automated haematology analysers are based on following principle:

- **Electrical impedance (Coulter principle)**
- **Light scattering**
- **Fluorescence**
- **Light absorption**
- **Radiofrequency conductivity**

Most analysers are based on a combination of different principle:

- **Electrical impedance (Coulter principle)** (Fig. 15.3 and Table 15.3)

  In 1956, Wallace Coulter described the Coulter principle. It is the classic and time-tested technology used for counting of blood cells. It is the basis of the cell count and volume measurements on most of the automated haematology instruments. It was first developed and marketed by Coulter electronics, hence also called Coulter principle (Fig. 15.3).

  Electrical impedance procedure: Whole blood is passed between two electrodes through an aperture so narrow that only one cell can pass through at a time. The impedance changes as a cell pass through. The change in impedance is proportional to cell volume, resulting in a cell count and measure of volume. Impedance analysis returns CBCs and three-part WBC differentials granulocytes, lymphocytes, and monocytes) but cannot distinguish between the similarly sized granular leucocytes: Eosinophils, basophils and neutrophils. Counting rates of up to 10,000 cells per second can be achieved and a typical impedance analysis can be carried out in less than a minute.

  Two electrodes placed in isotonic solutions are separated by a glass tube having a small aperture. A vacuum is applied and as a cell passes through the aperture, flow of current is impeded and a voltage pulse is generated.

  The requisite condition for cell counting by this method is high dilution of sample, so that minimal numbers of cell pass

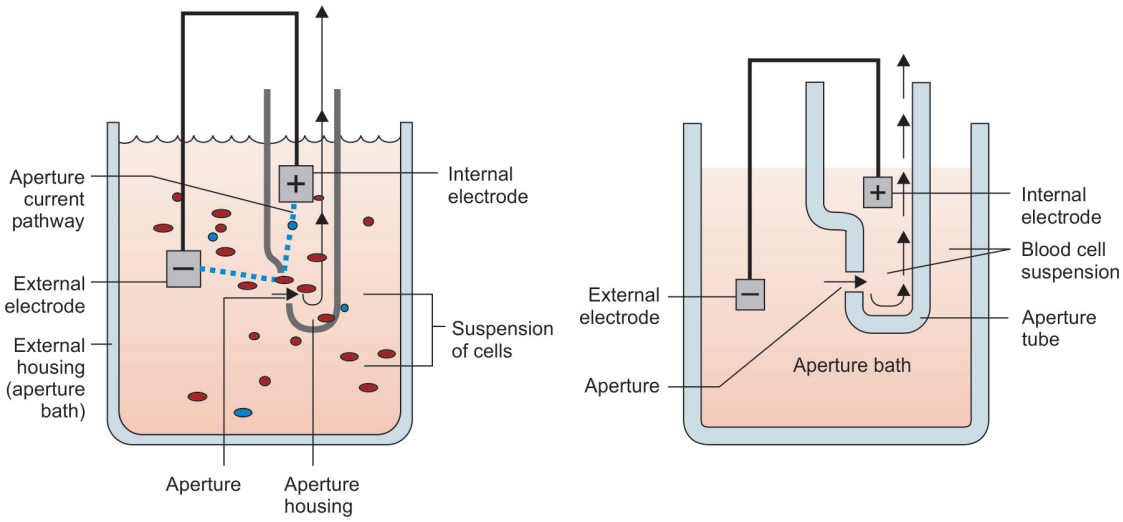


Fig. 15.3: Electrical impedance (Coulter principle)

through the aperture at one point of time. There are two electrodes on either side of the aperture, as the solution in which the cells are suspended is an electrolyte solution, an electric current is generated between the two electrodes.

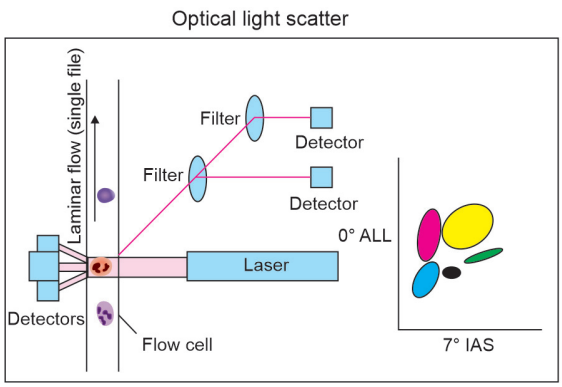
When a cell pass through this narrow aperture, across which a electrical current is flowing, change in electrical resistance (i.e. momentary, interruption of electrical current between the two electrodes) occurs. A small pulse is generated due to a temporary increase in impedance. This pulse is amplified, measured and counted. The height of the pulse is proportional to cell volume. The width of the pulse corresponds with the time required for the cell to traverse the aperture. Cells that do not pass through the center of the aperture generate a distorted pulse that is not representative of the cell volume.

Some analysers use hydrodynamic focusing to force the cell through the central path so that all cells take the same path for volume measurement.

An anticoagulated whole blood (EDTA mixed) sample is aspirated into the system, divided into two portions and mixed with a diluent. One diluent is passed to the red cell aperture bath (for red cell and platelet counting), and the other is delivered to the WBC aperture bath (where a reagent is added for the lysis of red cells and release of haemoglobin; this portion is used for leucocyte counting followed by estimation of haemoglobin). **Particles between 2 and 20 fl are counted as platelets, while those between 36 and 360 fl are counted as red cells**. Haemoglobin is estimated by light transmission at 535 nm.

The number of pulses corresponds with the number of cells counted. The data are plotted on a frequency distribution graph with cell size on X-axis and relative cell number on Y-axis. Cell populations are separated on histograms by size thresholds and the cell count represents the number of cells counted between the upper and lower thresholds for each population (set by the manufacturer).

- **Light scatter:** Each cell flows in a single line through a flow cell. A laser device is focused on the flow cell; as the laser light beam strikes a cell which is scattered in various directions. One detector captures the forward scatter light (**forward angle light scatter or FALS**) that is proportional to **cell size** and a second detector captures **side scatter (ss)** light (90°) that corresponds to the **nuclear complexity and granularity of cytoplasm**. This simultaneous measurement of light scattered in two directions is used for distinguishing between granulocytes, lymphocytes and monocytes (Fig. 15.4).
- **Fluorescence:** Measurement of emission of fluorescent light is sensitive and broadly used for analysis of multiple cellular characteristics, e.g. cell surface antigens using labeled antibodies (immunophenotyping), DNA (nucleated RBC or normoblasts,) RNA (reticulocytes) and other cellular characteristics.
- **Light absorption:** Concentration of haemoglobin is measured by absorption spectrophotometry, after conversion of haemoglobin to cyanmethaemoglobin or some other compound. In some analysers, peroxidase cytochemistry is used to classify leucocytes. The peroxidase activity is determined by absorbance (Fig. 15.5).
- **Radiofrequency conductivity:** This is a newer technique conductivity is measured by using a high frequency electromagnetic probe. This provides information on the cells internal constituents (chemical composition, nuclear characteristics and granular constituents) by permeating the lipid layer of a cell's membrane. Conductivity is helpful mainly in differentiating cells of same size, like small lymphocytes and basophils.



- Each cell flows in a single line through a flow cell.
- A laser device is focused on the flow cell.
- As laser light beam strikes a cell, it is scattered in various directions.
- Photodetectors capture the light.
- Forward scatter light (FALS) ∝ to cell size.
- Side scatter (SS) light (90°) corresponds to nuclear complexity and granularity of cytoplasm.
- Used to distinguish between granulocytes, lymphocytes and monocytes.

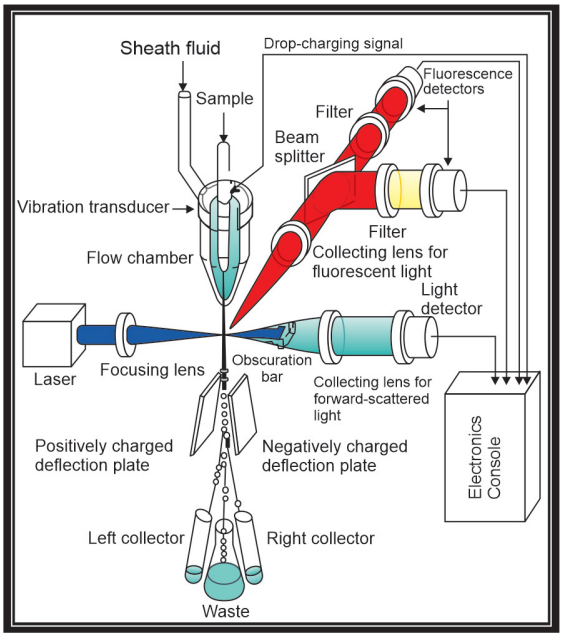


Fig. 15.4: Principles of forward and side scatter (light scatter)

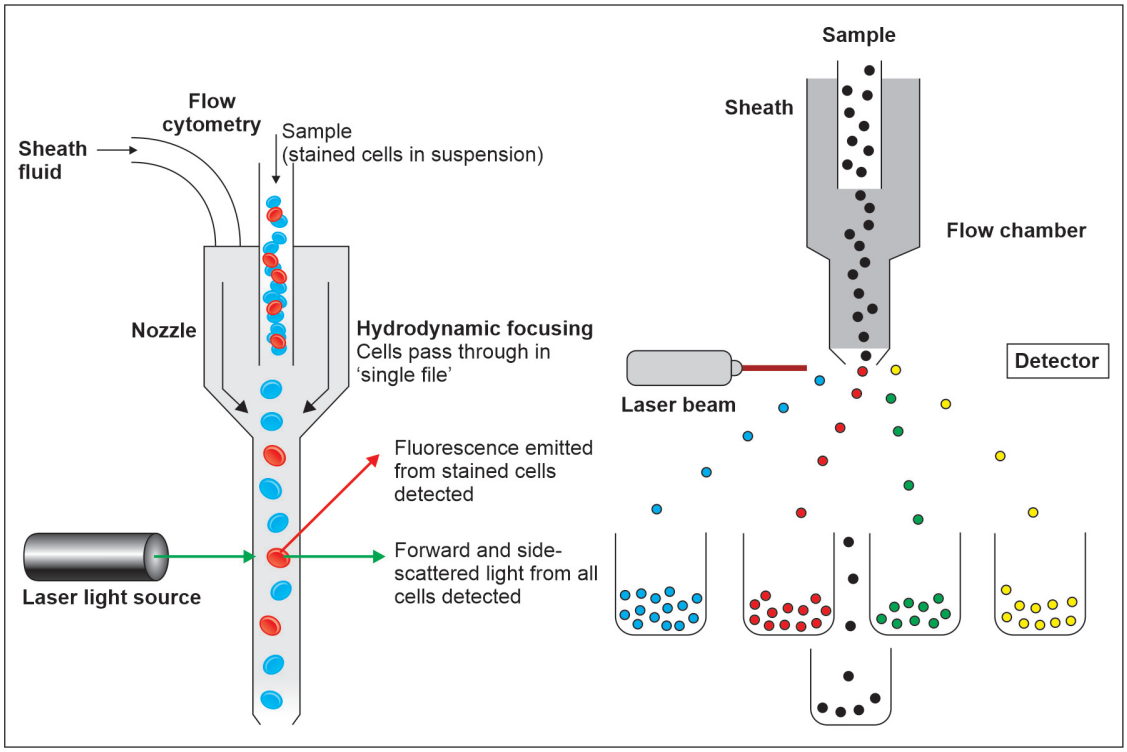


Fig. 15.5: Principles of fluorescence

Table 15.3: Reportable parameters and analysis methods (5-part differentials/ Unicel Coulter D × H 800 system)

Parameter	Method	Description
WBC	Coulter principle	White blood cell count or total leucocyte count (TLC) • Measure directly, multiplied by the calibration factor • Corrected for interference if necessary. If no correction is required, then WBC = UWBC (uncorrected WBC) • WBC = N × 10^3 cells/µl
UWBC	Coulter principle	Uncorrected WBC • Measure directly, multiplied by the calibration factor • UWBC = N × 10^3 cells/µl
RBC	Coulter principle	Red blood cell count or erythrocyte count • Measure directly, multiplied by the calibration factor • Corrected for very high WBC count, if necessary • RBC = N × 10^6 cells/µl
Hgb	Photometric	Haemoglobin or haemoglobin concentration • Transmittance of light at 525 nm through a lysed WBC solution in the Hgb cuvette, compared to the transmittance of the same light through a reagent blank. The system converts this ratio to the Hb value using a calibration factor • Weight (mass) of Hb determined from the degree of absorbance found through photo current transmittance expressed in g/dl • Corrected for WBC interference • Hb (g/dl) = [constant × $\log^{10}$ (Reference%T/sample%T)]
Hct	Calculated	Hematocrit or PCV • The relative volume of packed erythrocytes to whole blood. • Hct (%) = RBC × MCVV[10]
MCV	Derived from RBC histogram	Mean corpuscular volume • The average volume of individual erythrocyte derived from the RBC histogram • The system multiples the number of RBC in each channel by the size of the RBC in that channel. The products of each channel between 36 and 360 femtoliters (fl) are added. The sum is divided by the total number of RBC between 36 and 360 fl. The analyser then multiplies by a calibration factor • Corrected for WBC interference • Expressed in fl

- **Cytochemistry:** A cytochemical reaction determines peroxidase activity of leucocytes. Relative positivity seen in neutrophils, eosinophils, and monocytes along with light scatter data are used to determine differential leucocyte count (DLC).

The above machine (Unicel D × H 800 analyser) measures the following parameters:

WBC, UWBC (uncorrected WBC), RBC count (for whole blood and body fluids), Hgb (haemoglobin), Hct (haematocrit), MCV, MCH, MCHC, RDW, RDW-SD (red cell distribution width standard deviation), PLT (platelet), MPV (mean platelet volume), NE percent (neutrophil percent), LY percent (lymphocyte percent), MO percent (monocyte percent), EO percent (eosinophil percent), BA percent (basophil percent), NE# (neutrophil absolute number), EO# (eosinophil absolute number), BA# (basophil absolute number), NRBC (nucleated RBC), NRBC# (nucleated RBC absolute number), RET percent (reticulocyte percent), RET# (reticulocyte absolute number), MRV (mean reticulocyte volume), IRF (immature reticulocyte fraction), TNC (total nucleated cell in body fluids).

In the dataplots, different colours represent different memberships. Shades of colours represent density: Dark colours for low density and bright colours for high density: Dark colours for low density and bright colours for high density, as shown in the following listing.

nRBC analysis	Differential analysis (DLC)
nRBC: Red Others*: Green WBC: Blue	Lymphocyte: Blue Monocyte: Green Neutrophil: Purple Eosinophil: Orange Basophil: White Non-white cells: Red

*Other includes RBC debris, platelet debris, etc.

Table 15.4: Different automated haematology analyser (5-part or more differential counting capacity) and technology used in them

Instrument and manufacturer	Technology used for differential count
• Beckman-Coulter instrumentation (Coulter STKS, GEN-S, LH700 series)	VCS (volume, conductivity, and scatter) technology 1. Impedance with low frequency electromagnetic current 2. Impedance with high frequency 3. Laser light scattering
Sysmex instrumentation (Roche diagnostic corporation) (SE series, XE 2100)	1. Impedance with low frequency direct current 2. Impedance with radio frequency current 3. Hydrodynamic focusing
• Cell Dyne Technology (Abbott diagnostic instrumentation)	• The Coulter principle
• Cell-Dyn 1800 • Cell-Dyn 3500, 3700	• Multiple-angle polarized scatter separation (MAPSS): 1. Four light-scattering parameters: Forward light scatter, orthogonal light scatter, narrow-angle light scatter, and depolarized orthogonal light scatter 2. Hydrodynamic focusing 3. Use of flow cells
Cell-Dyn Ruby, Sapphire	1. Multiple-angle polarized scatter separation (MAPSS) 2. Hydrodynamic focusing 3. Use of flow cells 4. Fluorescence

Any deviation from the expected histogram is flagged by the analyser, mandating review of blood smear. A large proportion of 3-part differential counts are 'flagged' to avoid missing abnormal cells. Flags are signals that occur when an abnormal result is detected by the analyser (Table 15.6).

Definition of flags: A signal to the operator that the analyzed sample may have a significant abnormality/does not meet acceptance criteria/cannot be displayed.

Causes of error resulting in flags: Analyser, sample or random run error.

Table 15.5: Critical values in haematology analysers

Parameter	Critical value
WBC	≤1000 or ≥30,000/mm^3
Hgb	≤6.5 or 19.0 g/dl
Hct	20.0 or 60.0 %
PLT	30,000 or 1000,000/mm^3

Table 15.6: The region (R) flags and the abnormalities

Abnormality	Region	R flag
• Erythrocyte precursors (NRBCs) • Nonlysed erythrocytes • Giant and/or clumped platelets • Heinz body • Malaria	Far left (<35 fl)	R1
• Blasts • Basophilia • Eosinophilia • Plasma cells • Abnormal/variant lymphocytes • Abnormal cell populations • Eosinophils	Between lymphocytes and monocytes	R2
• Immature granulocytes	Between monocytes and granulocytes	R3
• Increased absolute granulocytes	Far right (>450 fl)	R4
• Multiple flags		RM

Table 15.7: Effect of haemolysis on CBC parameters

Parameter	Effects
WBC	Probably unaffected
RBC	Falsely low due to RBC lysis and/or RBC fragments not to be counted as RBCs
Platelet	Falsely high due to RBC fragments being incorrectly counted as platelets
Haemoglobin	Reportable-accurate measurement relies on RBCs being completely lysed
MCV	Invalid falsely low or depending on the degrees of haemolysis. May be falsely low if RBC fragments cause smaller pulses to be produced in the RBC aperture or falsely high if fragmented RBCs fall below the RBC threshold and are not counted
MCH	Invalid—calculated with a falsely low RBC
MCHC	Invalid—calculated with a falsely low Hct
RDW	Falsely high due to RBC fragments increasing the CV of the RNC histogram

Red Blood Cell Analytic Parameters

RBCs have three quantitative values, namely the volume of packed red cells (Hct), Hb, RBC count and red cell indices (MCV, MCH and MCHC). All these six values are calculated by haematology analyser.

- **RBC count:** This is directly measured by aperture impedance or light scatter analysis. In a red cell histogram, number of RBCs is plotted on Y-axis while X-axis plots cell volume of RBCs. But if there is marked leukocytosis (WBC >50,000/mm^3), the RBC count may be erroneous.
- **Haematocrit (PCV):** The analyser measures it by directly measuring RBC number and RBC volume (Hct or PCV = RBC number/RBC volume). But if there is polycythaemia, or abnormal plasma osmotic pressure, there may be errors.
- **MCV:** It is calculated by analyser and the result is obtained by dividing the summation of the RBC volume by the RBC count. Paraproteinemias and agglutination of RBCs (e.g. cold agglutinin disease) may cause falsely raised MCV. Severe hyperglycaemia (blood glucose >600 mg/dl), also may cause falsely raised MCV due to osmotic swelling of RBCs.
- **Haemoglobin concentration:** It is measured directly by a modified cyanomethaemoglobin method. A non-hazardous reagent (e.g. sodium lauryl sulfate) is used by some analysers. Sometimes a non-ionic detergent is used for rapid lysis of RBC and to reduce turbidity caused by cell membranes and plasma lipids.
- **MCH and MCHC:** These two parameters reobtained indirectly by calculations.
- **Red cell distribution width (RDW):** RDW is a quantitative measure of anisocytosis (variation in sizes of RBCs). Normal value is 11.5–14.5. RDW is raised (>14.5) in iron deficiency anaemia. But it is not raised in thalassaemia minor or trait and in anaemia of chronic diseases (two important causes of microcytic anaemia). In thalassaemia major there may be raised RDW.

RED CELL HISTOGRAM

The principle of RBC histogram is aperture impedance. In the analysers, blood cells in 36–360 fl volume are counted as RBCs. WBCs are also counted along with RBCs in the diluting fluid. But number of WBCs is statistically insignificant (thousands as opposed to millions of RBCs). If there is very high WBC count (>50,000/mm^3), then RBC count and histogram will give errors or flags.

- Peak between 60 and 125 fl: Calculation of MCV and RDW.
- Left shift of the curve: Microcytosis
- Right shift of the curve: Macrocytosis
- Bimodal peak of the curve: Dimorphic anaemia or double population of RBCs.
- Bimodal histogram skewed to the right: Reticulocytosis, initial response to iron therapy.
- Trail of RBC population on the far right of histogram: Cold agglutinates of red cells.
- Normal red cell histogram (Fig. 15.6) displays cells from 36 to 360 fl
- (24–36 fl) flag may be due to:
  1. RBC framents
  2. WBC fragments
  3. Giant platelets
  4. Microcyte
- Shift to right:
  - Leukaemia
  - Macrocytic anaemia
  - Megaloblastic anaemia
- Bimodal
  - Cold agglutinin
  - IDA, megaloblastic anaemia with transfusion.
  - Sideroblastic anaemia
- Trimodal
  - Anaemia with transfusion

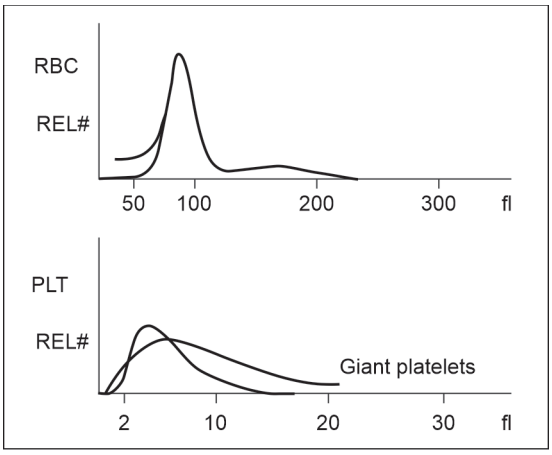


Fig. 15.6: Red cell histogram

Leucocyte Analysis

- **WBC count and leucocyte differentials:** The analysers may give 3-part differential (lymphocytes, monocytes and granulocyte) or may give 5-part differential (3-part + eosinophils + basophils). The principle of 3-part differential counting is based on electrical impedance volume measurement of leucocytes. The principle of 5-part differential counting is based on a combination of different principles, e.g. impedance, light scatter and electrical conductivity. It may also be a combination of light scatter, peroxidase staining and resistance of basophils to lysis in acid buffer, etc.
- **Histogram:** In WBC volume histogram, cell size is plotted on X-axis and the number of WBCs are plotted on Y-axis. Cells with volume 35–90 fl: Lymphocytes; 60–160 fl: Mononuclear cells; and 145–160 fl: Neutrophils. If there is any deviation, it is flagged by the analysers, when it requires blood smear examination. Many cases of 3-part differential counts are 'flagged' to avoid missing abnormal results (Fig. 15.7).

WBC Cytogram (Scattergram) (Fig. 15.8)

In this WBC cytogram or scattergram, each dot represents a cell of given volume and density. The positions of dots in scattergram (graph) depends on many things: The degree

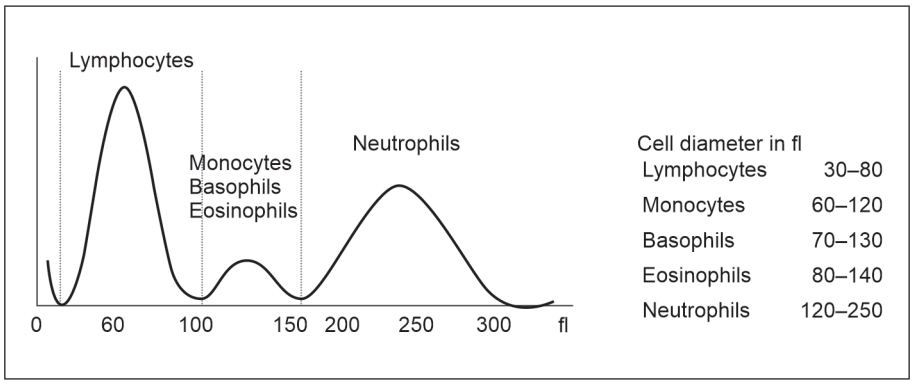


Fig. 15.7: WBC histogram

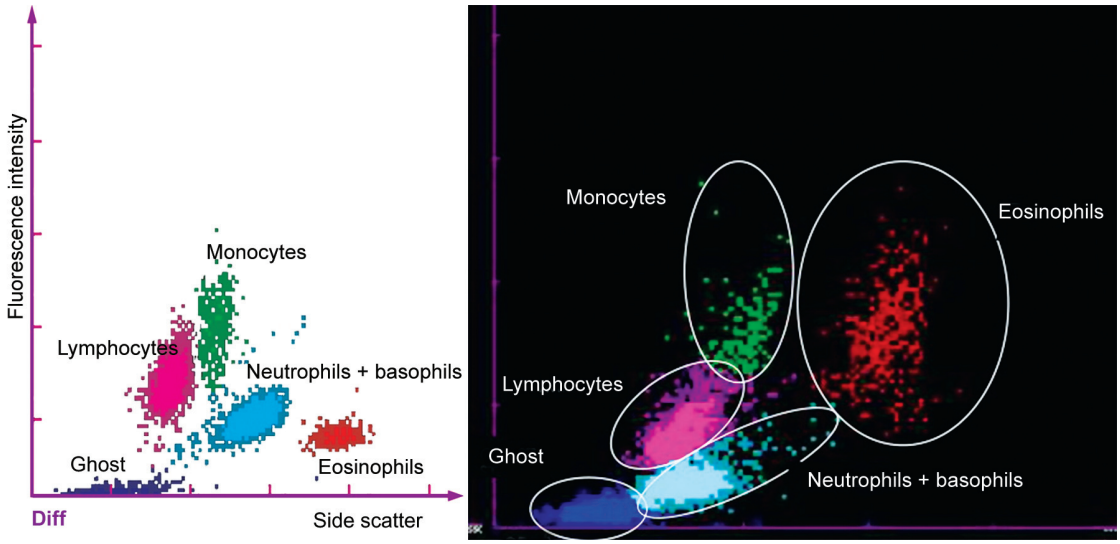


Fig. 15.8: WBC cytogram (scattergram)

of side scatter, degree of forward scatter, light absorption by the cell and cytochemical staining (if used). The side scatter (SS) is observed on X-axis, while forward angle light scatter (FALS) is observed on Y-axis. Low FALS and low SS indicate lymphocytes. Subsequent increase in FALS and SS give monocytes, neutrophils and lastly eosinophils. Basophil counting is done by a different technology.

Platelet Analysis

Platelet count: In automated haematology analysers, platelets are identified and counted by light scatter, electrical impedance or platelet antigen staining method in RBC aperture.

Falsely raised platelet count in analyser may be due to fragments of WBCs or RBCs. Falsely decreased platelet count may be due to the presence of platelet clumps or platelet agglutinins or adsorption of platelets to WBCs. But these false results usually give abnormal histogram and are easily identified.

Histogram: Platelet volume is plotted on X-axis and relative cell frequency on Y-axis. Particles with volume 2–20 fl are counted as platelets.

Platelet Histogram (Fig. 15.9)

- Normal platelet histogram displays cells from 2 to 20 fl).

(0–2)

- Air bubbles
- Dust
- Electronic and electrical noise

Over 20 fl

- Microcyte
- Schistocyte
- WBC fragments
- Clumped platelets

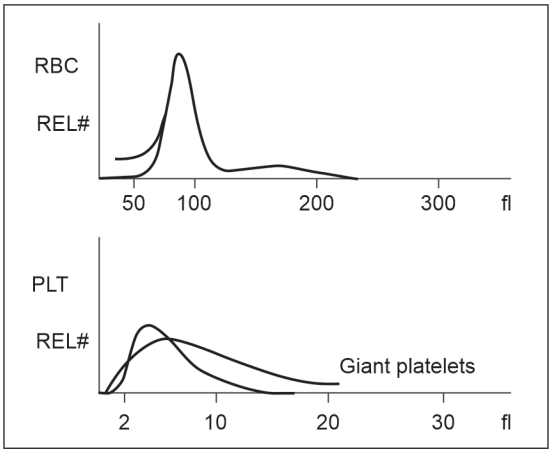


Fig. 15.9: Platelet histogram

Mean platelet volume (MPV): It indicates average size of platelets. Normal MPV is 7–10 fl. Usually, MPV has an inverse relationship with platelet number.

i. Increased MPV (>10 fl): Thrombocytopenia with peripheral destruction of platelets. It is seen in ITP and it stimulates megakaryocytes in the bone marrow to produce immature platelets.
ii. Decreased MPV (<7 fl): It is observed in bone marrow hypoplasia/aplasia (megakaryocytic hypoplasia) and anticancer or cytotoxic drug therapy. Decreased MPV is due to presence of small platelets in circulation in above mentioned conditions.

Platelet distribution width (PDW): This indicates variation in the size of platelets (normal <20%). Increased PDW is observed in megaloblastic anaemia, in CML and after chemotherapy. PDW is analogous to RDW (Fig. 15.10).

Reticulated platelets: Reticulated platelets are newly released or young platelets which contain residual RNA (analogous to RBC reticulocyte). It gives an estimate of thrombopoiesis. Normal range 3–20%.

Increased reticulated platelets: Observed in thrombocytopenia due to peripheral destruction of platelets production. So, it helps in distinguishing platelet destruction syndromes from hypoplastic platelet production.

Reticulated platelets can be detected by automated haematology analysers or by flow cytometric methods using thiazole orange dyes (which bind RNA) (Table 15.8).

NEWER PARAMETERS AVAILABLE ON SELECTED HAEMATOLOGY AUTOANALYSERS

1. **Automated red cell parameters**
   - **Nucleated RBCs:** In older analysers, nRBCs are included in the WBC count and corrections are needed to get actual WBC count. But some modern analysers can detect nRBC by using fluorescent dye and light scatter methodology. The number of nRBC is calculated from multiplying nRBC% by WBC count. Then this is calculated from multiplying nRBC% by WBC count. Then this number is subtracted from WBC count to get the corrected WBC count.

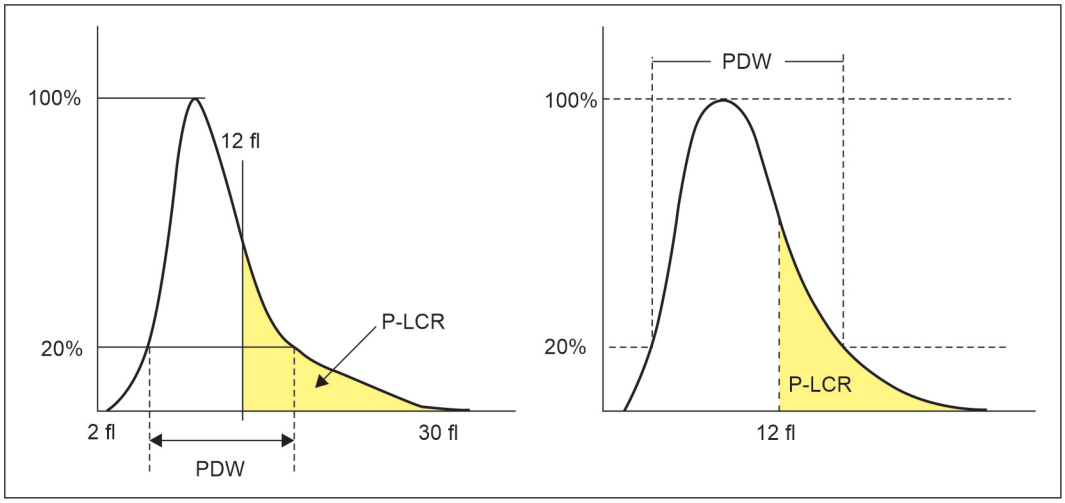


Fig. 15.10: Histogram of platelet size distribution and the definition of platelet size deviation width (PDW), and platelet-large cell ratio (P-LCR). The distribution width at the level of 20% was defined as PDW, and the percentage of the platelets with a size of more than 12 fl was defined as P-LCR

Table 15.8: Causes of errors in haematology autoanalysers

Parameter	Causes of erroneous decrease	Causes of erroneous increase
All parameters	Clotted sample	—
WBC count	Clotted sample	Large platelet clumps, nucleated RBCs, cryoglobulins, unlysed RBCs
RBC count	Clotted sample, autoagglutination, microcytic RBCs	Very low WBC count, large number of giant platelets
Platelets	Platelet clumping and platelet satellitism	Fragments of WBCs, microcytic RBCs, cryoglobulins
MCV	Cryoglobulins	Autoagglutinins (clod agglutinins), very high WBC count, hyperglycaemia
MCHC	Very high WBC count	Autoagglutination (cold agglutinins), hyperlipidaemia.
Haemoglobin (Hb)	Clotted sample	Very high WBC count, high bilirubin, hyperlipidaemia.

- Fragmented RBCs: Some modern analysers can detect and quantify red cell fragments. Fragmented red cell count can exclude schistocytes on peripheral blood smear.
- Percent hypochromic RBC (%HYPO): It refers to proportion of hypochromic RBCs (normally <6%). It is useful for identifying early iron deficiency.
- Low haemoglobin density (LHD): It is produced by mathematically transformation of MCHC and is useful for identification of reduced iron availability. It correlates with % HYPO also.

2. **Automated reticulocyte parameter**
   - Reticulocyte count: Various fluorescent dyes are used which combine with RNA of reticulocytes. The fluorescence emitted by the fluorescence dyes is then counted by a flow cytometer. More immature reticulocytes fluoresce more strongly as they contain more RNA.
   - Immature reticulocyte fraction (IRF): This parameter assesses reticulocyte maturity level based on RNA content (intensity of staining) and is an early indicator of response to specific replacement therapy for anaemia. IRF increases earlier than reticulocyte count and haemoglobin level.
   - Reticulocyte haemoglobin content: This parameter estimates haemoglobinization of most recently produced RBCs. This is a marker of functional iron deficiency.
3. **Automated platelet parameter**
   - Reticulated platelets: Already described above
   - Platelet-large cell ratio (P-LCR): This measures percentage of platelets >12 fl (normal value 15–35%). Increased P-LCR is observed in thrombocytopenia due to platelet destruction, microcytes, platelet aggregates and giant platelets.
   - Immature platelet fraction or IPF (reticulated platelets): It indicates young platelets containing large amount of RNA. This is a marker of thrombopoietic activity (normal value 3–20%).
     i. Increased IPF (reticulated platelets): Thrombocytopenia due to decreased platelets production, and in inherited thrombocytopenia with giant platelets.
     ii. Normal on decreased IPF: Thrombocytopenia due to decreased platelet production.

Advantage of this parameter is that bone marrow examination can be avoided because increased IPF indicates responsive marrow (especially in conditions with increased platelet destruction).

Table 15.9: Limits and acceptable imprecision: Current state-of-the-art (As per ISO:15189)

Parameter	State-of-the-art [CV (%)] reproducibility	State-of-the-art [CV (%)] between batch	'Ricos' criteria [CV (%)]
Hb	0.9	1.0	1.43
Hct (PCV)	1.2	1.4	1.35
RBC	1.1	1.1	1.60
MCV	0.6	0.8	0.70
MCH	1.1	1.5	0.70
RDW	2.0	2.0	1.80
WBC			
• High level (>10 × 10^9/L)	1.5	1.5	—
• Normal level (1–10 × 10^9/L)	2.5	2.5	5.73
• Low level (<1.0 × 10^9/L)	6.0	6.0	–
Neutrophils (absolute)			
• Normal level (0.5–8.0 × 10^9/L)	2.5	2.5	8.55
• Low level (<0.5 × 10^9/L)	10	10	–
Eosinophils (absolute)	10	10	10.5
Basophils (absolute)	20	20	14.0
Lymphocytes (absolute)	3.5	3.5	5.10
Monocytes (absolute)	8.5	8.5	8.9
Platelets			
• Normal range	3.5	3.0	4.6
• Low (~ 50 × 10^9/L)	4.5	4.5	–
• Very low (10–20 × 10^9/L)	5.0	5.0	–
MPV	2.5	2.5	2.15

In a verification report, the results for imprecision (repeatability and between batch) should be reported including the mean and CV (%). The state-of-the-art CV (%) for imprecision (reproducibility and between batch) is based on the current literature. The fourth column represents the 'Ricos criteria' that illustrate biological variation, and these performance criteria are based on biological variability. There is controversy regarding the use of these criteria for method verification. Therefore, use of the current state-of-the-art CV (%) (columns 2 and 3) as criteria for acceptability of the **haematology analyser (HA)** under verification (Table 15.9).

For reticulocytes, the 'Ricos criteria' depend on the method used, for a fluorescent method the % varies between 0.8 and 6.5. In clinical laboratories or settings where the HA are not used to guide platelet transfusion therapy or for example, therapy with antibiotics in case of neutropenia a higher % CV can be considered for platelet count and neutrophil count in the lower range with a maximum of 10% CV for both.

Quality Control of Haematology Analyser

For intra-instrument quality control (internal quality control), the measurement of quality control samples on a daily basis (or more often) is obligatory. Usually these quality control samples and obtained from the manufacturers and they consist of two or three levels (low or L1, normal or L2 and high L3). In India, several companies also supply controls, e.g. Bio Rad, Sysmex, Horiba, Mindray, Siemens, Abbott, Beckman Coulter, etc.

There are several pitfalls. **Firstly**, these samples are usually manipulated to lengthen the shelf life; therefore, they may behave differently than ordinary patient material. **Secondly,** the manufacturer's, target's limits are often very broad, and subtle changes in analyser behaviour may thus be missed. So, it is recommended to adjust the target range after a run-in period of several measurements, for example, to the mean 2 standard deviations. An advantage in using these quality control samples is that they may be used to judge the instrument precision over time (i.e. drift) using a **Levey-Jennings graphs**. However, it must be kept in mind that at the end of the shelf life, the quality of the control samples may deteriorate (Fig. 15.11).

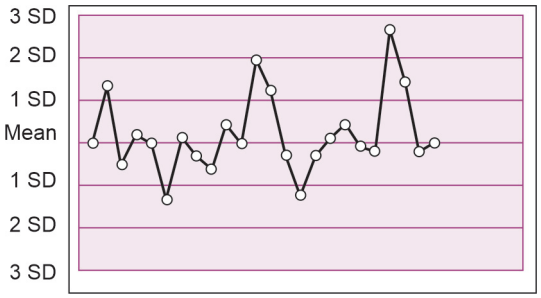


Fig. 15.11: Levey-Jennings chart

If a laboratory has multiple haematology analysers (HAs) and/or different analytical techniques that are used for the same parameter inter-instrument quality control should be performed. All methods should lead to the same result in each sample. If different methods lead to unexpected discrepancies between reported results, this may lead to misinterpretation and possibly unnecessary clinical intervention. It is recommended that multiple HAs are compared at least on a weekly basis, using at least three samples or more.

The participation in an **External Quality Assessment (EQA) Scheme or EQAS** (also known as **proficiency testing or PT scheme**) is mandatory. In India, for haematology EQAS is available from AIIMS, New Delhi, or CMC, Vellore. The EQA samples, should be handled in the same way as routine patient samples to acquire a fair comparison. Laboratories must use the EQA results to compare their results with (inter) national consensus or reference results. They should use these to improve their quality and to harmonize their HA with a consensus group. EQA material should not be used for calibration purposes (because a EQA consensus value and a true value (calibrator) are not necessarily similar.

Indicators that may appear after the date

i. @: Data is outside the linearity limit
ii. * : Data is doubtful
iii. + or –: Data is outside the reference limits
iv. –––––: Data does not appear due to analysis error or abnormal sample
v. ++++: Data exceeds display limit (Fig. 15.12)

Flags in Haematology Analysers

(Fig. 15.12)

Table 15.10: Parameters measured in haematology analysers

Directly measured	Derived from histograms	Calculated
• RBC count • WBC count • Platelet count • Haemoglobin level • Reticulocyte	• MCV • RDW • DLC • PDW	• Haematocrit of PCV (MCV/RBC count) • MCH (Hb/RBC count) • MCHC (Hb/Hct)

Sysmex XT-4000i Analyser

This analyser uses the electric resistance detecting method (impedance technology) with hydrodynamic focusing to measure RBC, PLT, MPV, MCV, and haematocrit (Hct). Fluoresce flow cytometry is used to measure WBC, differential count, optical PLT count, and reticulocyte count. The system employs a 633 nm semiconductor laser for flow cytometry analysis. For the

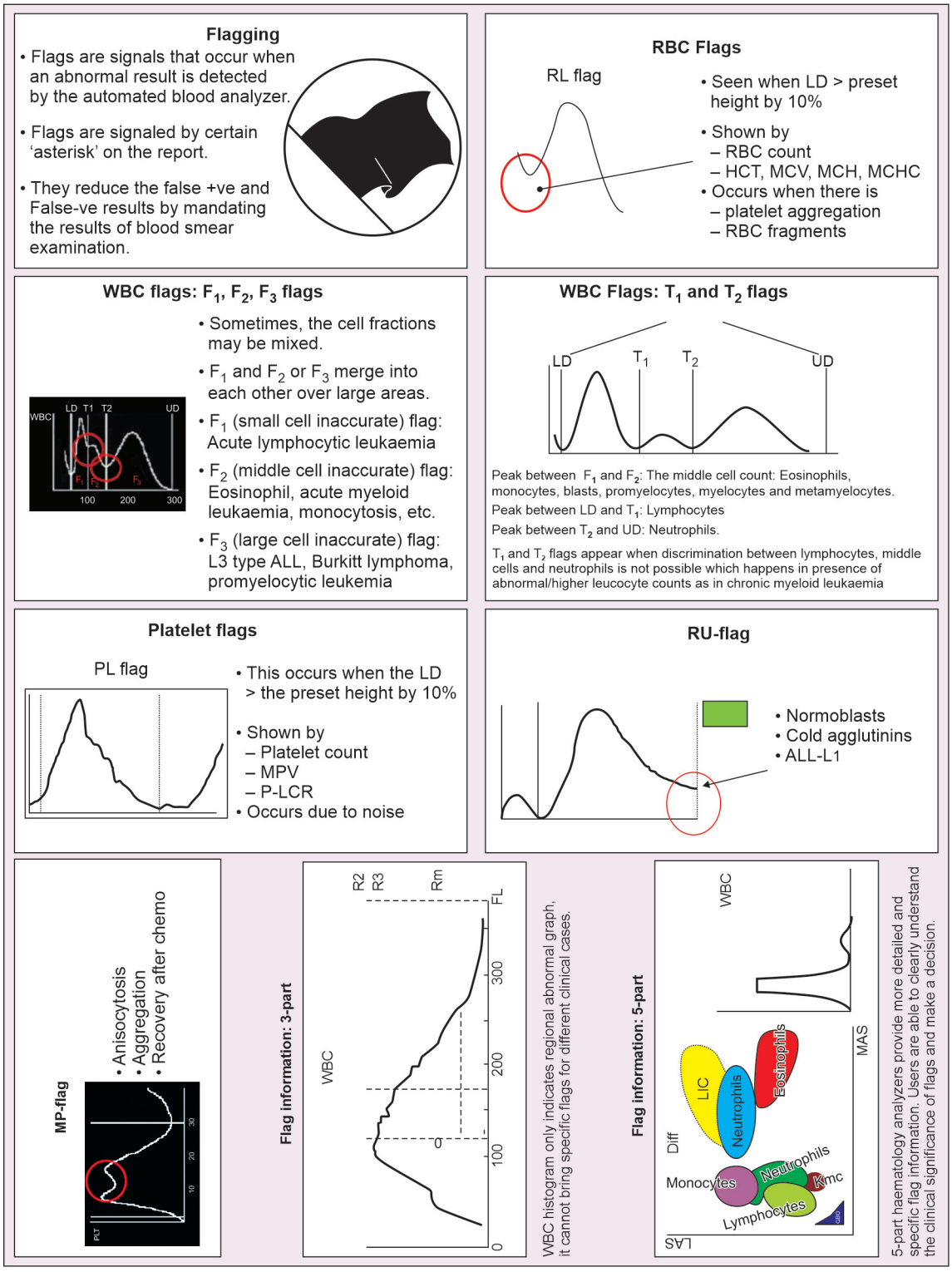


Fig. 15.12: Flags in haematology analysers

measurement by flow cytometry of the proportional count, expressed as percent of the total WBC, of neutrophils (NEUT), lymphocytes (LYMPH), monocytes (MONO), and eosinophils (EOS). Basophils (BASO) are measured separately using cell size, and side scatter (SSC) properties. Haemoglobin (HGB) is measured photocolourimetrically using SLS-HGB (sodium lauryl sulphate), a cyanide-free method (Fig. 15.13 and Tables 15.11 and 15.12).

Fig. 15.13: Sysmex XT-4000i analyser

Table 15.11: Reagents used in Sysmex XT-4000i analyser

Reagents	Function
Cellpack	RBC/PLT and HGB diluent, rinsing of the instrument; hydrodynamic focussing
Stromatolyser-4DL	Differential count lysing reagent
Stromatolyser-4dS	Differential count stain
Stromatolyser-FB	Diluent for WBC count and lyses all cells except BASO
Sulfolyser	Noncyanide HGB lyse (sodium lauryl sulphate)
Ret-Search (II)	Dilutes sample for reticulocyte analysis
Ret-Search (II) dye	Stains reticulocytes and platelets for analysis

Table 15.12: Advantages and disadvantages of haematology analysers

Advantages	Disadvantages
• Speed with efficient handling of large number of samples	• Flagging of a laboratory test result demands labor intensive manual examination of a blood smear
• Accuracy and precision in quantitative blood tests	• Comments on red cell morphology cannot be generated
• Ability to perform multiple tests on a single platform	• Platelet clumps are counted as single. Hence, spurious low platelet count
• Significant reduction of labor requirements	• Erroneously increased or decreased results due to interfering factors
• Invaluable for accurate determination of cell indices	• Expensive with high running costs

Terminologies in Quality Control

- **Quality assessment:** Adequate control of the pre- and post-analytical from sample collection to report dispatch.
- **Quality control:** Measures that must be included during each assay run to verify the test working properly.
- **Proficiency testing (PT):** Determines the quality of results generated by laboratory.
- **Internal quality control:** Continuous evaluation of the reliability of the daily works of the laboratory with validation of tests.
- **External quality control:** Evaluation by an outside agency of between laboratory and method comparability.
- **Accuracy:** Refers to closeness to true value.
- **Precision:** Refers to reproducibility of test.

- **Controls:** Substances used to check the precision. Analyzed either daily or along each batch. Should have same test, properties as blood samples. Stabilized anticoagulated whole blood or pooled red cells with known test parameters are used as controls. Three levels of control, e.g. low (L1), normal (L2) and high (L3) are used.
- **Calibrators:** Check the accuracy. Value assigned to them by a reliable reference centre.
- **Use of controls:** Most convenient and accurate procedure to check the quality of a particular test. Controls are supplied by manufacturer of a machine or by an external agency. Ten consecutive values of control recorded and mean and SD are calculated. Three concentrations of control should be analyzed. These are plotted on Levey-Jennings (LJ) chart.

Control Values and Decision

- Consider using Westgard Control Rules
- Use premise that 995.5% of control values should fall within + 2 SD.
- Commonly applied when two levels of control are used
- Use in a sequential fashion

Westgard Rules

- 1_{2s} Rule
- 1_{3s} Rule
- 2_{2s} Rule
- R – 4s Rule
- 4 – 1s Rule
- 10 × Rule

What to do when control value is out of limit?

- This means value "out of control"
- Stop testing
- Identify and correct problem. There may be machinery problem.
- Repeat testing on patient samples and controls.
- Do not report patient results until problem is solved and controls indicate proper performance.

FLOW CYTOMETRY

'Flow cytometry' as the name suggests is a technique for cell counting and measurement of different properties of the cell ('*cyto*' = cell, '*metry*' = count/measurement).

It is a laser-based technology which measures and analyzes different physical and chemical properties of the cells particles flowing in a stream of fluid through a beam of light. So, by definition flow cytometry measures properties of cell as they flow in a fluid suspension across an illuminated property of cell as they flow in a fluid suspension across an illuminated light path.

Principles of Flow Cytometry (FCM)

- A specimen of monodisperse suspension of cells in isotonic fluid is forced under pressure into a tube which delivers it to the flow cell, where a fluid column with laminar flow and a high flow rate is generated (so-called **sheath fluid**).
- The specimen sample is introduced into the flow cell by a computer-driven syringe in the center of the sheath fluid. This creates a coaxial stream within a stream (called a sample core stream). The pressure of the sheath stream aligns the cells or particles in such a way that they are presented to the light beam one at a time. Flow cytometer measures the amount of light emitted by fluorochromes associated with individual cell or particle. New flow cytometers have three to four lasers (Figs 15.14 and 15.15).

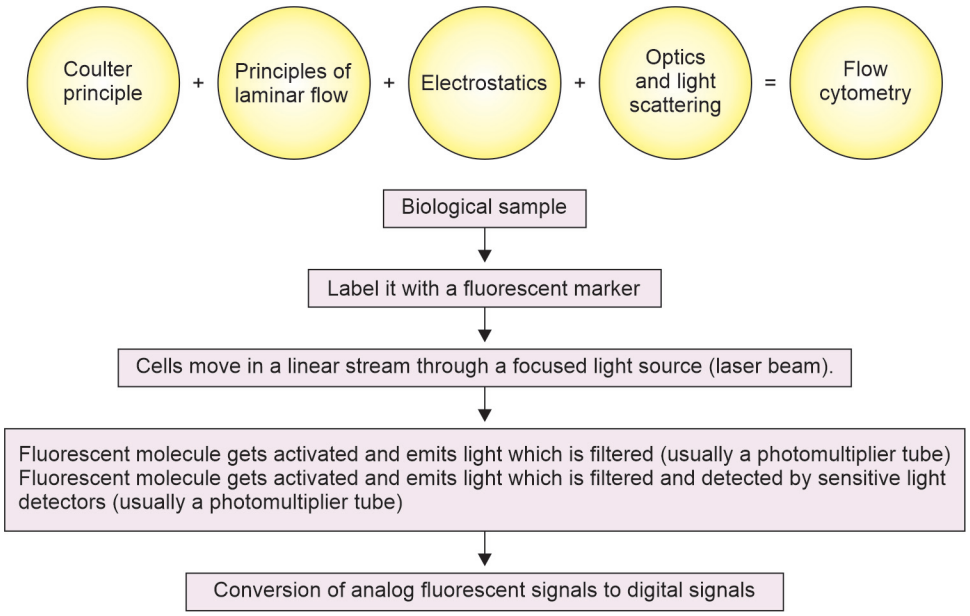


Fig. 15.14: Basic mechanism in a flow cytometry

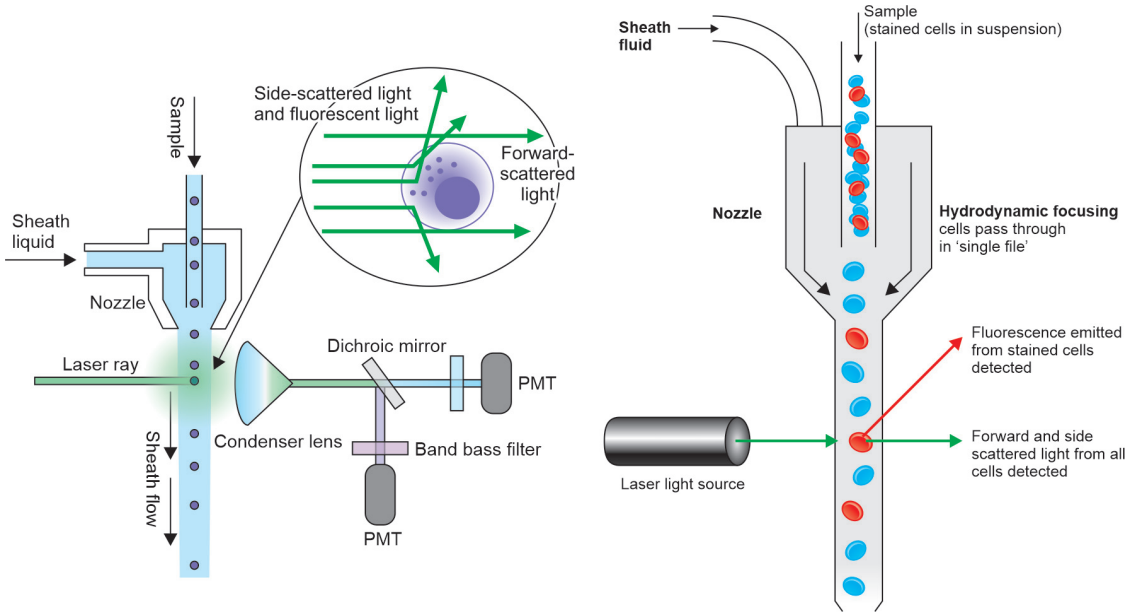


Fig. 15.15: Basic mechanism in a flow cytometry

Component of Flow Cytometry

Usually flow cytometry (FCM) has four major components:

- Fluidics or flow system
- Optical system (light source and light detectors)
- Electronic system
- Computer

FLUIDICS SYSTEM

- One of the fundamentals of flow cytometry is the ability to measure the properties of the individual cell or particle, which is managed by the fluidics system.
- When a sample is injected into a flow cytometer, it is ordered into a stream of single particles.
- The fluidic system consists of a flow cell (quartz chamber):
  - i. Central channel/core: Through which the sample is injected.
  - ii. Outer sheath contains faster flowing fluid which surrounds the central channel or specimens' stream. This sheath fluid is composed of buffered saline (0.9% saline).

Once the sample is injected into a stream of sheath fluid within the flow chamber, they are forced into the center of the stream forming a single file by the principle of hydrodynamic focusing.

One cell or particle can pass through the laser beam at a given moment.

- The sample pressure in the central channel/core is always higher than the sheath fluid pressure, ensuring a high flow rate allowing more cells to enter the stream at a given moment.
- High flow rate: Used for immunophenotyping of cells
- Low flow rate: Used for DNA analysis.

Optical system (light source and light detectors)

- After the cell delivery, the need is to excite the cells using a light source.
- The light source used in a FCM is either laser (more) (commonly) on arc lamp
- Lasers are commonly used because they are highly coherent, and uniform. They can be easily focused on a very small area (like a sample stream). They are monochromatic, emitting single wavelengths of light. Argon laser is popular and it has 488 nm wavelength (blue to blue-green).
- When a light intersects a laser beam at the so-called "interrogation point" two events occur:
  - a. Light scattering
  - b. Emission of light (fluorescent)

Fluorescence is light emitted during decay of excited electron to its basal state.

a. Light scatter

- When light from a laser interrogates a cell, that cell scatters light in all directions.
- The scattered light can travel from the interrogation point down a path to a detector.
- Light which is scattered in the forward direction (along the same axis the laser is travelling) is detected in the forward scatter channel and is known as **forward scatter (FSc).**
- The intensity of FSc signal has been attributed to **cell size, and refractive index (membrane permeability).**
- Laser light which is scattered 90° to the axis of the laser path is detected in the side scatter channel and is known as side scatters (SSc). Side scatter detects cell granularity.
- Study of FSc and SSc allows us to know the differentiations of different types of cells.
- The light scattered in the forward direction is proportional to the square of the radius of a sphere, and so to the size of the cell or particle.
- The cells are labeled with fluorochrome-linked antibodies or stained with fluorescent membrane, cytoplasmic or nuclear dye. A fluorochrome is a fluorescent marker (Table 15.13).

Table 15.13: Commonly used fluorochromes and wavelengths of their maximum emission

Fluorochrome	Maximum emission at wavelength
Fluorescin isothiocyanate (FITC)	560 nm (observed colour green)
Phycoerythrin (PE)	576 nm (observed colour orange/red)
Peridin–chlorophyll alpha (PerCP) complex	680 nm
Allophycocyanin (APC)	660 nm
Texas red	620 nm (observed colour red)
ECD (PE—Texas Red Tandem)	615 nm
PC5 (PE—cyanin 5 tandem)	667 nm

b. Emission of light (fluorescence) (Fig. 15.16)**:** For FCM, the most common fluorophores are FITC and PE. FITC is a small, charged molecule that can be easily conjugated to protein through an isothiocyanate group. It has an excitation maximum at 495 nm and emits green light (530 nm). It can be excited by a 488 nm argon-laser. R-phycoerythrin is a phycobiliprotein extracted from red algae. It is excited at 495 nm and 564 nm but maximum emission occurs at 576 nm. This is also excited by 488 nm argon-ion laser.

So, fluorochrome is a molecule that fluoresces (i.e. gives out a particular colour) when a laser hits it. Fluorescence occurs when a molecule excited by light of one wavelength loses its energy by emitting light of a longer wavelength. The exciting and emitting light being of different wavelengths, can be separated from one another using optical filters.

Light detectors: The light scattered or emitted by the cells as cells flow through the laser beam is measured by detectors. As mentioned before, light scatter is collected at two angles: Forward scatter (FSC) and side scatter (SSC). A photodiode collects the FSC signals. Photomultiplier tubes (PMTs) collect the emitted SSC and fluorescence signals. A system mirrors and optical filters routes these signals to their detectors.

Band pass (BP) filters are placed in front of PMTs. This optimizes the specificity of the detector for a particular fluorescent dye by allowing only a narrow range of wavelengths to reach the detector.

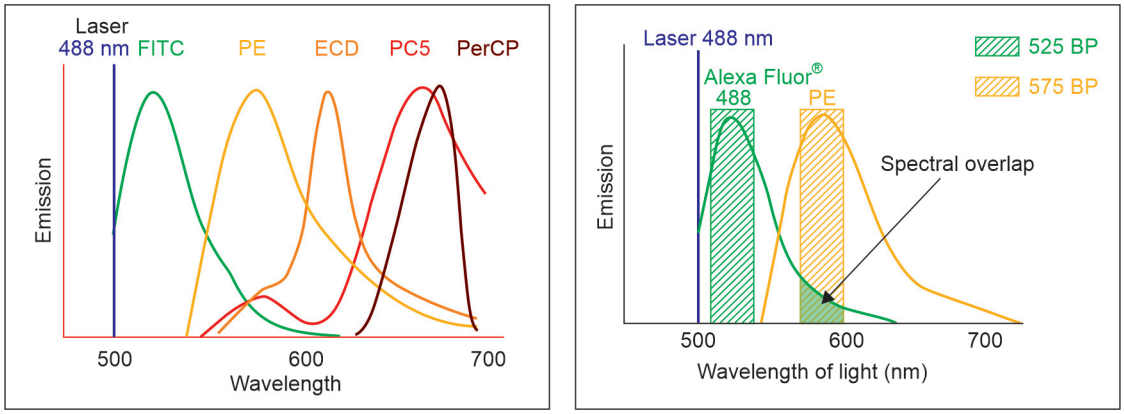


Fig. 15.16: Emission of light (fluorescence)

There are also **long pass (LP) and short pass (SP) filters** present. LP filters permit longer wavelength transmission and SP filters permit shorter wavelength transmission.

Detectors convert the detected light into electrical impulses, the magnitude of which are proportional to the amout of light and gives information about the cross-sectional area of the cell.

Electronic System

The optical signals (photons) are convened to corresponding electronic signals or impulses (electrons) by the photo detectors mentioned above (photodiodes and photomultiplier tubes or PMTs). The electric current travels to the amplifier and is converted to a voltage pulse. The voltage pulse is assigned a digital value which represents a channel by the Analog-to-digital converter (ADC). The channel number is now transferred to the computer which displays it to the appropriate position on data plot.

Computer

Computer controls the overall functioning of the flow cytometer. It stores, analyzes and presents the data. It also provides interface with the user.

Data analysis: Once a data file has been saved, the data can be displayed in a number of different plot types. These range from histograms, to 2-D plots such as dotplots, contour and density plots to 3-D plots such as a tomogram plot.

Gating: *Gating or cell sorting* is used to identify subsets of data or populations. Gates defined populations can be used to generate statistics and limit the number of events collected is saved. For example, if you are interested in analyzing lymphocytes in a sample of blood containing a mixed population of cells, you can place a gate on the lymphocytes and this will therefore show only data from within that gate, i.e. lymphocyte.

APPLICATIONS OF FLOW CYTOMETRY

1. **Diagnosis and classifications**
   a. Classification of hematopoietic neoplasms: Leukaemia, lymphoma, myelodysplastic syndrome (MDS), mastocytosis.
   b. Diagnosis of paroxysmal nocturnal haemoglobinuria (PNH): Deficiency of CD55 and CD59.
2. **Prognosis of diseases**
   a. Prognosis of different hematological malignancies like CLL/SLL and acute leukemia.
   b. Prognosis and stage of HIV by counting CD4, CD8 and CD4:CD8 ratio.
3. **Identification of clonality:** Immunoglobulin light chain class restriction (either kappa or lambda) is frequently used as a surrogate marker of clonally in mature B cell neoplasms.
4. **Detection of minimal residual disease**
   Aberrant antigen expression on neoplastic cells is helpful in monitoring patients following chemotherapy, e.g. expression of CD5 (a pan-T cell marker) in mantle cell lymphoma and CLL .
5. **Monitoring effect of drug therapy**
   a. Detect multiple drug resistance in cancer chemotherapy
   b. Therapeutic targets: FCM can identify potential therapeutic targets which is helpful mainly in hematolymphoid malignancies.
6. **Others**
   a. Analysis of DNA ploidy, proliferation (S-phase), and programmed cell death (apoptosis).
   b. Transplantation: Histocompatibility cross-match of organs for transplantation and in stem cell transplantation.
   c. Detection of antibodies to platelets and neutrophils.
   d. Enumeration of reticulocytes and platelets.
   e. Foetomaternal hemorrhage: Detection and quantitation of foetal haemoglobin in maternal blood sample.

Table 15.14: Monoclonal antibodies used in different leukaemia

Lineage	Primary panel	Secondary panel
Myeloid	CD13, CD33, CD117 (c-Kit), MPO (cyt)	CD14, CD64, CD41, CD61, lysozyme, glycophorin A
B-lymphoid	CD19, CD79a (cyt), CD10 or CALLA, CD22 (cyt)	Cyt IgM, surface Ig (κ/λ)
T-lymphoid	CD3 (cyt), CD2, CD7,	CD1a, membrane CD3, CD5, CD4, CD8
Non-lineage restricted (primitive stem cell)	HLA-DR, TdT (nuclear)	CD34

Advantages of Flow Cytometry (FCM)
(Table 15.15)

- Highly sensitive rapid and accurate.
- Separation of cell groups is archived through electronic gating and tedious manual isolation is not required.
- Allows simultaneous evaluation of multiple cellular parameters (forward scatter or FSC, side scatter or SSC and fluorescent)
- Only a small sample volume is required.
- Both surface and intracellular antigens can be identified.
- Highly reproducible and so it can be compared between laboratories.
- Quantitation of cellular subsets can be done.

Limitations of Flow Cytometry (FCM)
(Table 15.15)

1. High price of the instrument and reagents.
2. Special training is required for operation and expertise is needed for interpretation.
3. Often unsuitable for samples with low cellular yield-like CSF and FNAC. Because a minimum of 10,000–20,000 cells/events are required for FCM analysis.
4. Sometimes, instrument fail to differentiate between normal cells and abnormal cells. Hence, incorrect interpretation may be done if sufficient number of tumor cells and parameters are not analyzed.
5. Loss of morphological correlation with immunophenotype.

Table 15.15: Advantages and disadvantages of multicolour flow cytometric (MFC) immunophenotyping

Advantages	Disadvantages
• Increased accuracy: Using large number of flurochromes is associated with an exponential increase in the information, permitting a more reliable identification • Smaller sample size: Increased number of antibodies per tube means fewer tubes and less sample but allows acqition of more cellular events resulting in smaller coefficients of variation and increased data precision. This is particularly valuable for paucicellular sample like CSF or FNAC and pediatric samples	• Increased need for expertise in data analysis and interpretation • Human error associated with pipetting a high number of antibodies into a single tube. This can be overcome by preparing in-house monoclonal antibodies which have been shown to be stable for up to 4 weeks • Increased complexity of compensation: Inaccurate compensation is probably the main source of erroneous data in MFC. It can be solved by applying compensation matrices

Contd.

Table 15.15: Advantages and disadvantages of multicolour flow cytometric (MFC) immunophenotyping (Contd.)

Advantages	Disadvantages
• Increased efficiency: Less time is required for sample processing and acquisition • Increased sensitivity for minimal residual disease monitoring	• Challenges of antibody panel validation: This is crucial to run fluorescence minus one control for all new antibody combinations and to check for stearic hindrance between antibodies used to label antigens which are in close proximity on the cell • Tandem dye conjugate issues: Tandem dyes are conjugates of two fluorochromes which can leads to problems in resonance excitation transfer if exposed to light

Table 15.16: Comparison of immunohistochemistry and flow cytometry

Parameter	Immunohistochemistry	Flow cytometry
Nature of sample	Formalin fixed paraffin embedded (FFPE) tissue, frozen section	Blood, bone marrow aspirates, body fluids, FNAC, fresh unfixed tissue
Number of cell required	Fewer	Many
Morphology and tissue architecture	Preserved and thus cytology can be appreciated	Lost
Dual or multiple staining on same group of cells	Laborious	Possible
Ability to perform on archive material	Yes	No
Distinction between surface versus cytoplasmic antigens	No	Yes
Gating or cell sorting	Not possible	Possible
Quantitative analysis	No	Yes
Availability of antibodies	Limited	Abundant
Hodgkin lymphoma	Useful	Not useful

Table 15.17: Comparison of molecular techniques and flow cytometry

Parameter	Molecular techniques	Flow cytometry
Speed	2–3 days (up to 1 week)	1–2 hours
Target	DNA or RNA (RNA is an unstable target)	Protein/cells (end product)
Applicability	Depends on disease (chromosome aberration)	Broad
Multiplexing	Technically demanding	Relatively easy (even 25–100 test/tube)
Accuracy	Quantitative/semiquantitative	Quantitative
Focus	All cells in sample, unless prior purification	Any subpopulation
Facilities	Special lab needed (pre-PCR lab, PCR lab, etc.)	Only standard lab needed (+ flow cytometry)

Automated Coagulometer

Coagulation tests can be done by:

- Manual method
- Coagulometers:
  - i. Semiautomated method
  - ii. Automated method (Fig. 15.17)

Manual Method

- All reagent and samples are added manually by the operators.
- Temperature is maintained by a water bath or heat block
- May require external measurement by operator, most often using a stopwatch.
- Human error may be present. Difficult to do when there is large workload.

Semiautomated method

- All reagents and samples are added manually by the operators
- Usually contains a device for maintaining, constant 37°C temperature. Analyser may or may not internally monitor temperature.
- Has mechanism to automatically initiate, timing device upon addition of final reagent and external mechanism for detecting clot formation.

Automated method

- All reagents are automatically pipetted by the instrument; Samples may or may not be automatically pipetted.
- Contains monitoring devices, and internal mechanism to maintain and monitor constant 37°C temperature throughout testing sequence.
- Timers are initiated and clot formation detected automatically.
- Perform autodilutions mainly for the single factor assays.
- Storage of large number of patient data and control result.
- Generates flagging for sample abnormality and instrumental malfunctioning.
- Batch processing of multiple samples for single test or multiple tests on a single sample can be performed.

Clotting Assay

In this method, sample and reagent are mixed together to form a clot. End point of the reaction, i.e. clot is detected by:

- Mechanical method:
  - i. Electromechanical (impedance method)
  - ii. Magnetic, steel ball method
- Optical method
  - i. Photo-optical
  - ii. Nephlometric
  - iii. Chromogenic
  - iv. Immunologic
- Electrochemical

Fig. 15.17: Semiautomated and fully automated coagulometer

Mechanical Method

Electromechanical method (impedance method)

i. When coagulation process takes place, the concentration of clotting factors (charges) and inorganic ions will change along the time and the measured impedance or conductance will be also change correspondingly at the same time.
ii. During the reaction, one probe, moves in and out of the solution at constant intervals. The electrical circuit between the two probes is not maintained as the moving probe rise in and out of the solution.
iii. When a clot (fibrin) is formed in the solution, the fibrin strands maintain electrical contact between the two probes when the moving probe leaves the solution, which stops the timer.

Magnetic, Steel Ball Method

Mechanical clot detection involves, monitoring the movement of a steel ball within the test solution using a magnetic sensor. As clot formation occurs, the movement of the ball changes, which is detected by the sensor, there are two variations of this principle used in current instrumentation.

i. A change in the movement of the steel ball may be detected when there is increased viscosity of the test solution, changing its range of motion.
ii. Or by a break in contact with the magnetic sensors when the steel ball becomes incorporated into a fibrin clot as the cuvette rotates.

Photo-optical Method

i. Detection of clot formation measured by a change in OD of a test sample which is the basis of photo-optical instrumentation. This is also known as **turbidometric methodology.**
ii. When a light source of a specified wavelength is passed through a test solution (plasma), a certain amount of light is detected by a photodetector or photocell located on the other side of the solution.
iii. The amount of light detected is dependent on the colour and clarity of the plasma sample and is considered to be the baseline light transmission value.
iv. When soluble fibrinogen begins to polymerize into a fibrin clot, formation of fibrin strands causes light to scatter, allowing less light to fall on the photodetector (i.e. the plasma becomes more opaque, decreasing the amount of light detected).
v. When the amount of light reaching the photodetector decreases to an exact point from the baseline value as predetermined by the instrument, this change in OD triggers the timer to stop, indicating clot formation.

Nephelometric Method

i. Quantifying plasma proteins based on the specific reaction of the protein being measured with highly specific antisera.
ii. Precipitants are antigen–antibody complexes, which show up in solutions as turbidity and scatter indent light.
iii. The nephelometer uses a light-emitting diode at a high wavelength (usually >600 nm) to detect variations in light scatter as antigen–antibody complexes are found. When the light rays encounter insoluble complexes such as fibrin strands, they are scattered in both forwards (180 degree) and side (90 degree) angles.
iv. Instruments employing this type of measuring system detect the amount of agglutination of particles by reading the increasing amount of light scattered at 90 degree angle as agglutinates are formed. The timer is triggered to stop when the amount of light scatter reaches a specific predetermined level.
v. This method of endpoint detection is in contrast to the photo-optical systems, which sense decreased light transmission 180 degrees due to the opaqueness of the sample in a cuvette when fibrin is formed.

Chromogenic Method

Chromogenic or amidolytic methodology is based on the use of a specific colour-producing substance known as chromophore. The chromophore normally used in the coagulation laboratory is para-nitroaniline (p-nitroaniline or PNA), which has an optical absorbance peak at 405 nm on a spectrophotometer.

Immunologic Method

i. Immunologic assays are based on antigen–antibody reactions.
ii. Latex microparticles are coated with a specific antibody directed against the analyte (antigen) to be measured.
iii. A beam of monochromatic light is then passed through the suspension of microlatex particles. When the wavelength of light, greater than the diameter of the particles in suspension, only small amount of light will be absorbed by the particles.
iv. When the latex microparticles coated with specific antibody come in contact with the antigen present in the solution, the antigen attaches to the antibody and form bridges between the articles, causing them to agglutinate.
v. As the diameters of the agglutinates become larger and closer to the wavelength of the monochromatic light beam, the greater the amount of light that is absorbed.
vi. The increase in light absorbance is proportional to the size of the agglutinates, which in turn, is proportional to the antigen level present in the sample, which is read from a standard curve.

Electrochemical Method

This is also known as **INRatio Meter (HemoSense)** or near patient testing device.

The INRatio single-use test strip is made of laminated layers of transparent plastic. Each test strip has a sample well where blood is applied, three channels through which the blood sample flows to reach the testing areas, reagents to start the coagulation process, and electrodes that interface with ratio meter. The device detects a change in electrical resistance when blood clots (Table 15.18).

Table 15.18: Advantages and disadvantages of different methods used in coagulometer

Method	Advantages	Disadvantages
Mechanical Method	• No interference due to physical characteristics such as lipaemia or haemolysis • May use small sample volumes • Some can analyses whole blood for same tests, removing the need for centrifugation	• Impossible to observe graphics of clot formation • May present problems of endpoint detection in some sample with low fibrinogen
Photo-optic method	• Possibility of graphics on clot formation • Optical checks for haemolysis/ lipaemia/icterus on some optical systems	• Interference due to lipaemia, haemolysis, hyperbilirubinaemia, or protein increase on some systems • Some systems may present difficulties with clot detection when using some completely transparent reagents

Contd.

Table 15.18: Advantages and disadvantages of different methods used in coagulometer (Contd.)

Method	Advantages	Disadvantages
		• Very short coagulation periods may go undetected owing to delay prior to initiation of monitoring
Nephelometric method	• Can measure antigen–antibody reactions in proteins present in very small amounts	• Limits number of available tests • Cost of reagents
Chromogenic method	• Fully specific assays may be easier. • Additional parameters (which may not be suitable for measurement by clot detection), may be measured by this method • Increase the repertoire of possible tests • Possible improvements in precision compared to clot based analyses	• Limited by the instrument's wavelength • Requires large test volumes for positive cost-benefit ratio • Cost of instrument and reagents
Immunological method	• Can automate time consuming, manual methods • Increases the number of possible tests	• Limited number of tests available • Cost of instruments • Cost of reagents

Platelet Function Analyser

- Uses stimulators of platelet adhesion and aggregation in an environment that stimulates an injured blood vessel wall.
- More sensitive screening test than the bleeding time method.
- Nonspecific test: Not diagnostic for any single disorder.
- Principle of the test: The instrument adds citrated blood to is a reservoir with collagen/epinephrine is a bioactive membrane. A pressure sensor detects the formation of a platelet plug on the membrane. The time it takes to close the aperture, in the membrane with the platelet plug is recorded; the result is a function of platelet count, platelet activity, vWF activity (Fig. 15.18).

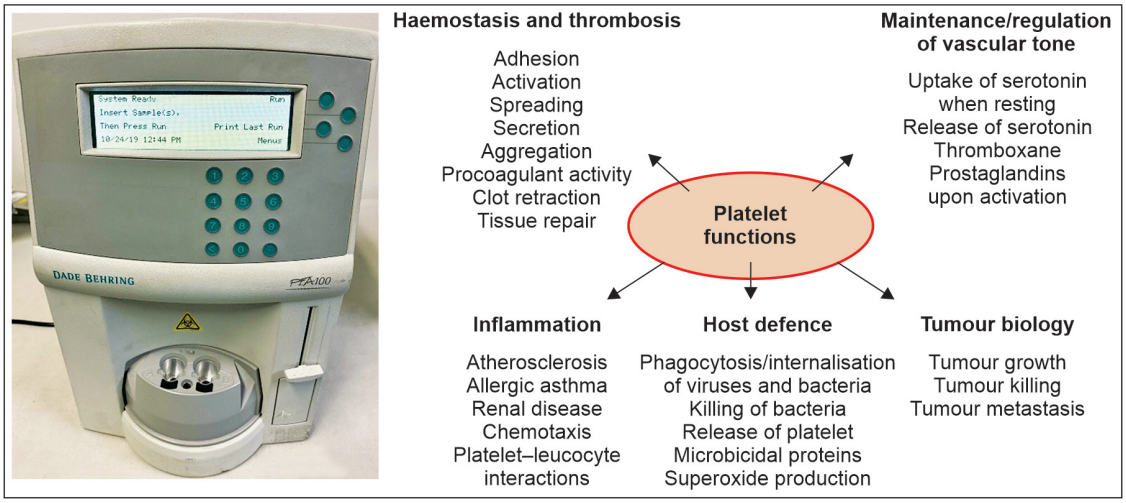


Fig. 15.18: Platelet function analyser

Platelet Aggregometry (Fig. 15.19)

- Platelet aggregometry involves a series of tests performed on whole blood or platelet-rich plasma, using several agonists or platelet activators.
- The agonist is added to the suspension and a dynamic measure of platelet clumping is recorded.
- Simultaneously to platelet aggregation, **luminometry test** can be performed. In that case, ATP release is assayed using a *luminescent marker*.

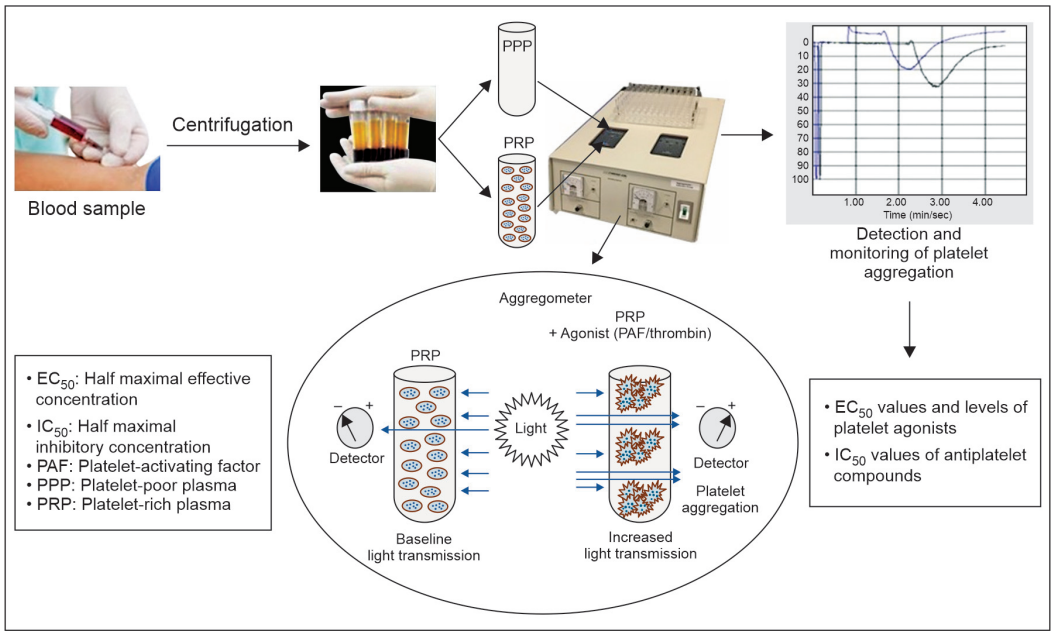


Fig. 15.19: Platelet aggregometry. PPP: Platelet-poor plasma; PRP: Platelet-rich plasma

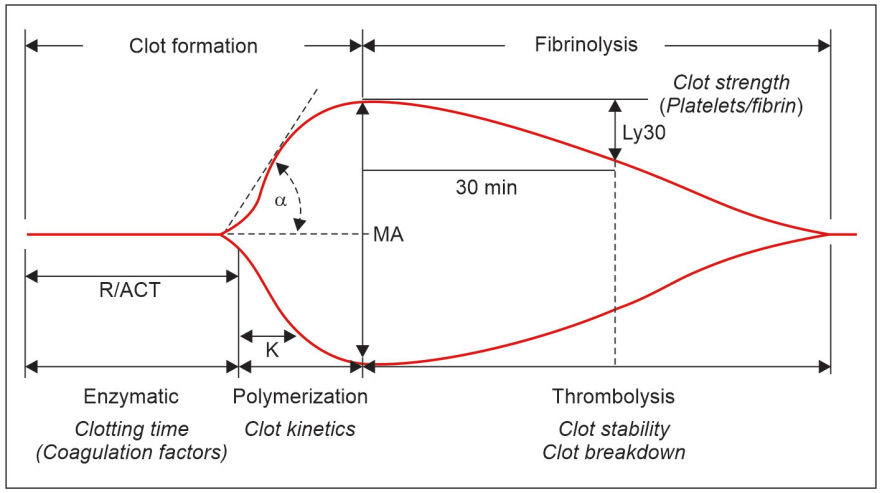


Fig. 15.20: Illustration of a thromboelastography (TEG) tracing and the accompanying parameters. The depiction of the TEG tracing and the parameters were measured throughout the lifetime of a clot. Abbreviations: α: Alpha angle; ACT: Activated clotting time; K: Coagulation time; Ly30: Percentage of lysis 30 min after MA; MA: Maximum amplitude; R: Reaction time

Thromboelastography (TEG) (Fig. 15.20)

- Sample of citrated whole blood is placed in a cup which has a pin carefully connected to a torsion wire.
- As the cup rotated in a back and forth movement, the aggregates formed within the cup and the wire to become more rigidly placed and reflects the strength of the aggregates formed within the cup.
- The movement or lack of movement is reflected via either an optical or magnetic detector.
- A graphic presentation is produced (Fig. 15.19).

Uses of TEG (Table 15.19)

i. Illustrate function and dysfunction in the haemostatic system.
ii. Allows physicians to give appropriate amounts of FFP, cryo and platelets to control haemorrhage.
iii. Reduces unnecessary use of blood products.
iv. Allows effective management of hypercoagulability.
v. Differentiates surgical from pathological bleeding.

Automated ESR (Fig. 15.21 and Table 15.20)

The erythrocyte sedimentation rate (ESR) is a most widely used test in clinical practice. The Westergren method is considered as the reference methodology. Subsequently modifications have been made and ICSH guidelines allow for the use of alternative newer and safer ESR techniques. The Ves-matic analyser is a new automated instrument for measuring ESR and the advantage conferred by this method is that it can generate ESR readings within 25 minutes. Additionally, the same EDTA sample collected for complete blood counts can also be used for ESR analysis by this machine. One such instrument is Ver-Matic cube 30.

Instrument overview of Ves-Matic Cube 30

This is an automatic bench top analyser designed and programmed to determine the ESR on whole blood samples anti-coagulated with EDTA. It can analyze up to 30 blood samples per analytical cycle; the through put is roughly of 60 ESR results per hour. The instrument performs the analysis making use of full blood count samples and accepts any kind of brand/model of top lavender tubes available in the market. It is therefore, neither necessary to use a dedicated citrate tube, nor to transfer the blood from the tubes inside the instrument.

The main innovation of the system is that the sedimentation of red cells in autologous plasma is read directly in the original EDTA tubes used for the full blood count, by means

Table 15.19: Thromboelastogram (TEG)

Components	Definition	Normal values	Problem with	Treatment
R time	Time to start forming clot	5–10 minutes	Coagulation factors	FFP
K time	Time until clot reaches a fixed strength	1–3 minutes	Fibrinogen	Cryoprecipitate
Alpha angle	Speed of fibrin accumulation	53–72 degrees	Fibrinogen	Cryoprecipitate
Maximum amplitude (MA)	Highest vertical amplitude of the TEG	50–70 mm	Platelets	Platelets and/or DDAVP
Lysis at 30 minutes (Ly30)	Percentage of amplitude reduction 30 minutes after maximum amplitude	0–8%	Excess fibrinogen	Tranexamic acid and/or amino-caproic acid

Fig. 15.21: Automated ESR

of a specially designed optical system. Due to this features, no part of the system comes in contact with or consume any of the blood samples during its operation. As a result, there is no transfer of blood from the original tube into any part of the analyser and no production of waste fluids. The system is therefore designed to maximize the operator safety and protection. The Ves-Matic Cube 30 is environment-friendly as it eliminates the possibility of biological contamination of the environment via biological waste and reduces the amount of plastic tubes that have to be disposed of.

This instrument is a true walk-away system. Sample analysis is executed completely automatically (mixing of the samples and reading of the sedimentation) and the results, obtained in only 15 minutes of sedimentation, correlate with those obtained with the Westergren citrate standard method (60 minutes sedimentation performed at 18°C using dedicated glass pipettes of 200 mm, with an internal bore with a diameter of 2.5 mm. The total duration of the analysis is 33 minutes, including the mixing of the samples ensuring the careful resuspension and disaggregation of red blood cells (RBCs spontaneously aggregate at rest).

The analytical cycle: The full count EDTA tubes are inserted sample holding rotor. ESR and non-ESR samples are sorted by reading the bar-code label applied on the tube. The bidirectional connection to the LIS and the complete host integration allow the automatic selection of the sample for which ESR testing is required.

Once the tubes have been loaded in the 30 position rotor, the analytical cycle is started pushing the START key; in case all of the 30 positions are occupied by samples, the cycle is started by closing the lid. The sample holding rotor bends of 90° on its shaft and start rotating to thoroughly mix the samples. The mixing phase takes 15 minutes. At the end of mixing phase, the rotor goes back to home position and the level of blood at time 0 of sedimentation is read for each sample. After 15 minutes of sedimentation, the final level reached by the settling red cells is read and recorded to give the final ESR result (1st

hour, according to the Westergren citrate method).

The user interface is through a keyboard, and the software is extremely simple and intuitive: To start a run it is sufficient just to load the samples and press the START key.

A very important feature of the system is the possibility of using EDTA tubes of different brands and models simultaneously with the only restriction of rubber cap tuber; for this kind of tubes a special adaptor—to be placed on the rotor is provided with the instrument.

Host computer connection: The Ves-Matic Cube 30 can be connected to a host computer via the RS 232C serial interface. The bidirectional communication to the LIS allows the instrument to receive the work list containing the barcodes of the samples for which ESR is requested.

Temperature correction: The Ves-Matic Cube 30 contains a temperature sensor. It measures the working temperature inside the instrument and is positioned inside the analysis module. The actual temperature is displayed in the 'temperature window' on the screen, either in Celcius (°C) or in Fahrenheit (°F) degrees. The instrument reports the results corrected to the standard temperature of 18°C according to Manley's normogram. Nevertheless, it is possible to deactivate the temperature correction feature for differing laboratory needs.

Quality control: This instrument has a built-in internal QC function. A bi-level (normal and abnormal) ESR control blood is available to verify the proper functioning of the instrument. The ESR control is modified and stabilised human blood that can be used to monitor the accuracy and precision of this instrument.

Transponder: The 'transponder' is an electronic device that allows the instrument to have a defined number of executable tests available. For every result the transponder will automatically undergo a decrease in the number of available tests. Once number of available tests is exhausted, the operator must reload the instrument with a new transponder. The transponder has the dimensions and appearance of a normal CBC test tube. To reload the instrument simply insert the new transponder in the dedicated holder inside the analyser. Transponder tube will automatically transfer the reload to the instrument.

At the end of the operation the transponder tube is empty and cannot be used again.

Table 15.20: Advantages and disadvantages of automated ESR method

Advantages	Disadvantages
• Provide more rapid result • Uses small volumes of sample • The sample anti-coagulated blood (EDTA) can be used for other tests like CBC • No mouth pipetting, hence greater safety. • Saves technician time • Interface with the laboratory information system (LIS)	• Automated system tends to underestimate when the ESR value is high (>20 mm/hr). But discrepancy is not found when ESR value is normal • Cost of the analyser is high compared to ESR pipette used in Westergren method

Ves-Matic 20 Instruments

- It is designed to measure 20 blood sample
- Results are comparable to Westergren method
- Results are available to approximately 22 minutes
- Working principle: Blood is collected in a special cuvette. Sample is mixed by instrument. Sample is allowed to stand at 18° slant from vertical. Opto-electrical sensor measures ESR. Data are elaborated and printed.

Factors Affecting ESR

Physiological factors	Laboratory factors
• Plasma factors	Temperature
• Red cell factors	Time
• Rouleaux formation	Anticoagulants
• Age	Tube factor
• Sex	Tilting of tube
• Pregnancy	Vibration
	Sunlight

Plasma factors

i. Fibrinogen, globulin and cholesterol increase ESR by decreasing negative charge of RBC. Negative change of RBC prevents the RBC coming together (repulsion of RBCs).
ii. Plasma albumin retards sedimentation of RBCs.
iii. Lecithin retards ESR.

Red cell factors

i. Number: If there is increased in red cell mass, there is decrease in ESR. If there is decreased red call mass, there is increased in ESR.
ii. Size: Microcyte sediment slowly and macrocyte sediment rapidly than normal normocyte.
iii. Shape: The shape of the RBCs can also affect the ESR.

Sickle cell retard ESR because of abnormal cell hamper rouleaux formation, spherocytes also sediment at a slow rate.

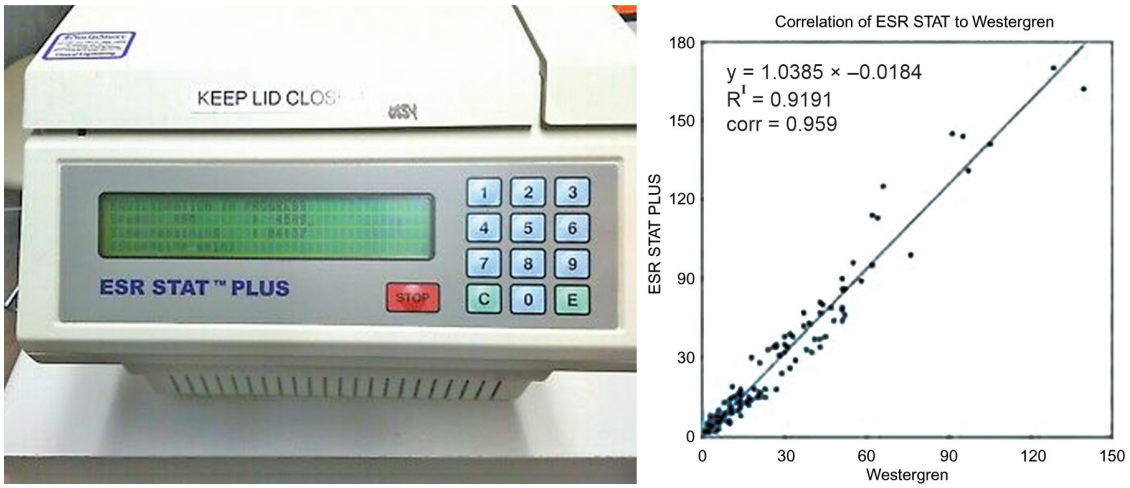
Rouleaux formation: When RBCs aggregate to form a rouleaux and settle down, then the area is much less than sum of the area of constituent corpuscle. Hence, it is important factor which increases ESR.

- **Age:** ESR is low in infants. In adults, ESR gradually increases and in old age ESR is higher.
- **Sex:** Women have slightly higher ESR then men because of their low PCV and increased fibrinogen content.
- **Pregnancy:** The ESR begins to increase from third month onwards and return to normal, about 4 weeks after delivery. Because there is increase in plasma volume leading to haemodilution.
- **Temperature:** Test should be done at 20–25°C. Higher temperature causes false high results due to reduction in a plasma viscosity. Always bring refrigerated blood to room temperature before doing ESR. Rise in every 3°C temperature will cause rise of ESR by 1 mm.
- **Time:** Test should be done within 2 hours of blood collection. EDTA sample should be kept at 4°C, if delayed but should be performed within 6 hours.
- **Anticoagulant:** K_2 EDTA or tri-sodium citrate should be used. Heparin alters cell membrane potential and should not be used.
- **Tube factor**: ESR is greater with longer tube. Inner diameter should be 2.5 mm or more to overcome capillary attraction.
- **Position of the tube:** Tube should be perfectly vertical. ESR increases as the RBC slide down along the lower side. Angle of 3° from vertical can increased ESR by 30%.
- **Vibration:** Vibration can reduce ESR.
- **Haemolysed or clotted sample:** It retards ESR. Clot traps fibrinogen, hence no rouleaux formation occurs.
- **Sunlight:** Direct sunlight on ESR tube increases ESR.
- **Drugs:** Drugs like dextran, methyldopa, oral contraceptives, penicillamine, procainamide, theophylline, and vitamin A can increase ESR; while aspirin, cortisone and quinine may decrease it (Table 15.21).

Table 15.21: Causes of increased and decreased ESR

Increased ESR	Decreased ESR
• **Infections**: Acute rheumatic fever, tuberculosis, osteomyelitis, pyogenic arthritis, pelvic inflammatory disease, bacterial endocarditis. • **Inflammatory diseases**: SLE, rheumatoid arthritis, polymyalgia rheumatica, temporal arteritis • Acute myocardial infarction • Paraproteinemias: Multiple myeloma, Waldenström's macroglobulinaemia, cryoglobulinaemia • Malignancies and metastasis • Hyperfibrinogenaemia • Others: Anaemia, ruptured ectopic pregnancy, renal disease **Very high ESR (100 mm/hr)** Tuberculosis, multiple myeloma, hyperfibrinogenaemia, myocardial infarction, rheumatoid arthritis, temporal arteritis and polymyalgia rheumatica, SLE, severe anaemia	• Polycythaemia • Afibrinogenaemia or decreased fibrinogen level (hypofibrinogenaemia) • Sickle cell anaemia • Spherocytosis • Microcytosis • Congestive cardiac failure (CCF) • Corticosteroid use

ESR STAT PLUS (Fig. 15.22)



This is centrifugation based method. It provides result in 5 minutes.

Working principle: Sample is placed in centrifuge

Infrared laser tracks the erythrocyte plasma interface and takes multiple measurements

↓

Liner portion of sedimentation is identified

↓

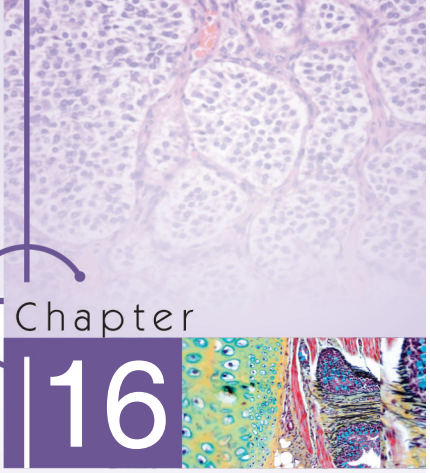
Software algorithm to determine ESR result

Fig. 15.22: ESR STAT PLUS

SEDIMAT (Fig. 15.23)

- The filled Sediplast Westergren pipette is placed into the SEDIMAT automated ESR reader, which accelerates sedimentation under controlled conditions.
- The reader displays the result of each sample on an LCD displays after 15 minutes.
- The results are also stored in memory and can be printed out.

Fig. 15.23: SEDIMAT



Chapter

16

HPLC, Capillary Electrophoresis and Haemoglobinopathies

Chromatography is a laboratory technique for the separation of a mixture. This separation method is based on the different interactions of the specimen compounds with the **mobile phase**, and with the **stationary phase**, as the compounds travel through a support medium.

The compounds interacting more strongly with the stationary phase are retained longer in the medium than those that favour the mobile phase. The mixture is dissolved in a fluid (mobile phase) which carries the mixture through a structure holding another material called the stationary phase. The various constituents of the mixture travel at different speeds and hence get separated. Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase which affects this separation.

History: Chromatography was first applied by Italian-born scientist Mikhail Tsvet in Russia in 1900. He primarily separated plant pigments like carotene, chlorophyll, and xanthophylls. As these compounds, have different colours (orange, green and yellow respectively), this separation technique was named as **chromatography**. Subsequently Anchor John Porter Martin and Richard Laurence Millington Synge made significant developments in the technique and they won Nobel Prize in chemistry in 1952. Their principles and basic techniques of partition chromatography later used to develop several chromatography methods: Paper chromatography, gas chromatography and **high-performance liquid chromatography (HPLC).**

Different Terms of Chromatography

- **Chromatograph:** Equipment that enables a sophisticated separation, e.g. gas chromatographic or liquid chromatographic separation.
- **Chromatography:** It is a physical method of separation which distributes components to separate between two phases, one stationary (stationary phase), the other (mobile phase) moving in a definite direction.
- **Chromatogram:** It is the visual output of the chromatograph. In the case of an optional separation, different peak of patterns on the chromatogram correspond to different components of the separated mixture.
- **Analyte:** It is the substance to be separated during chromatography.
- **Analytical chromatography:** It is used to determine the existence and possibly also the concentration of analyte(s) in a sample.
- **Bonded phase:** This is a stationary phase which is covalently bonded to the support

particles as to the inside wall of the column tubing.

- **Mobile phase:** It is the phase that moves in a definite direction. This may be a liquid (liquid chromatography and capillary chromatography), a gas (GC) or supercritical fluid (supercritical-fluid chromatography, SFC). In this phase, the mixture sample is separated/analyzed and the solvent moves the sample through column (column chromatography). In the case of HPLC method, the mobile phase consists of a non-polar solvent(s) such as hexane on normal phase or a polar solvent like methanol in reverse phase chromatography and the sample gets separated. The mobile phase moves through the chromatography column (the stationary phase) where the sample interacts with the stationary phase and is separated.
- **Stationary phase:** This phase fixes the substance in place for the chromatography procedure. As for example, silica layer in thin layer chromatography.
- **Immobilized phase:** It is the stationary phase that is mobilized on the support particles, or the inner wall of the column tubing.
- **Retention time:** It is the characteristic time which takes for a particular analyte to pass through the system (from the column inlet to detector) under set conditions.
- **Detector:** It refers to the instrument used for qualitative and quantitative detection of analytes after separation.
- **Sample:** It is the specimen or matter which is analyzed in chromatography. This may consist of a single component of it may be a mixture of components. When the sample is treated in the course of an analysis, the phase on to the phases containing the analytes of interest is/are referred to as the **sample,** whereas everything out of interest separated from the sample before or in the course of the analysis is referred to as **waste**.
- **Solute:** It refers to the sample components in partition chromatography.
- **Solvent:** It refers to any substance capable of solubilizing another substance, and especially the liquid mobile phase in liquid chromatography.

Retention time (Rt) is this time it takes a compound to elute. This value is characteristic of a compound, and is related to the strength of its interaction with the stationary phase and the mobile phase. The retention time, therefore can be used to determine a compound's identity. As for example, if two compounds are separated and their retention times are represented by Rt1 and Rt2. There are uncorrected retention times which are measured from the injection times, T = 0.

A column's ability to separate two compounds depends on two factors: (i) The difference in the retention of the compounds or capacity factor, k′ and (ii) the width of their peaks, Wb. The value of k′ can be calculated by the following equation:

$$k^1 = (Rt1 - Tm)/Tm \text{ or } Rt1'/TM$$

where;

Tm = Retention time of a non-retained compound

Rt1′ = Corrected retention time of first sample

Another measurement derived from the calculated capacity factor is the selectivity factor (α) or relative retention of two solutes. A ratio of both capacity factors is used to calculate the selectivity factor. To measure the width of each peak, draw tangents along the side of the peak to the baseline. The distance between the two intersected lines is represented by Wb. To calculate the number of theoretical plates (N), use the following equation:

$$N = 16\ (Rt/Wb)^2$$

A plate number has no units and larger the value of (N) for a column, the greater its separation efficiency. The combined effects of solvent efficiency and column efficiency are expressed in the resolution (Rs) of the column.

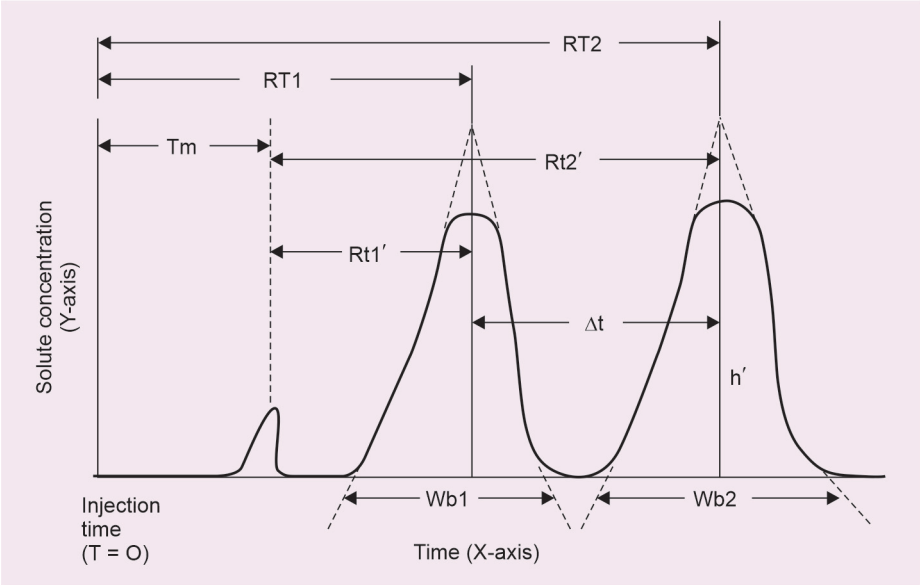


Fig. 16.1: Chromatogram for the separation of two compounds (1st and 2nd) in a mixture of sample. RT1: Retention time of 1st component, RT2: Retention time of 2nd component, Tm: Retention time of nonretained compound, Injection time or T = 0 means starting point, Wb1: Width of 1st component, Wb2: Width of 2nd component, h′: Peak height, Δt: Time interval between two peak heights

$$Rs = 2 (Rt2 - Rt1)/(Wb1 + Wb2)$$

The concentration of unknown compound is found out from the peak height (h′) and may be calculated using an integrator of the method of internal standardization.

Techniques by Chromatographic Bed Shape

Column chromatography: This is a separation technique in which the stationary bed is within a tube. The particles of the solid stationary phase or the support coated with a liquid stationary phase may fill the whole inside volume of the tube (packed column) as be concentrated on or along the inside tube wall leaving an open, unrestricted path for the mobile phase in the middle part of the tube (open tubular column). The differences in rates of movement through the medium are calculated to different retention times of the sample. The liquid solvent (the eluent) is passed through the column by gravity on by the application of air pressure.

Silica gel (SiO_2) and alumina (Al_2O_3) are two adsorbents commonly used by column chromatography.

Uses/Application

- To isolate active ingredients
- To separate compound mixtures
- To determine drug estimation from drug formulations
- To remove impurities
- To isolate metabolites from biological fluids (Fig. 16.2).

Paper chromatography: This is a technique that involves placing a small dot or line of sample solution onto a strip of chromatography paper. The paper is placed in a container with a shallow layer of solvent and sealed. As the solvent rises through the paper, it meets the sample mixture that travels up the paper with the solvent. This paper is made of cellulose a polar substance and the compounds within the mixture travel further if they are less polar.

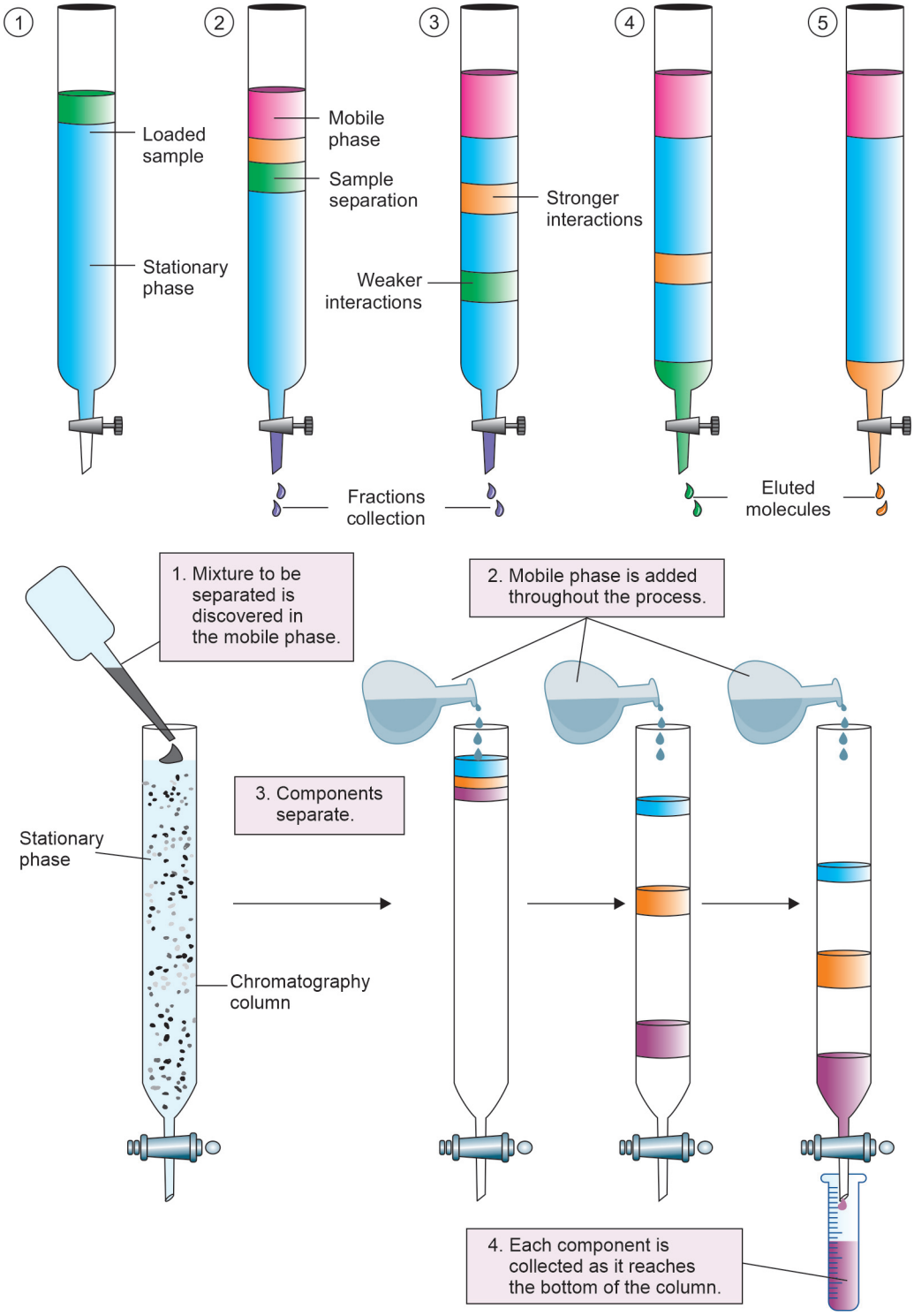


Fig. 16.2: Column chromatography

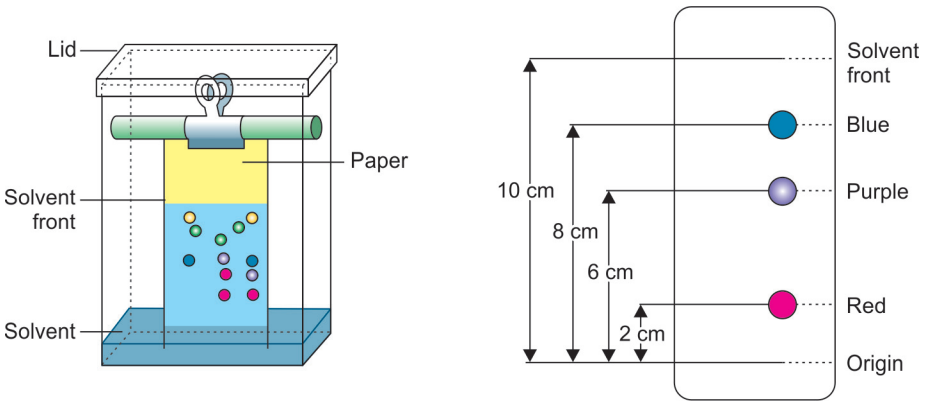


Fig. 16.3: Paper chromatography

Use: To separate coloured constituents in a substance. The stationary phase is solid cellulose and mobile phase is liquid (Fig. 16.3).

Thin-layer chromatography (TLC): Thin-layer chromatography is a widely employed laboratory technique used to separate different biochemical on the basis of their relative attractions to the stationary and mobile phases. Unlike using a stationary phase of paper as in paper chromatography, TLC involves a stationary phase of a thin layer of absorbent like silica gel, alumina, or cellulose on a flat, inert substance. This is a very versatile; multiple samples can be separated simultaneously on the same layer. So, it is very useful for screening applications such as testing drug levels and water purity. For even better resolution and faster separation which utilizes less solvent, high-performance TLC can be used (Fig. 16.4).

Techniques by Physical State of Mobile Phase

Gas chromatography: Gas chromatography (GC) is useful for compounds which are naturally volatile or can be easily converted into a volatile form. GC has been a widely used method owing to its high resolution, low detection limits, accuracy, and short analytic time.

Gas chromatographic separation is always carried out in a column, which is typically "packed" or capillary". Packed columns are

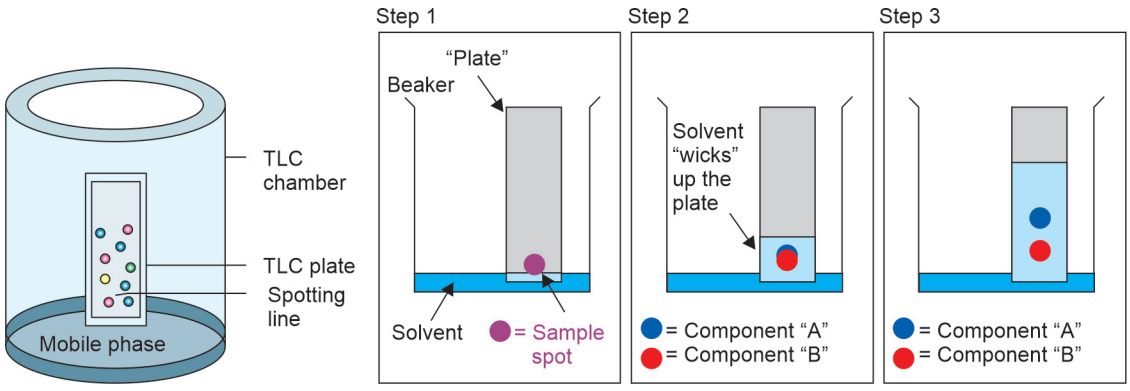


Fig. 16.4: Thin-layer chromatography (TLC)

routinely used and are cheaper as well as easier to perform. Capillary columns generally give far superior resolution and is now preferred, especially for complex mixtures. Both types of column are made from non-adsorbent and chemically inert materials. Stainless steel and glass are the usual materials for packed columns and quartz or fused silica for capillary columns.

Retention of a compound in GC is determined by its vapour pressure and volatility, which, in turn, depend on its interaction with the stationary phase.

Uses

- It is widely used in analytical chemistry. It is well suited for use in the petrochemical, environmental monitoring and remediation and industrial chemical fields.
- It is also widely used in chemical research (Fig. 16.5).

Liquid chromatography: Liquid chromatography (LC) is a separation technique in which the mobile phase is a liquid. Liquid chromatography (LC) techniques use lower temperature for separation, thereby archiving better separation of thermolabile compounds. LC is easier to recover sample in liquid chromatography than in GC. The mobile phase can be removed, and the sample can be processed further or reanalyzed under different conditions. Presently, LC which generally utilizes very small packing particles and a relatively high pressure referred to as **high performance liquid chromatography (HPLC).** HPLC emerged in the late 1960s.

In HPLC, the sample is forced by a liquid at high pressure (the mobile phase) through a column that is packed with a stationary phase composed of irregularly or spherically shaped particles, known as **porous monolithic layer**, or a porous membrane. HPLC is subdivided into two groups. Methods in which stationary phase is more polar than the mobile phase (e.g. toluene as the mobile phase and silica as the stationary phase) are termed **normal phase liquid chromatography (NPLC)** and the opposite (e.g. water-methanol mixture as the mobile phase and C l8 or octadecyl silyl as the stationary phase) as termed **reversed-phase liquid chromatography (RPLC).**

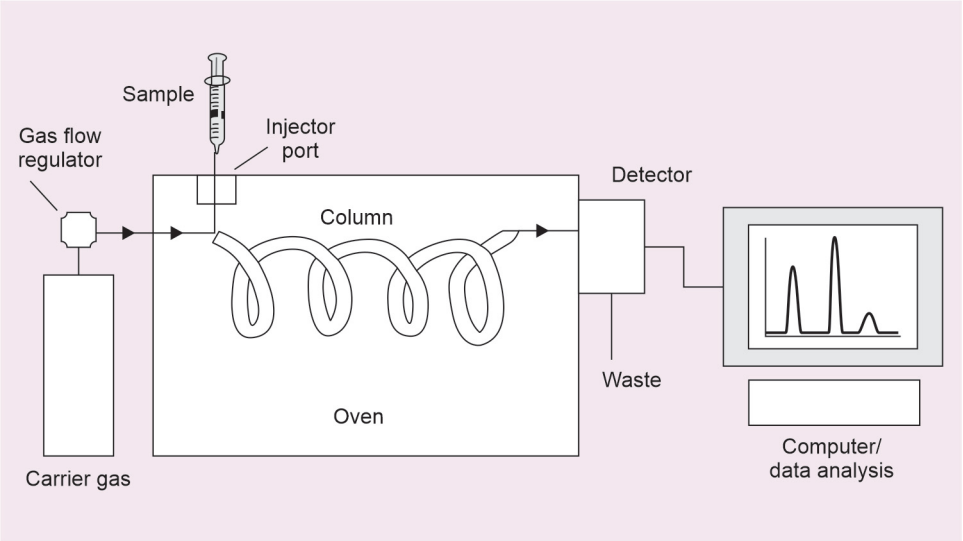


Fig. 16.5: Gas chromatography

There are five commonly used separation techniques in liquid chromatography

1. Adsorption (liquid-solid) chromatography
2. Partition (liquid-liquid) chromatography
3. Ion/exchange chromatography
4. Affinity chromatography
5. Size exclusion chromatography

Of these five separation techniques, ion-exchange chromatography is very popular in medical fraternity. Ion exchange chromatography is used to diagnose haemoglobinopathies and to measure HbA1C level in diabetes (Fig. 16.6).

Ion exchange chromatography: In exchange chromatography (or ion chromatography) uses an ion exchange mechanism to separate analytes based on their respective charges. It is usually performed in columns but can also be useful in planer mode. It uses a charged stationary phase to separate charged compounds including ions, cations, amino acids, peptides and proteins. In conventional methods, the stationary phase is an ion exchange resin that carries changed functional groups which interact with oppositely charged groups of the compound to retain. There are two types of ion exchange chromatography: Cation exchange and anion exchange. In **cation exchange chromatography**, the stationary phase has negative charge and exchangeable ion is a cation. In **anion exchange chromatography**, the stationary phase has positive charge and the exchangeable ion is an anion (Fig. 16.7).

ION EXCHANGE CHROMATOGRAPHY TO DETECT HAEMOGLOBINOPATHIES

Introduction

Ion exchange high-performance liquid chromatography is a very popular technique to detect thalassaemias and other haemoglobinopathies. **Cation exchange HPLC or CE-HPLC** is preferred over, anion exchange. CE-HPLC of red blood cell lysate is now established as a rapid, accurate and reproducible diagnostic procedure to separate various human haemoglobin fractions. It is performed routinely in many laboratories, mainly by fully automated HPLC machine. Interpretation of the HPLC chromatograms is a complex process requiring inputs from the clinical background of the case as well as data of complete blood count (Fig. 16.8).

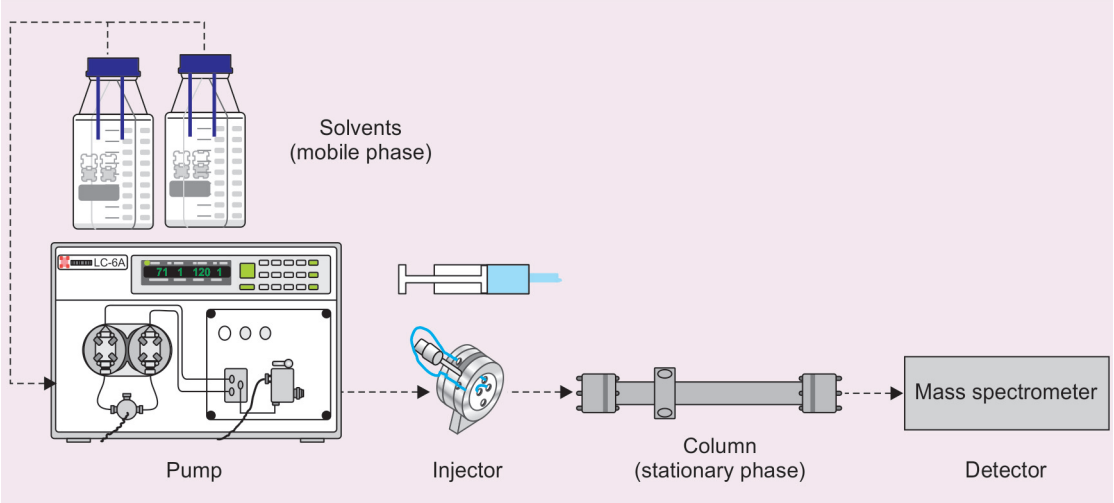


Fig. 16.6: Liquid chromatography

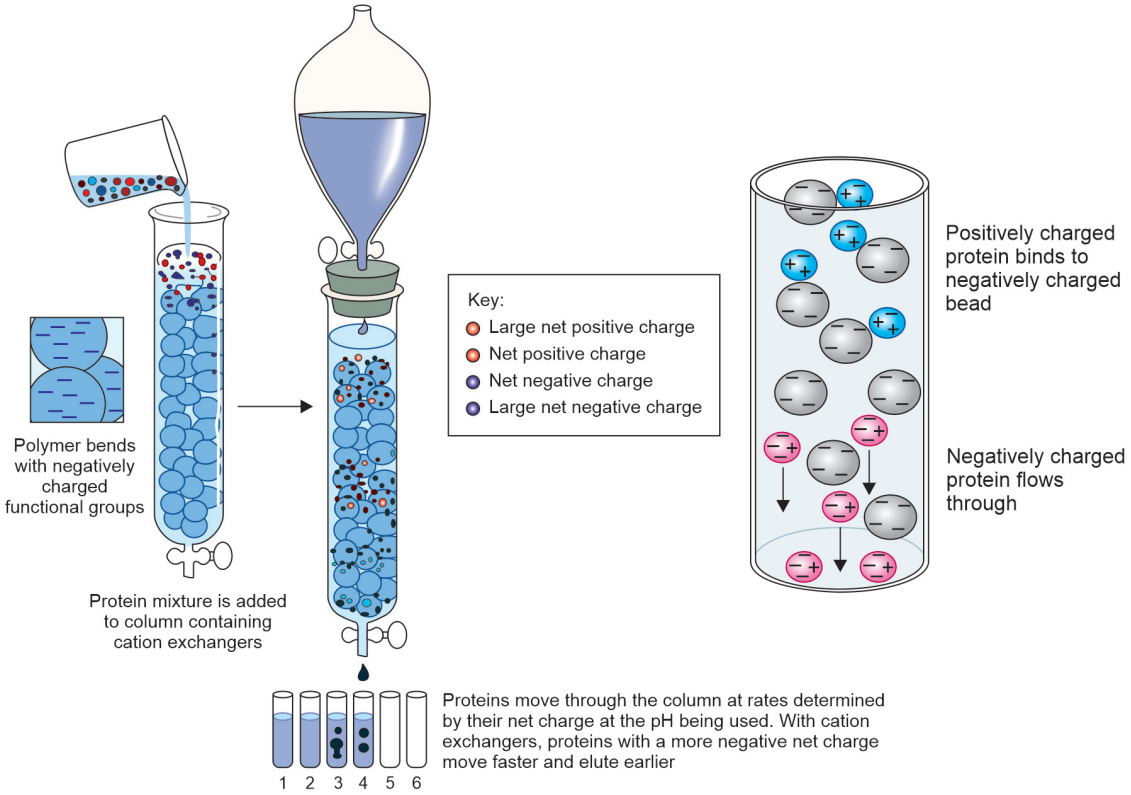
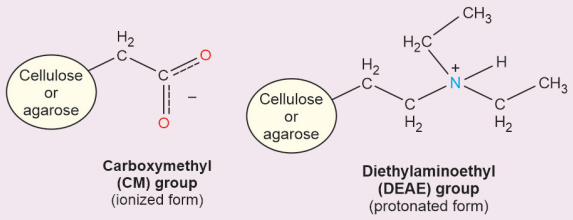


Fig. 16.7: Ion exchange chromatography

Ion exchangers: Functional groups

Cation exchangers	Anion exchangers
• Methylsulfonate (S-) • Sulphopropyl (SP-) • Carboxymethyl (CM-)	• Quaternary aminoethyl (QAE-) • Diethylaminopropyl (DEPE-) • Diethylaminoethyl (DEAE-)



Ion exchange chromatography

Anion exchangers	Cation exchangers
They contain immobilized cationic groups that bind to anions. The most common: A matrix with attached diethylaminoethyl (DEAE) DEAE: Matrix-CH_2-CH_2-$N^+H(CH_2CH_3)_2$	They contain immobilized anionic groups that bind to cations, e.g. a matrix with carboxymethyl (CM) goup CM: matrix-CH_2-COO^-

Fig. 16.8: Ion exchange chromatography

Principle of Cation Exchange HPLC or CE-HPLC

The basic principle of CE-HPLC involves passing the analyte of interest (a mixture of haemoglobins in solution) at a high pressure (approximately 100–200 kg/cm^2) through a cylindrical column, packed with a small spherical particle (typically 5 µm diameter **silica gel, known as stationary phase**). Very small sample volumes (usually approximately 5 µl) are applied to the column. Different types of haemoglobin (viz. HbA, HbF, HbA_2,

etc.) adsorb onto the silica packing with different intensities based on their ionic interactions. The column is then perfused by a **buffer (known as mobile phase)** which constantly varies in pH and ionic strength. Different haemoglobins elute out with the perfusing buffer at different but characteristic time points in response to the continually changing salt gradient. Another variable that affects elution or retention time is the column temperature which is kept fixed during procedure (5-6 min run approx.)

Materials and Methods

Firstly, patient suspected of thalassaemia or any structural variant is screened. These include cases of microcytic hypochromic anaemia (MCV<80 fl, MCH <27 pg, and RBC count >5 million/mm^3) not responding to conventional treatment, clinically suspected cases of haemoglobinopathy, antenatal, and other cases coming for thalassaemia screening (Fig. 16.9).

Blood: 5 ml intravenous blood sample was collected in EDTA anticoagulant. Red cell indices were measured on an automated haematology analyzer.

There are many HPLC machines in the market. One of them is variant haemoglobin testing system (Variant II Beta-thalassaemia Short Program, Bio-Rad Laboratories Inc. Hercules, CA, USA) which is very popular.

Principle of the HPLC Machine (Fig. 16.9)

The Variant II Beta Thalassaemia Short Program utilizes principles of ion exchange HPLC. The blood samples are automatically mixed and diluted on the Variant II Sampling Station (VSS) and injected to the analytical cartridge. The Variant II Chromatographic station (VCS) dual pumps deliver a programmed buffer gradient of increasing ionic strength to the cartridge, where HbA_2/F are separated based on their ionic interaction with the cartridge material.

The separated HbA_2/F then pass through the flow cell of the filter promoter where the changes in the absorbance at 415 nm are measured. An additional filter at 690 nm corrects the background absorbance. The Software (Variant II Clinical Data Management CDM) performs reduction of raw data collected from each analysis. To aid in the interpretation of results, windows have been established for the most frequently occurring haemoglobin based on the characteristic retention time. For each sample, a sample report and a chromatogram are generated by CDM showing all haemoglobin fractions eluted their retention times, the area of the peaks and values of the fractions.

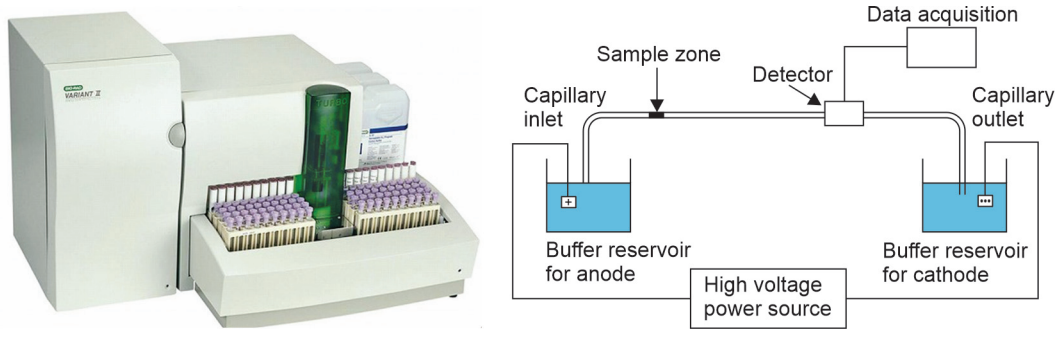


Fig. 16.9: Principle of the HPLC machine

Reagents for Bio-Rad Variant II, Short Program (Fig. 16.10)

1. Elution buffers (1, 2) sodium phosphate buffer.
2. Whole blood primer: Lyophilized human red blood cell haemolysate with gentamicin, tobramycin and EDTA as preservative.
3. HbA_2/F calibrator/diluent set: Lyophilized human red blood cell haemolysate with gentamicin, tobramycin, and EDTA as preservative analytical cartridge. Diluent contains deionized water.
4. Wash/diluent solution: Deionized water
5. Control: Normal (HbF 1–2%, Hb A_2, 1.8–3.2%) and abnormal (HbF, 5–10%, HbA_2, 4–6%).

Fig. 16.10: Reagents for Bio-Rad Variant II, Short Program

CALIBRATORS AND CONTROLS

All HPLC runs are preceded by priming and then calibration of instrument. Separate calibration factors are obtained for HbA_2 and HbF as ratios of expected to obtain values. Since the two values should ideally be equal (i.e. a ratio of 1) these are deemed to have passed if they lie between 0.7 and 1.3. These calibration factors are then applied for all subsequent patient samples. The retention time of HbA_2 in the calibration is also a useful indicator of run reliability. Normally, it lies between 2.60 and 2.70 min, and the instrument may need temperature adjustments if wider deviations occur. This is especially common as the column cartridge ages. Usual cartridge lifetimes being around 250 injections. Bilevel controls, one normal (HbF 12%, HbA_2 1.8–3.2%) and one elevated (HbF 5–10%, HbA_2 4–6%) should be analyzed at the beginning as well as the end of each set of patient specimens. The high control in case of Bio-Rad instruments also contain a variant peak that must elute in the window. All peaks must be symmetrical, temperature variations being the most common cause again of asymmetry.

Commonly buffers used

Buffer	pH value
Phosphate buffer	around 7.0
Tris-borate-EDTA buffer (TBE)	around 8.0
Tris-acetate-EDTA buffer (TAE)	above 8.0
Tris-glycine buffer (TG)	more than 8.5
Tris-citrate EDTA buffer (TCE)	around 7.0
Tris-EDTA buffer (TE)	around 8.0
Tris-maleic acid buffer (TME)	around 7.5
Lithium borate buffer (LB)	around 8.6

Sample Collection and Preparation

Five milliliters (5 ml) of whole blood is collected in a vacuum collection tube containing EDTA which can be stored at 2–8°C for maximum 7 days if processing is delayed. No preparation is required unless the sample is in a tube other than the recommended tube or there is less than 500 µl of sample in the tube. In such case, sample is manually prediluted. Predilution is carried out by mixing 1 ml wash/diluents with 5 µl of whole blood sample.

Interpretation of HPLC Reports (Table 16.1, Figs 16.11 and 16.14 to 16.22)

Reports and chromatograms are studied and interpreted by observing HbA_2 and F concentration for beta-thalassaemia and retention time and area percentage of other peaks and windows for structural variants. Each chromatogram shows peaks of HbA_0, A_2, and HbF along with C window, D window, S window, and two minor peaks, P2 and P3.

Several haemoglobin variants elute same window; they were provisionally diagnosed by ethnicity of the patients.

Other relevant tests are done when required. As for example, sickling test as supporting evidence of HbS. Family study is carried out whenever possible and correlation

Table 16.1: Interpretation of different chromatogram regions and peaks in HPLC chromatography windows (retention time ranges collected from Bio-Rad kit)

Region/Window	Retention time	Interpretation
Pre-integration region	<1 minute	Bilirubin, HbH, Hb Bart, modified HbF
P1 peak	0.63–0.85	A miniscule peak usually found in specimens with increased HbF
F window	0.98–1.22	HbF, Hb Okayama
P2 window	1.28–1.50	Glycated HbA or HbA1C
P3 window	1.50–1.90	Aged samples, HbJ-Meerut
A window	1.90–3.10	HbA, glycated HbS, intact Hb-Koln
A2 window	3.30–3.90	HbE, HbD-Iran, Hb-Lepore, HbG-Koushatta, Hb-Zurich, Hb-Korle Bu
D window	3.90–4.30	HbD-Punjab, HbG-Philadelphia
S window	4.30–4.70	HbS, HbQ-Thailand, Hb-Manitoba
C window	4.90–5.30	HbC, Hb constant spring, Hb-Agenogi
Further unknown peaks	>5.30	HbQ-India

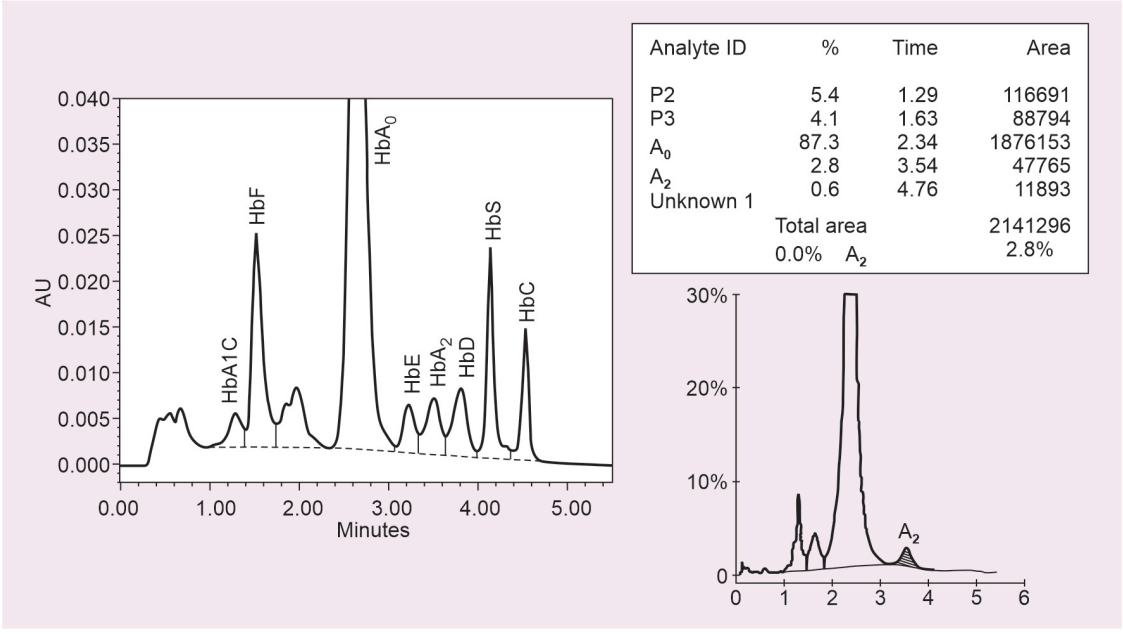


Fig. 16.11: Interpretation of HPLC report

with findings of Hb electrophoresis result is done in few cases (Table 16.1).

Table 16.2: Genotypes of some common structural variants

Abnormal variants	Genotype
HbE	Beta 26 (B8) Glu → Lys, GAG → AAG
HbD-Punjab	Beta 121 (GH4) Glu → Gln, GAA → CAA
HbS	Beta 6 (A3) Glu → Val, GAG → GTG
HbD-Iran	Beta 22 Glu → Gln, GAA → CAA
Hb-Hope	Beta 136 (H14) Gly → Asp (GGT → GAT)
HbO-Arab	Beta 121 (GH4) Glu → Lys, (GAA → AAA)
HbC	Beta 6 (A3) Glu → Lys, GAG → AAG
HbJ-Meerut	Alpha 120 (H3) Ala → Glu, GCG → GAG
HbQ-India	Alpha 64 (E13) Asp → His, GAC → CAC

Materials and Methods of HPLC (Bio-Rad Variant II)

Samples are drawn into tubes containing dipotassium EDTA (Becton Dickinson Vacutainer systems). All Samples are analyzed on the Bio-Rad Variant II HPLC system with use of the Variant II β-Thalassaemia Short Program Reorder Pack (Bio-Rad Laboratories, USA) as described in the instruction manual for the assay. Briefly, in this system the samples are mixed by the Variant II Sampling Station, diluted with the specific hemolyzing/wash buffer, and injected into an assay-specific analytic cartridge. The Variant II dual pumps deliver a programmed buffer gradient of increasing ionic strength to the cartridge, where the haemoglobin fractions are separated based on their ionic interaction with the cartridge material. The separated haemoglobin fractions pass through a flow cell, where absorbance is measured at 415 nm; background noise is reduced with the use of a secondary wavelength at 690 nm. The raw data are integrated by the **Clinical Data Management (CDM)** software of Bio-Rad laboratories, and a chromatogram/sample report is generated. The integrated peaks are assigned to manufacturer-defined windows derived from the retention time, i.e. the time in minutes from sample injection to the maximum point of the elution peak, of normal haemoglobin and common variants. If a peak elutes at a retention time not predefined, it is labeled as unknown peak.

Evaluation of Retention Times

The CVs (coefficient of variation) or imprecision of the retention times of HbF, HbA, HbA_2 and each of the 15 variants seen on three or more occasions are calculated. Hb-New York is not included in these calculations because it coelutes with HbA. It is presumed that normalisation of the retention time of the haemoglobin variant to that of either HbA or HbA_2 at the time of assay may minimise the imprecision of the assay attributable to changes in lots of reagents and columns.

The CVs of the retention time normalised to the retention time for HbA or the retention time for HbA_2 at each occurrence for each of the 15 variants are calculated. For each incident of six variants with an retention time in the HbA_2 window, the mean retention time for HbA_2 in the preceding five samples and the following five samples is determined and is used in the calculation.

The mean (SD or standard deviation) of the CVs for the retention time, the retention time normalized to the retention time for HbA, and the retention time normalized to the retention time for HbA_2 are calculated. There is no statistical difference in imprecision between the mean (SD) CV for the retention time [1.0 (0.7) %] and the mean CV for the retention time normalized to the retention time for HbA [1.3(0.5) %; P = 0·10] or normalized to the retention time for HbA_2 [1.0 (0.4) %, P =0.51]. For this reason, subsequent analysis of the data is done with only the retention time.

The SD for the retention times of HbF, HbA, HbA_2 and the 15 variants seen on 3 or more occasions do not correlate with either the retention time (p = 0.889) or the % Hb (p = 0·228), demonstrating that the SD is independent of those two variables. The SD, the measure of the variation around the mean for the retention time, was therefore used to predict the statistical difference of the retention time of a variant seen fewer than 3 times from that of another variant. The mean (SD) for the individual SDs of the retention times observed in this different haemoglobin was 0.026 (0.016) min. A difference in the retention time of two haemoglobin variants greater than the mean of the individual SDs + 2 SD [0.026+ (2 × 0.016) = 0.058] is considered significant.

Haemoglobin Variants with Retention Times <0·63 min

The CDM (Clinical Data Management) software does not integrate elution peaks which occur at <0.63 minute. The tetramers Hb Bart (α4) and HbH (α4) and HbF1 (the acetylated form of HbF), all elute before chromatogram integration. So, they are not indicated on the chromatogram report. The elution peaks are detected by visual analysis of the chromatogram. Hb Bart is seen in newborns at risk for at least two gene deletions of the α-globin.

Table 16.3: Manufacturer-assigned windows for Bio-Rad Variant II HPLC system

Peak name	Retention time, min
P1 window	0.63–0.85
F window	0.98–1.20
P2 window	1.24–1.40
A_0 window	1.40–1.90
A_2 window	3.30–3.90
D window	3.90–4.30
S window	4.30–4.70
C window	4.90–5.30

Haemoglobin variants with retention times in the P1 window (0.63–0.85 min)

No Hb variant is usually found in this window. A miniscule peak may be found in specimens with increased HbF.

Haemoglobin variants with retention times in the F window (0.98–1.20 min)

At least seven haemoglobin variants (4β and 3α-variants) are expected to elute in this window, all in quantities >10%.

Haemoglobin variants with retention times in the P2 window (1.24–1.40 min)

HbA_1C elutes in the P2 window. When the elution peak is >7% of the total haemoglobin, the patient records are checked for indication of diabetes and HbA1C quantification. If the % Hb in the P2 window and HbA1C values are concordant, i.e. within 15% of each other, no further studies are performed. The only haemoglobin variant which elutes in this window is Hb Hope, which has a mean (SD) % Hb [45.9 (2.2) %] much greater than would be expected for HbA1C.

Haemoglobin variants with retention times in the P3 window (1.40–1.90 min)

Nine haemoglobin variants (4α and 5β variants) have elution peaks in the P3 window. It is predicted that Hb-Camden (d = 0.10 from Hb Hope) and HbJ-Oxform (d = 0·11 min from Hb-Camden) can be differentiated and identified based solely on their retention times. Hb-Austin, Hb-Baltimore, and Hb-Fukuyama cannot be differentiated from each other by their respective retention times, in that case Hb electrophoresis is required. The % Hb is sufficient. However, to distinguish these variants from Hb-Fannin-Lubbock, HbJ-Anatolia, and HbJ-Mexico. To distinguish these three latter variants, however, retention times and electrophoresis were required. It is presumed that HbJ-Meerut can be differentiated and identified based solely on its retention time (d = 0.12 min from HbJ-Mexico).

Haemoglobin variants with retention time in the A window (1.90–3.10 min)

Six haemoglobin variants (2α and 4β variants) have elution peaks in the A window. HbJ-Toronto (d = 0.06 min from HbJ-Meerut), HbJ- Bangkok (d = 0·08 min from HbJ-Toronto) and Hb Ty Gard (d = 0.18 min from HbJ-Bangkok) can be differentiated and identified solely by their retention times. Hb-Köln (d=0:06 min from Hb Ty Gard), an unstable haemoglobin, can be identified by its retention time alone with its characteristic chromatogram. In addition to the elution peak for the intact haemoglobin at retention time 2.26 min, there is a secondary peak at retention time ~4.90 min representing the denatured Hb-Köln. Hb Ty Gard and Hb Twin Peaks do not separate from HbA_0 on haemoglobin electrophoresis and are detected only by HPLC. Hb Ty Gard appears to have a unique retention time, whereas Hb Twin Peaks has a characteristic chromatogram in which there is a hump on the downward slope of the HbA elution peak. Hb-New York appears to have a retention time identical to that of HbA. Alkaline electrophoresis is required to detect this β-variant; it moves more anodal than HbA under these conditions.

Haemoglobin variants with retention times in the A_2 window (3.30–3.90 min)

Five haemoglobin variants (one δβ-hydride, one α- and three β-variants) have elution peaks in the A_2 window. Hb-Lepore can be differentiated identified based solely on its on-retention time. The % Hb and the characteristic hump on the downward slope of the elution peak are additional distinguishing features of Hb-Lepore. The retention time for HbD-Iran appears to be significantly different from those of both Hb Lepore (d = 0.12 min) and HbA_2 (d = 0.14 min). In addition, The % Hb of HbD-Iran (47.7%) is significantly greater than either of these variants [Hb-Lepore, 12.1(1.5) %; HbA_2, 3.63 (0.04%]. The retention times and % Hb for HbA_2 and HbE are significantly different (p = 0.001 for both). The retention time for HbOsu-Christiansborg appears to be significantly different from that for HbE (d = 0.08 min) in addition to the % Hb [44.0% vs. 30.3 (4.0) %, respectively]. HbG-Honolulu appears to have a retention time (d = 0.09 min) and % Hb significantly different from those of Hbosu-Christiansborg (27.4% vs 44.0% respectively). Although Hb-Honolulu and Hb-Korle-Bu appear to have significantly different retention times (d = 0.06 min) the lower % Hb [27.4% vs. 46.5 (3.7) %, respectively] and characteristic chromatogram of HbG-Honolulu allows further differentiation of the two variants. HbG-Honolulu, an α-variant, shows the presence of the characteristic minor HbA_2 variant peak ($\alpha 2^{G\text{-}Honolulu}$ δ2) immediately after the variation peak, which is missing in HbOsu-Christiansborg, a β-variant.

Haemoglobin variants with retention times in the D window (3.90–4.30 min)

Three haemoglobin variants have elution peaks in the D window, all of which are β variants. The retention times, along with the % Hb are statistically different from Hb-Korle-Bu vs. HbD-Punjab (p <0.001, respectively) and Hb-Korle-Bu vs. HbG-Philadelphia (p <0.001, respectively). Although the retention times for HbD-Punjab and HbG-Philadelphia are statistically significant (p = 0.015), there is no statistically difference in % Hb (p = 0.21). The mean HbA_2 values for HbD-Punjab trait [1.4 (0.4) %] and HbG-Philadelphia trait [1.3 (0.04) %] are significantly lower (p <0.001, respectively) than the range for HbA_2 in the normal specimens. The chromatogram for HbG Philadelphia, an α-variant shows the presence of the characteristic minor HbA_2 variant peak ($\alpha 2^{G\text{-}Philadelphia}$ δ2) in all heterozygous cases. Because the range of retention times for Hb-Korle-Bu straddled both the A_2 and D windows, one-half of the specimens seen in a large series fell in the A_2 window although the meant retention time is in the D window.

Haemoglobin variants with retention times in the S window (4.30–4.70 min)

Six haemoglobin variants (three α, two β, and one δ-variant) have elution peaks in the S window. HbE-Seskatoon and HbS appear to have significantly different retention times (d = 0.18 min). Although, Hb-Manitoba, Hb-Montgomery, and HbA_2' all appear to have identical retention times, their retention times and % Hb are statically different from those of HbS ($p < 0.001$ for all). The % Hb values for Hb Manitoba and Hb-Montgomery do not appear to be statistically different [16.5%, vs. 15.7(2.2) %, respectively]. However, the % Hb for HbA_2' is statistically different from these two variants [1.2 (0.1)%; $p < 0.001$]. The retention time for HoQ-Thailand appears to be different from the retention times of Hb-Manitoba, Hb-Montgomery, and HbA_2 (d = 0.09, 0.09 and 0.08 min, respectively). In patients with heterozygous for HbA_2', the % Hb for HbA_2 [1.64 (0.17) %] is significantly lower $p < 0.001$ than the value for normal specimens.

Haemoglobin variants with retention times in the unknown window (4.70–4.90 min)

The elution peak for Hb-Hasharon, an α-variant fall in the time interval for unknowns. Although its retention time appears to be significantly different from that of HbQ-Thailand (d = 0.16 min), the retention time and the % Hb are statistically different ($p < 0.001$ for both) from those for HbO-Arab. This variant also has a characteristic chromatogram. In addition to the expected HbA_2 variant peak ($\alpha 2^{Hasharon}\delta 2$), immediately after the variant peak there are two small peaks in all examples of Hb-Hasharon. One minor peak appears at a retention time of ~4.27 min, presumably glycated or degraded Hb, and another appears immediately preceding the election peak.

Haemoglobin variants with retention times in the C window (4.90–5.30 min)

Three haemoglobin variants (three β variants) have elution peaks in the C window. HbO-Arab and HbC have statistically different retention times ($p < 0.001$), whereas the % Hb values are not statistically different ($p = 0.84$). In addition, all examples of HbO-Arab have a minor peak in the D window, which is not seen in the HbC trait. The retention time for HbG-Siriraj appears to be statistically different from that of HbO-Arab (d = 0:17 min) and that of HbC (d = 0.10 min). HbG-Sriraj, a β-variant, is reported to be 33–40% of the total haemoglobin in heterozygotes. This patient appears to have a concomitant α-thalassaemia, which would account for lower % Hb for the variant.

Haemoglobin detection and assignment of windows using Bio-Rad Variant II (β-Thalassaemia Short Programme)

The eluted haemoglobin fractions are detected by a flow-cell type photometer which records changes in the absorbance at 415 nm (haemoglobin) and 690 nm (background) on an integrating computer system. A chromatogram is generated displaying time on the X-axis and percentage on Y-axis. The area under the absorption peak approximates the percentage of the fraction detected, and each faction is assigned a window (i.e. range of retention times). The software control for overlapping/merging peaks by dropping vertical axes at the troughs. The report prepared incorporates numerical as well as graphical data, and their analysis is discussed next. Bio-Rad Variant II Turbo output (Bio-Rad Laboratories, Hercules, United states) using the β-Thalassaemia Short Programme for illustration. However, the principles remain similar even on other systems.

Methodological Considerations during Reporting

During reporting, attention must be given to the following areas:

- **Total area of analysis:** This must lie between 1 and 3 million μ volt-seconds. Specimens with lower areas (due to anaemia) an increased values (due to polycythaemia) must be reanalysed after

appropriate manual concentration by removing plasma or dilution by removing red cells respectively.

- **Cases with total area >100%:** The total area is the sum of all individual peaks' areas and can therefore, in the presence of overlapping peaks, can cross 100%. This is especially common in patients with beta-thalassaemia major, where the HbF peak overlaps and usually obliterates P2 and P3. In such situations, the percentages can be calculated manually, by taking the area of the peak of interest and dividing it by total area to get the proportion.
- **Small unknown peaks:** One or more unknown peaks often occur around P2/P3 window (retention timer 1.3–1.8). These may be safely ignored if 1% of total area. Unknown peaks at longer retention times and those >1% should be given greater attention as transfusion-transmitted peaks. HbA_2 may present as small peaks. One may also review the sample pun previously as carryover peaks are also usually small.
- **Misidentified haemoglobin fractions**
  In rare cases, with very large abnormal peaks, the entire fraction may be misassigned to either another category on as an unknown peak. This was commoner in older generation analyzers (like the variant), but can still occur with broad-based HbD and HbF peaks. Alternative techniques are then required to establish the identity of abnormal peak.
- **Pre-integration peaks:** These peaks, with retention time <1 min are not reflected in the tabular data and need to be looked for on the chromatogram. The causes of such peaks include HbH, Hb Bart, bilirubin and acetylated HbF. The clinical background and other HPLC findings usually indicate their nature, if found. In addition, bilirubin peaks are usually early, very sharp and thin, HbH peaks are usually dual, and of low to moderate height, while post-transnationally modified F are usually multiple with their height proportionate to the HbF%. If required, the software settings may be readjusted manually to include such peaks. This may be especially useful in cases with HbH disease.

P2 and P3 Peaks

These represent post-transnationally modified adult haemoglobin (HbA_0 or HbA) and show normal ranges of 3.8 ± 0.7 and 4.3 0.4 respectively (low and high normal values). P2 is comprised of glycated haemoglobin and levels 6.5% should be mentioned in the report with the suggestion to exclude diabetes mellitus. Low P2 levels are seen in cases with reticulocytosis. Elevated P3 may indicate HbJ-Meerut (an α-globin chain variant). This is also elevated in cases with the HbE variant (the elutes in the HbA_2 window) and in aged (old) specimens. Incidentally, the P1 peak is virtually always absent in normal specimens.

In conclusion, although CE-HPLC is a rapid, convenient and reliable investigation for haemoglobin disorders, it involves several methodological issues and nuances. Reporting pathologists must be aware those to extract maximum information from the technology of HPLC.

SCREENING OF THALASSAEMIA

Complete Blood Count

For screening of thalassaemia, mean, corpuscular volume (MCV) of <80 fl and/or mean corpuscular haemoglobin (MCH) value <27 pg can be generally used as cutoff levels for a positive screening result. These cutoff levels are derived from 2 standard deviations of the normal distribution of MCV and MCH from the normal population. The advantage of the thalassaemia screening by MCV and MCH is achievement of rapid, cost

effective, reproducible and accurate from good automated hematology analysers. But microcytic anaemias such as iron deficiency anaemia (IDA) will also have low MCV. Besides, a low MCV is not suitable for screening HbE carriers and the patients with single α-globin gene deletion (-$\alpha^{3.7}$ and -$\alpha^{4.2}$) or non-deletional α-globin gene mutations [i.e. Hb constant spring (Hb CS) and Hb Quong Sze]. Moreover, the interaction of heterozygous β-thalassaemia with α-thalassaemia trait alone or with glucose 6-phosphate dehydrogenase deficiency may lead to normal MCV and a false negative result during thalassaemia screening. Therefore, rather than using MCV only, it would be more rational to use both MCV and MCH.

The red cell distribution width (RDW) is a measure of degree of variations in red cell size. In case of iron deficiency anaemia, RDW is high (>14.5), whereas in most cases of thalassaemia it is usually normal (11.5–14.5). Although thalassaemia produces uniform microcytic red cells without a concomitant increase in RDW, this observation is variable among the thalassaemia syndromes and notable increase in RDW may be seen in HbH disease and β-thalassaemia minor.

Electrophoresis in Haemoglobinopathy

Electrophoresis is a technique used to separate molecules or compounds based on their migration pattern in a gel and electric field. It is still widely used in clinical laboratories for protein electrophoresis and differentiation of some enzymes. Manual preparation of gel and electrophoresis is rarely used in developed countries as more advanced and automated techniques like capillary electrophoresis are available.

Cellulose acetate electrophoresis is a representative custom electrophoresis technique. It is known to enable identification of HbA, F, S/G/D, C/E, and H and other variants. Automated, capillary electrophoresis is widely used and showed benefits in the identification of some variants indistinguishable in many automated HPLC systems.

Mass Spectrometry

Mass spectrometry is a technique to identity molecules based on their mass (molecular weight) to charge ratio. The unique advantage of the technique is that it uses minimal specific binding reagent for the molecules of interest. The simple analytical principle enables less interference and more accurate identification. The analysis of haemoglobin with mass spectrometry is not simple because the laboratory should have both technical expertise for analysis of proteins and very expensive instrumental. Besides identification of haemoglobin based on intact molecule's molecular weight, it can also analyze the sequence of amino acids in some degree. This is useful for the identification of new variants and confirmation of DNA sequencing (Fig. 16.12).

Prenatal Diagnosis for Haemoglobinopathy

To date, the prenatal diagnosis of thalassaemia and haemoglobinopathy represent one of the most frequent genetic analyses performed worldwide. The purpose of prenatal diagnosis is to identify and counsel asymptomatic individuals whose offspring at risk of an inherited haemoglobinopathy and to monitor the pregnancy for complications.

The clinical types of targets haemoglobinopathies which are targets of prenatal diagnosis are associated with potentially severe sequelae and intervene, such as sickle cell disease, β-thalassaemia major resulting from homozygosis of β-thalassaemia, and haemoglobin Bart's nonimmune hydrops foetalis caused by deletion or dysfunction of all 4α-globin genes.

The prenatal diagnosis involves the study of foetal material from chorionic villi, amniotic fluid, umbilical cord blood and foetal DNA in maternal circulation. Although analysis of

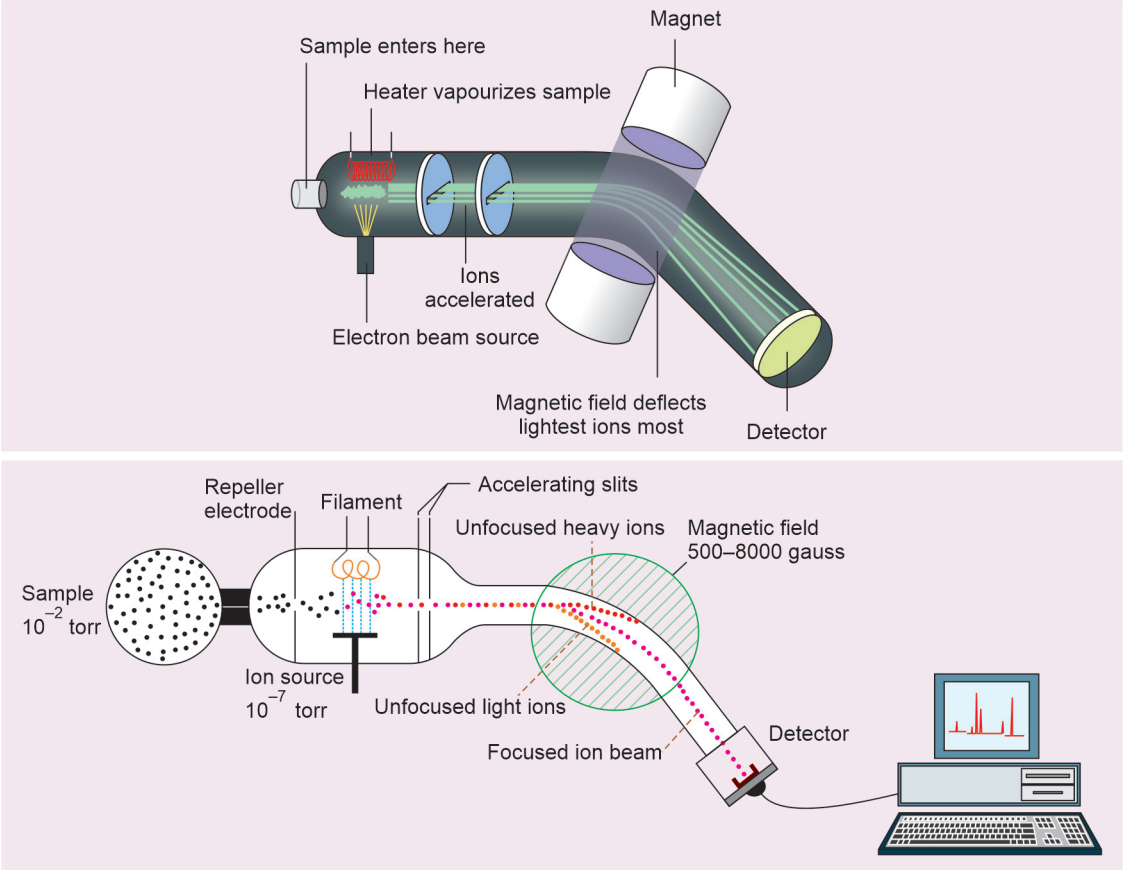


Fig. 16.12: Principle of mass spectrometry

foetal haemoglobin types is successfully performed by automated HPLC, but it is assessable through analysis of foetal blood obtained by cordocentesis and the procedure is prone to errors due to contamination by maternal tissue.

The recent development of noninvasive prenatal diagnostic testing using cell-free foetal DNA from maternal plasma allows active investigation of genetic analysis of foetus avoiding invasive procedure. Another advantage of this approach is that foetal DNA can be isolated from maternal blood earlier than using invasive procedures.

Various techniques have been applied to identify foetal haemoglobinopathies such as mass spectrometry, next generation sequencing and genotyping assay. These techniques are still developing and more studies are needed to develop and validate them to make them reliable noninvasive prenatal diagnosis of thalassaemia and haemoglobinopathies (Fig. 16.13).

Use of different analyzers and their performances (Table 16.4)

The Bio-Rad Variant II HPLC machine is very popular and widely accepted in laboratories worldwide. There are some other machines which are used to diagnose haemoglobinopathies. The role of four machines will be discussed briefly. These are: D-100 (Bio-Rad Laboratories, Hercules, CA, USA); HA

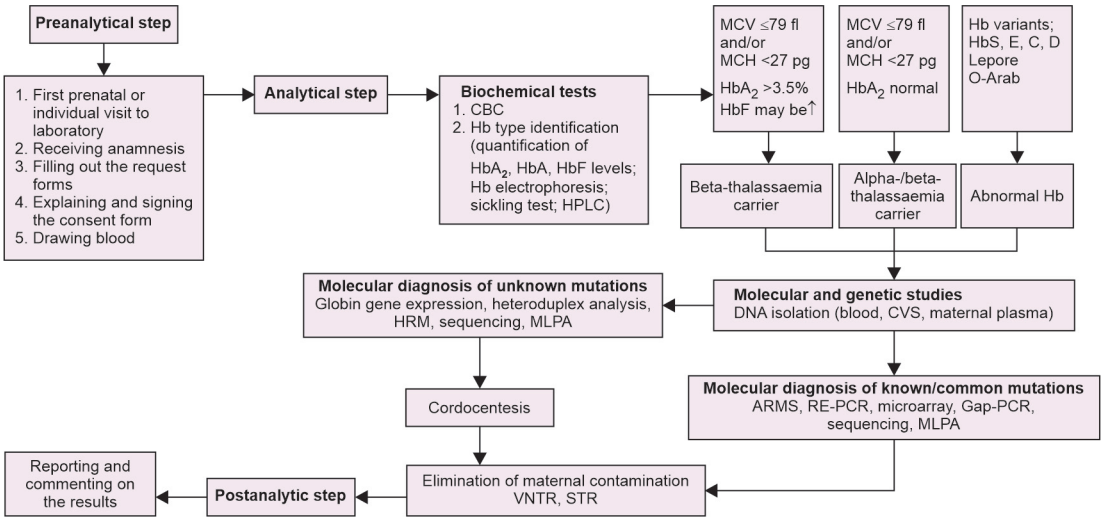


Fig. 16.13: Prenatal diagnosis of haemoglobinopathies and other genetic disorders

8180T (Menarini, Florence, Italy), HLC-723G8 (Tosoh Bioscience, Tokyo, Japan) and Capillaries 2 Flex piercing (Sebia, Lisses, France).

Table 16.4: Use of other four instruments in detecting haemoglobinopathy

Parameter	D-100	HA-8180T	HLC-723G8		Capillaries	
			HbA1C mode	Hb-pathy mode	HbA1C mode	Capillary electrophoresis
Measurement principle	HPLC	HPLC	HPLC	HPLC	Capillary electro-phoresis	Capillary electrophoresis
Throughput (test/ hour)	80	17	37	10	38	38
HbF estimation	Yes	Yes	Yes	Yes	Yes	Yes
HbA_2 quantification	No	Yes	No	Yes	Yes	Yes
Hb variant detection	Yes	Yes	Yes	Yes	Yes	Yes
Hb variant identification	Yes (S/C/E/D)	Yes (S/C/E/D)	Yes[a,b] (S/C/E/D)	Yes[a] (S/C/E/D)	No	Yes (S/C/E/D)
Hb variant quantification	Yes	Yes	No	Yes	Yes	Yes

[a]HbE co-eluates with HbA_2 which is indicated by an error flag. [b]Variants are identified as D, S and C but as HV0, HV1, and HV2 respectively.

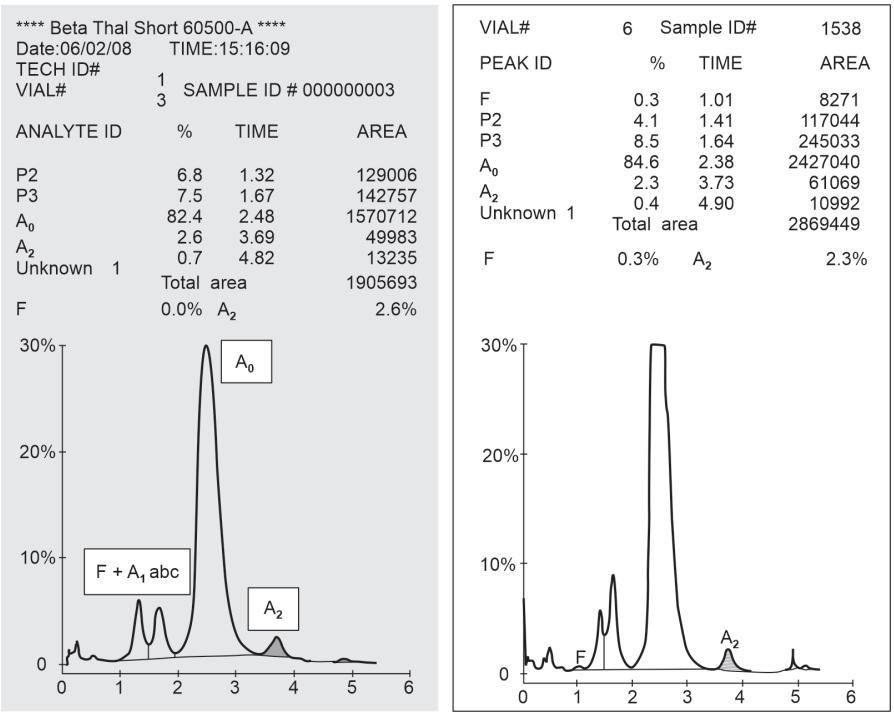


Fig. 16.14: Normal haemogolbin pattern in HPLC (cation exchange chromatography). HbA_2 is <3%, while adult Hb is maximum (>80%)

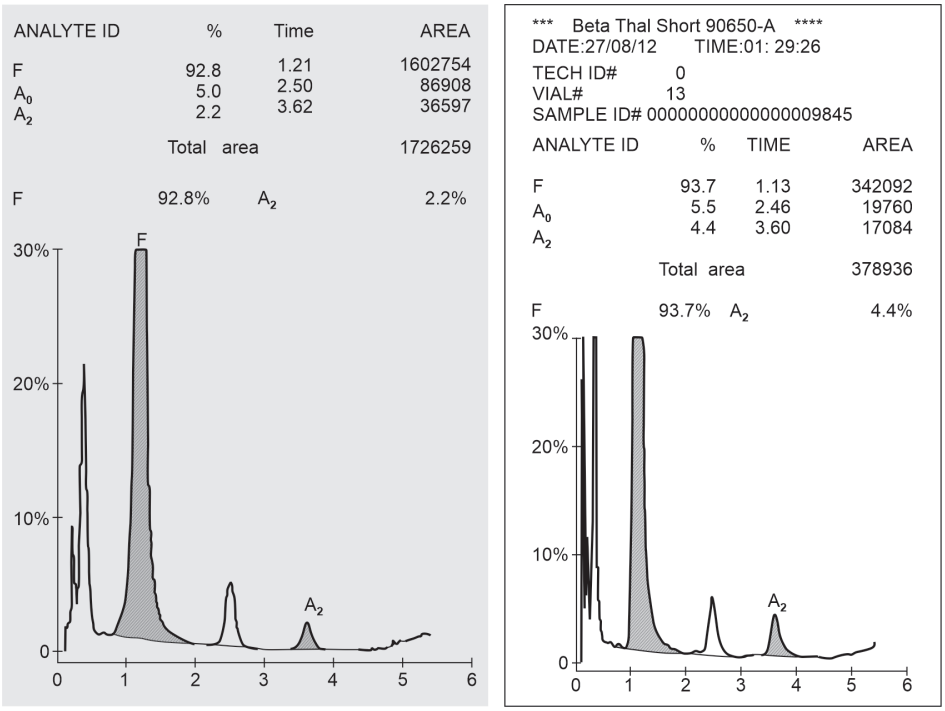


Fig. 16.15: HPLC showing β-thalassaemia major (HbF >90%)

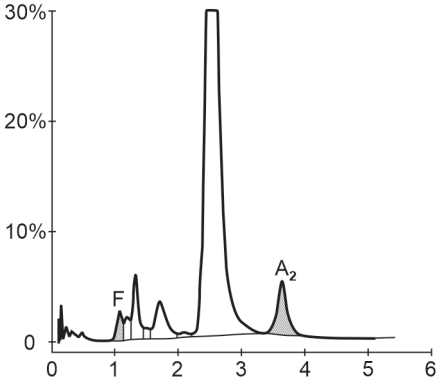
*** Beta Thal Short 90650-A ****
DATE:27/08/12 TIME:01: 49:22

TECH ID# 0
VIAL# 15
SAMPLE ID# 00000000000000009847

ANALYTE	ID	%	TIME	AREA
F		1.8	1.06	25807
unknown	1	1.4	1.18	19533
P2		4.0	1.32	57385
Unknown	2	0.7	1.48	9820
P3		4.6	1.70	66414
Unknown	3	0.5	2.07	6717
A_0		81.7	2.51	1175440
A_2		5.6	3.63	85690

Total area 1446806

F 1.8% A_2 5.6%



*** Beta Thal Short 60500-A ****
TEC ID# 1
VIAL# 14 SAMPLE ID# 000000059

ANALYTE	ID	%	TIME	AREA
P2		7.8	1.30	210069
P3		7.3	1.66	195716
A_0		78.6	2.44	2110738
A_2		5.9	3.70	159375
Unknown	1	0.4	4.82	10481

Total area 2686379

F 0.0% A_2 5.9%

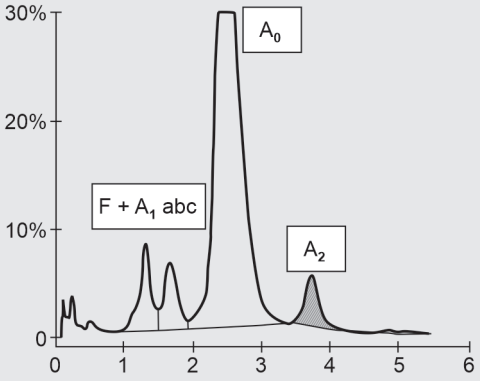


Fig. 16.16: Thalassaemia trait or thalassaemia minor (heterozygous). In the first case HbA_2 is 5.6% while in the second case HbA_2 is 5.9% (HbA_0 or adult haemoglobin, 81.7% and 78.6% respectively)

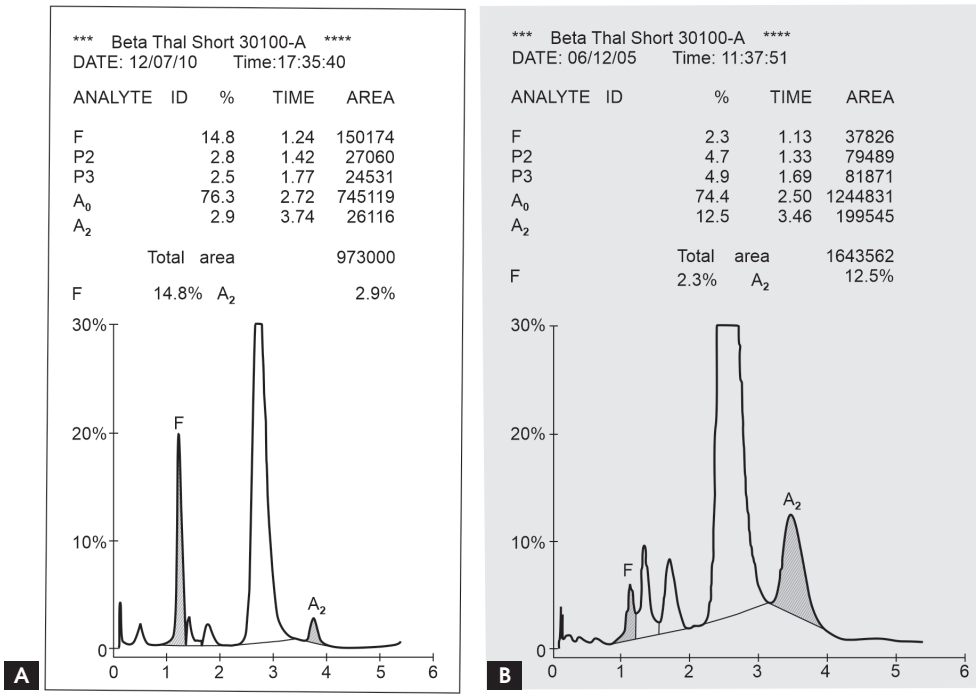


Fig. 16.17: (A) Delta-beta thalassaemia (δβ) with haemoglobin F level of 14.8%; (B) Hb-Lepore: HbA_2: 12.5% (RT-3.46'), HbF: 2.3%, HbA_0: 74.4%

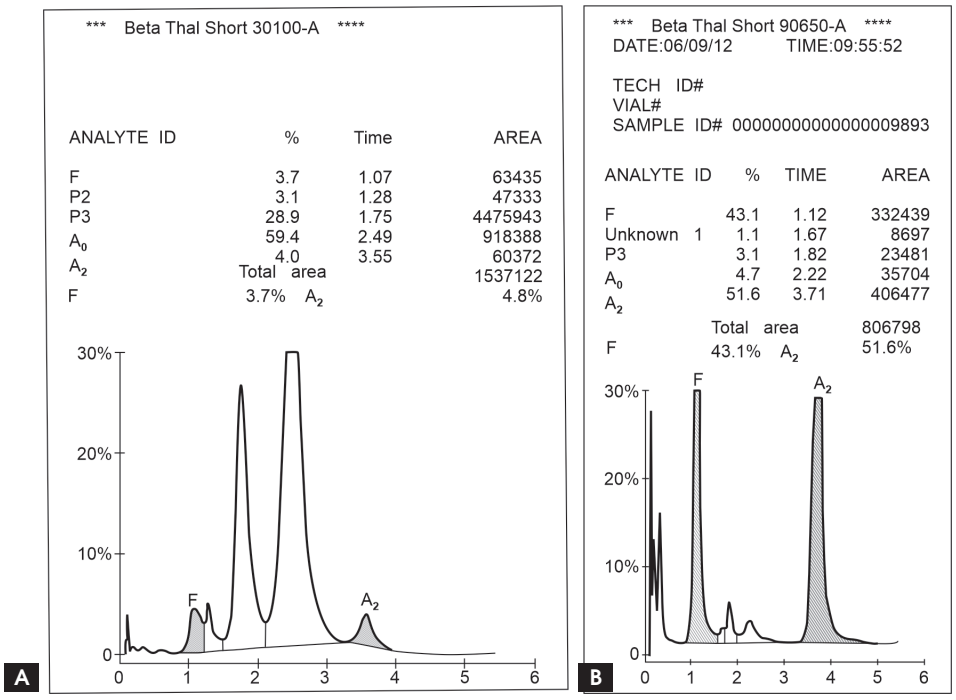


Fig. 16.18: (A) J chain haemoglobinopathy or HbJ-Meerut shows P3 peak 28.9% and A 59.4%.; (B) Haemoglobin E β-thalassaemia with A_2/E 51.6% and F 43.1%

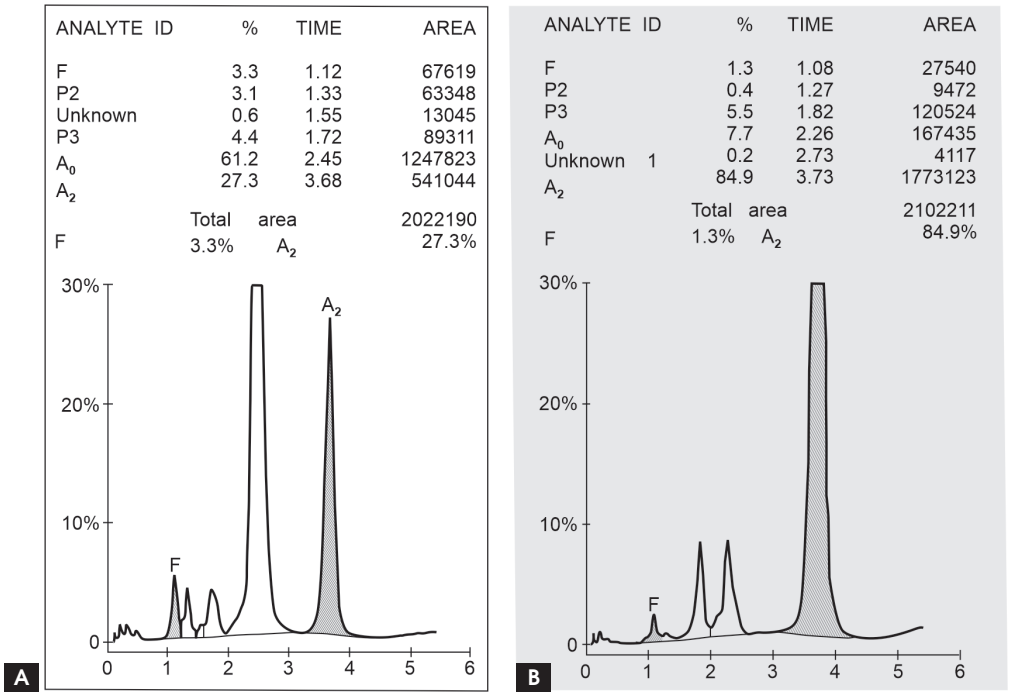


Fig. 16.19: (A) HbE trait; (B) HbE disease (HbA_2/E is high: in HbE trait 27.3% and in HbE 84.9% respectively)

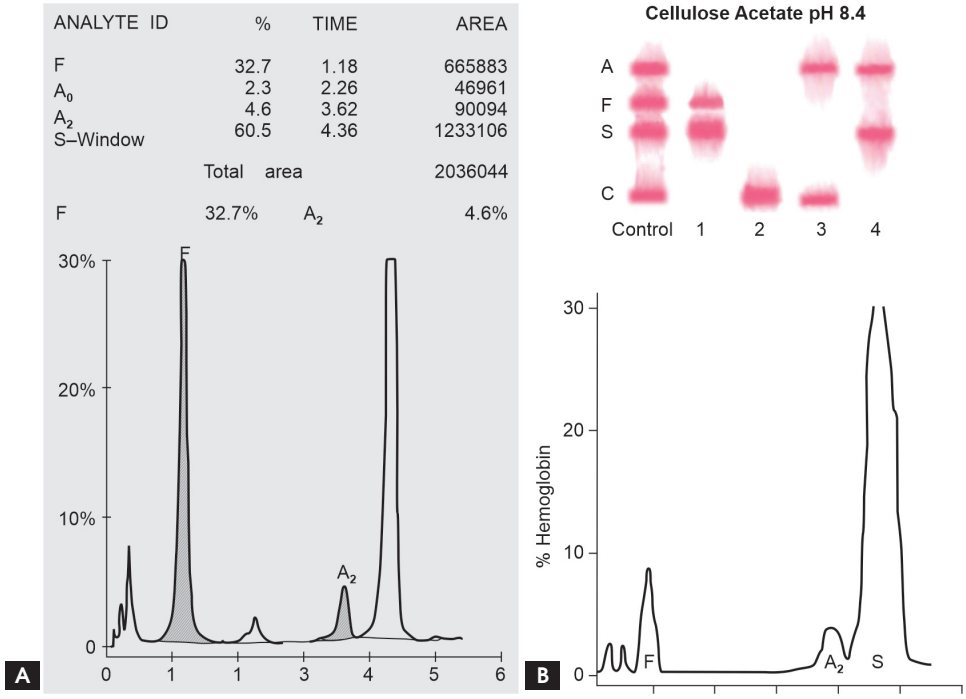


Fig. 16.20: (A) Double heterozygous HbS with β-thalassaemia (HbS 60.5% and HbF 32.7%); (B) Sickle cell anaemia (homozygous) where >90% HbS. Gel electrophoresis (cellulose acetate) shows a thick band of HbS in lane 1

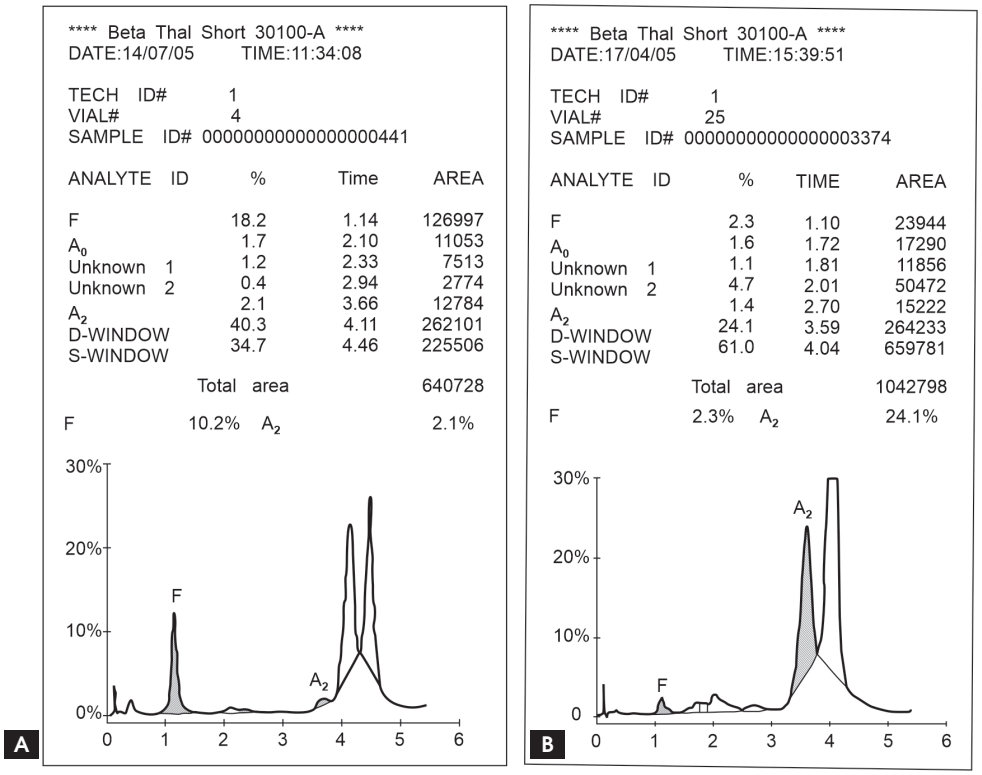


Fig. 16.21: (A) Haemoglobin (Hb) SD with HbS 34.7% and D 40.3%; (B) Haemoglobin (Hb) ED with HbE 24.1% and D 61.0%

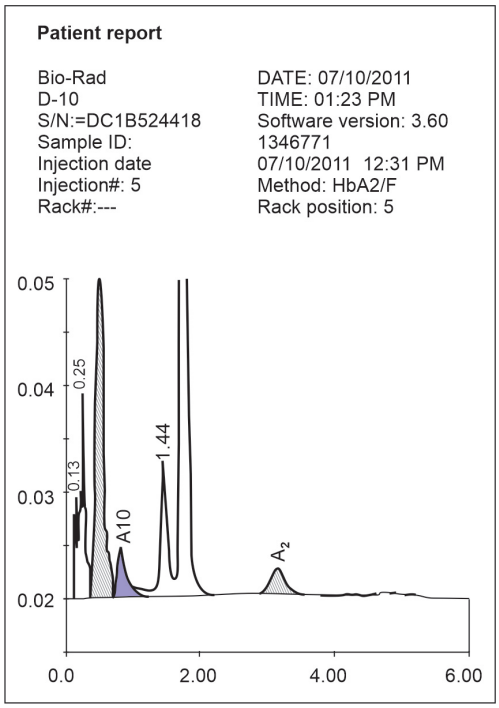


Fig. 16.22: Hereditary persistent of foetal haemoglobin (HPFH) in a 15-month old child (and persisted in adult life during follow up) who had elevated HbF of about 27% and normal HbA_2

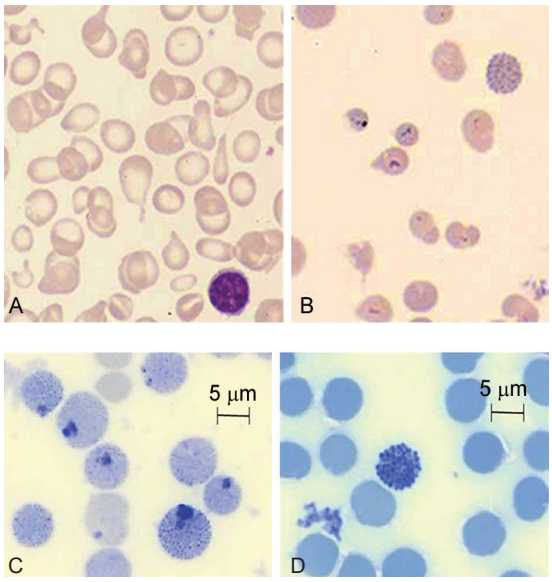


Fig. 16.23A to D: Blood smear and HbH (β4) in HbH disease. (A and B) Microscopic examination of the blood smear after staining with 1% brilliant cresyl blue at 1000 fold magnification; (C) Red cells of HbH disease patient showing inclusion body positive cells (1000 × magnification); (D) Red cells of α_0-thalassaemia carrier showing an occasional inclusion body positive cell after searching 10–20 fields (1000 × magnification)

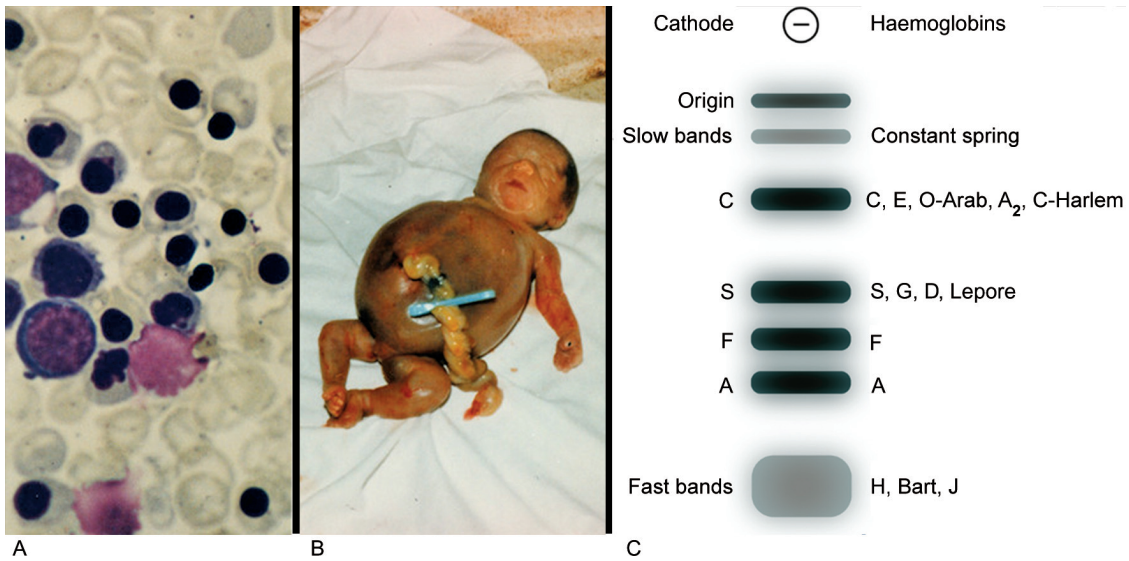


Fig. 16.24: (A) Peripheral blood smear (many nRBC or normoblasts) of hydrops foetalis (Hb Bart or γ4); (B) Clinical picture; (C) The mobility of haemoglobin variants on alkaline gel electrophoresis (cellulose acetate, pH 8.2–8.6) which helps to differentiate HbH and Hb Bart

Table 16.5: Classification of thalassaemia (major types)

Type	Haemoglobin	Genotype	Hb electrophoresis
β-Thalassaemia			
β-Thalassaemia major	<5 gm/dl	Homozygous β-thalassaemia (β^0/β^0, β^+/β^+, β^0/β^+)	HbA (0–50%), HbF (50–98%)
β-Thalassaemia	5–10 gm/dl	Variable; (β^0/β^+, β^+/β^+, β^0/β, β^+/β)	
β-Thalassaemia minor	10–12 gm/dl	Heterozygous β-thalassaemia; (β^0/β, β^+,β)	HbA_2 (4–9%), HbF (1–5%)
α-Thalassaemia			
Hydrops foetalis	3–10 gm/dl	Deletion of all four α genes (–/–,–/–)	Hb Bart or γ_4 (~100%)
HbH disease	2–12 gm/dl	Deletion of three α genes (–/–, –/α)	HbF (10%), HbH or β4 (2–4%)
α-Thalassaemia trait	10–14 gm/dl	Deletion of two α genes (–/α, –/α)	Almost normal, i.e. small amount of Hb Bart in neonatal
		black (–/–, α/α); Asian	period (5–6% in type 1 and 1–2% in type 2)
Silent carrier	12–14 gm/dl	Deletion of one α gene (–/α,α/α)	Almost normal

Table 16.6: Structural variants and mixed variants of thalassaemia

Type	Haemoglobin type diagnosis	Diagnosis	Gene type
Structural	HbS	HbS heterozygous	HbAS
variants		Sickle cell disease	HbSS
	HbC	HbC heterozygous	HbAC
		HbC disease	HbCC
	HbE	HbE heterozygous	HbAE
		HbE disease	HbEE
Mixed variants	β-Thalassaemia + HbS or HbE	Sickle cell β^+ thalassaemia	HbS β^+ thalassaemia
		Sickle cell β^0 thalassaemia	HbS β^0 thalassaemia
		HbE β^+ thalassaemia	HbE β^+ thalassaemia
		HbE β^0 thalassaemia	HbE β^0 thalassaemia
	HbS + HbC	HbSC	HbSC disease

Table 16.7: Different type of haemoglobin at birth and in adults

Haemoglobin	Structure	Level of birth	Level in adults	Comments
HbA	$\alpha_2\beta_2$	20–25%	97%	Reaches adult level by 1 year of age
HbA_2	$\alpha_2\delta_2$	0.50%	2.50%	Elevated in β-thalassaemia (minor)
HbF	$\alpha_2\gamma_2$	75–80%	<1%	Reaches adult level by 1 year of age; Elevated in β-Thalassaemia major
HbH	β4	15–20% in HbH disease	Not available	HbH produces Heinz bodies in erythrocytes and haemolysis
Hb Bart	γ4	100% in hydrops foetalis, 15–20% in HbH disease	Not available	Increased in carriers of α–thalassaemia trait at birth

Denaturing HPLC (DHPLC) (Fig. 16.25)

This is a recently described powerful technique for detection of mutations in DNA. This denaturing HPLC (DHPLC) is used for detection of single nucleotide substitutions or small insertions and deletions in both α and β globin genes. Small regions of the genes are amplified by PCR and analyzed using either partially or fully denaturing HPLC. The method is based on the differential retention time of homoduplexes and heteroduplexes in a mixture of denatured and reannealed PCR products. The eluted DNA is monitored using UV or fluorescent detectors. DHPLC has been shown to be a quick, sensitive and specific means of detecting mutations and polymorphisms. However, DNA sequencing may be required sometimes for a definitive characterization of the molecular defect (Table 16.8).

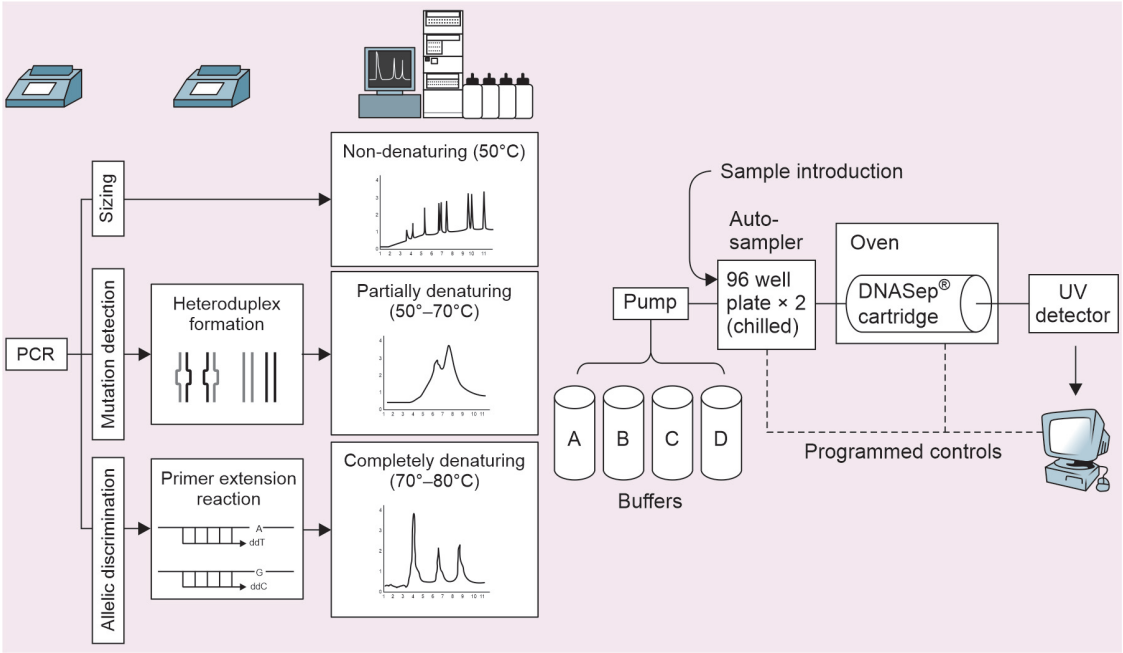


Fig. 16.25: Dentauring HPLC (DHPLC)

Table 16.8: Level of HbA_1, HbA_2 and HbF from birth to adult

Age	HbA_1%	HbA_2%	HbF%
0–1 month	17.7–54.0	0.0–1.3	46.0–81.0
2 months	37.1–70.36	0.4–1.9	29.0–61.0
3 months	41.0–84.0	1.0–3.0	15.0–56.0
4 months	68.2–88.6	2.0–2.8	9.4–29.0
5 months	74.9–95.6	2.1–3.1	2.3–22.0
6–8 months	83.5–95.8	1.9–3.5	2.3–13.0
9–12 months	91.7–96.7	2.0–3.3	1.3–5.0
13–24 months	94.5–98.2	1.6–3.5	0.2–2.0
25 months–adult	94.3–98.5	1.5–3.7	0.0–2.0

CAPILLARY ELECTROPHORESIS (CE)

The instrument, Capillary 2 Sebia, France, is very popular for capillary electrophoresis. It provides a more advantageous alternative for diagnosis of thalassaemia and haemoglobinopathies than HPLC. Its characteristics regarding precision, accuracy and cost are comparable to HPLC method. The main advantage of capillaries 2 system is the ability to separate and quantitate HbA_2, HbE, HbF, Hb-Lepore, HbH and Hb Bart. So, it can diagnose both α- and β-thalassaemias as well as other haemoglobinopathy. But Biorad Variant II can diagnose only β-thalassaemia and is unable to diagnose α-thalassaemia. Also, Biorad HPLC, machine cannot separate HbE and HbA_2 (elute in same region). In 2007, the CE system was approved by USA for evaluation of haemoglobinopathies.

Method in brief: The machine is equipped with 8 capillaries (so, 8 samples can be evaluated at a time). Manufacturer guidelines should be followed. Initial processing of the sample requires centrifugation of the EDTA whole blood at 5000 rpm for 5 minutes. Thereafter, the overlying plasma is removed and the erythrocyte pellet is vortexed for 5 seconds. Then it is added to lysis solution and an aliquot is introduced by aspiration at the anodic end into capillary tube before applying a voltage of 9,800V. It is run for 300 seconds (5 minutes) and detection of different haemoglobin is conducted by measuring absorbance at 415 nm at the cathodic end of the capillary tube. Separation is carried out in a microbore, fused-silica capillary, around 25–75 µm internal diameter. Electrophoretograms are recorded with the location of specific haemoglobin in specific zones. There are 15 zones (zone 1, zone 2 …… zone 15) in capillaries 2 system. The presence of adult haemoglobin or HbA is required for the appearance of the zone demarcations which help guide interpretation of structural variants. If HbA is not present, the test of the sample is repeated, being premixed with a 1:1 mixture of a normal control. Quantitative results for such a case are reported from the original sample alone and the 1.1 mixture is used for qualitative identification only. Manufacturer's recommended normal ranges for healthy adults are as follows: HbA 96.8% or more; HbF <0.5% and HbA_2 2.2–3.2% (Figs 16.26 and 16.27).

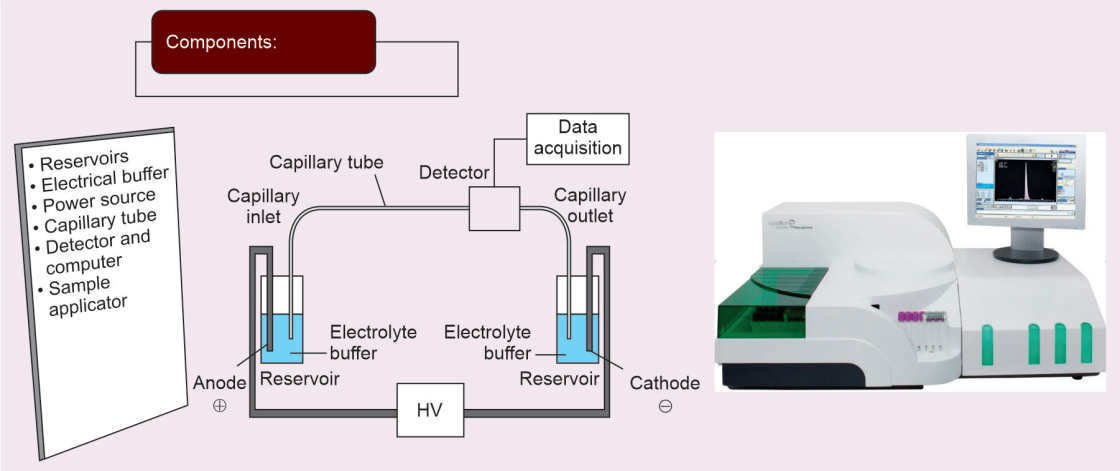


Fig. 16.26: Different components of capillary electrophoresis

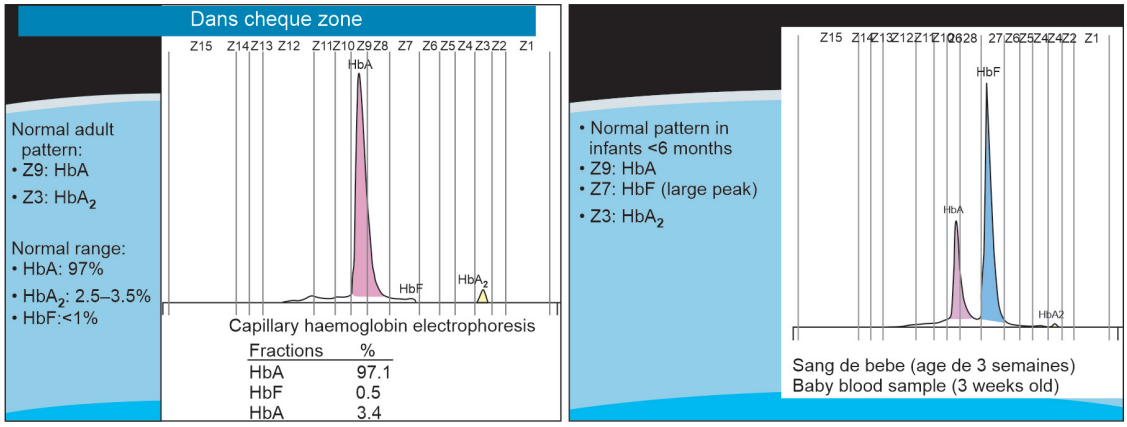


Fig. 16.27: Capillary electrophoresis in a normal adult (left side) and in an infant <6 months (right side)

Different Zones (Zone 1 to Zone 15) and Different Haemoglobins

Bracket indicates time in seconds

- **Zone 1 (260–290):** Hb delta A_2, Hb alpha A_2, HbA_2 variant
- **Zone 2 (250–260):** HbC, Hb constant spring, Setif HbA2 variant
- **Zone 3 (230–250):** HbA_2, HbO-Arab
- **Zone 4 (220–230):** HbE, Hb-Köln, denatured HbC
- **Zone 5 (210–220):** HbS, denatured HbO-Arab
- **Zone 6 (190–210):** HbD-Punjab, HbG-Philadelphia, HbOsu-Christiansborg, Hb D-Ouled Rabah, Hb Lapore, J-Toronto HbA_2 variant, J-Roviqo HbA_2 variant, denatured HbE.
- **Zone 7 (170–190):** HbF, HbO Thailand, Hb-Richmond, denatured HbS
- **Zone 8 (155–170):** Hb-Fac, Hb-Atlanta
- **Zone 9 (140–155):** HbA
- **Zone 10 (130–140):** Hb-Hope
- **Zone 11 (110–130):** Denatured HbA, HbJ-Kaohsiuna
- **Zone 12 (75–110):** Hb Bart, HbJ, HbJ-Mexico, HbJ-Baltimore
- **Zone 13 (64–75):** HbN-Baltimore, HbJ-Rovigo
- **Zone 15 (0–50):** HbH

Table 16.6: Different zones in capillary electrophoresis and important type of haemoglobins

Dans cheque zone: Potential variants located in each zone			
Zones	**Hb variant**	**Zone**	**Hb variant**
1	$HbdA_2$	9	HbA
2	HbC	10	HbM-Lwate, Hope
3	HbA_2	11	Denatured HbA, vassa, provience
4	HbE	12	Hb Bart
5	HbS	13	HbN-Baltimore
6	HbD-Punjab, Hb G-Norfolk	14	HbN-Seattle
7	HbF	15	HbH, I (I-Texas)
8	Hb-Lansing, Atlanta, Hinsdale		

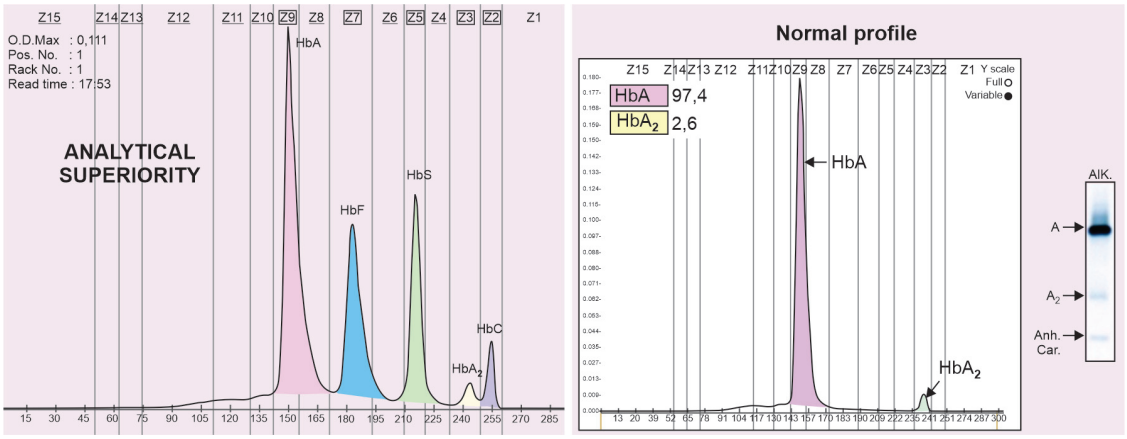


Fig. 16.28: Calibrators for capillary electrophoresis (CE) on left side. Normal pattern on right side

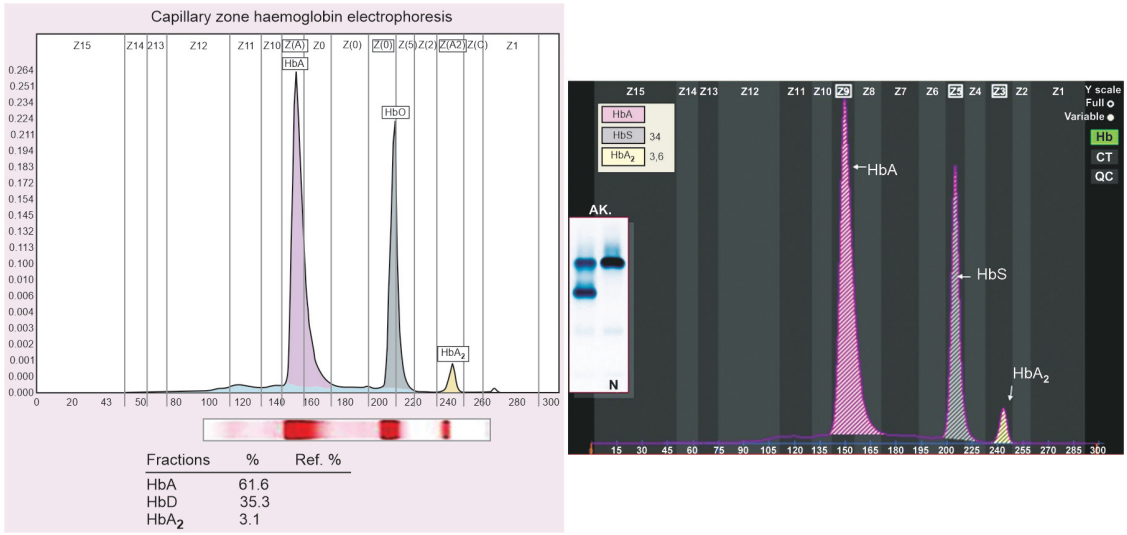


Fig. 16.29: CE shows HbD disease (left side). Heterozygous A/S disease (right side)

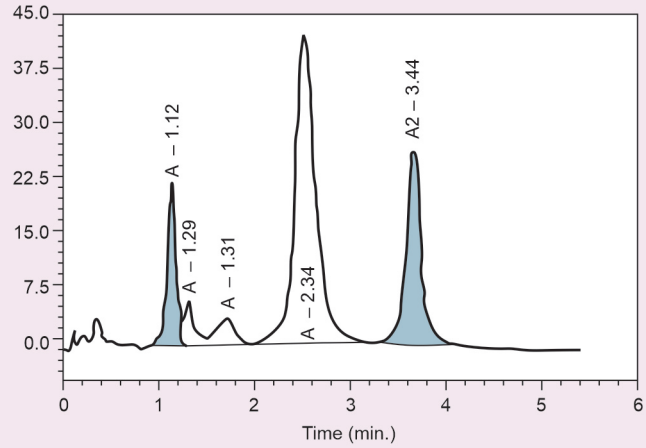
Peak name	Calibrated area 1	Area 1	Retention time (min)	Preak area
F	11.3*	---	1.17	151846
P2	---	3.3	1.29	51032
P3	---	4.0	1.70	55406
A_0	---	56.2	2.50	235792
A_2	26.2*	---	3.66	345620

Total area 1 1379455

F Concentration = 11.3*
A_2 Concentration = 26.1*

*Values outside of expected ranges

Analysis comments:



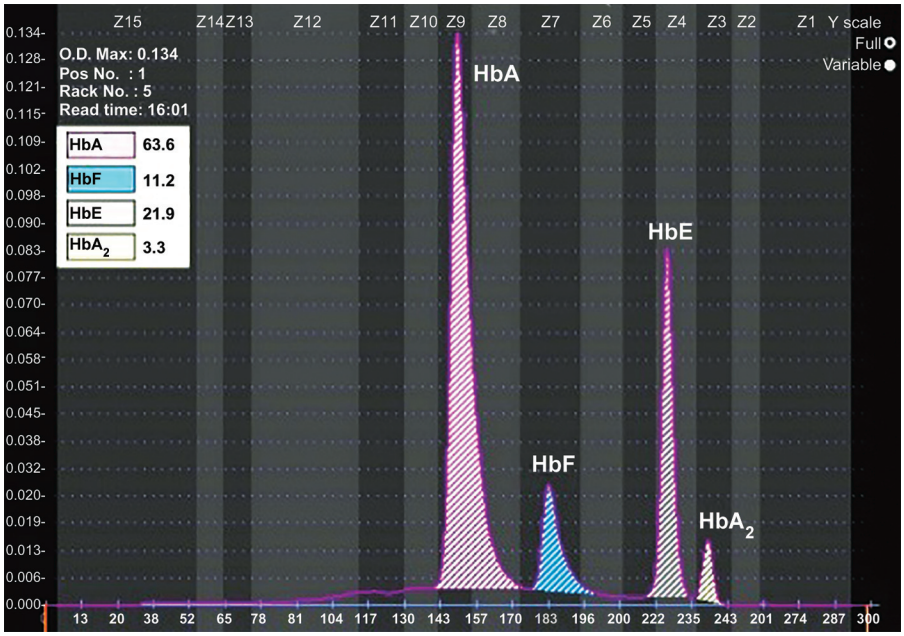
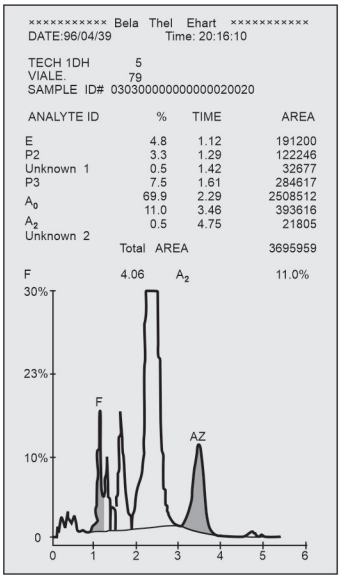
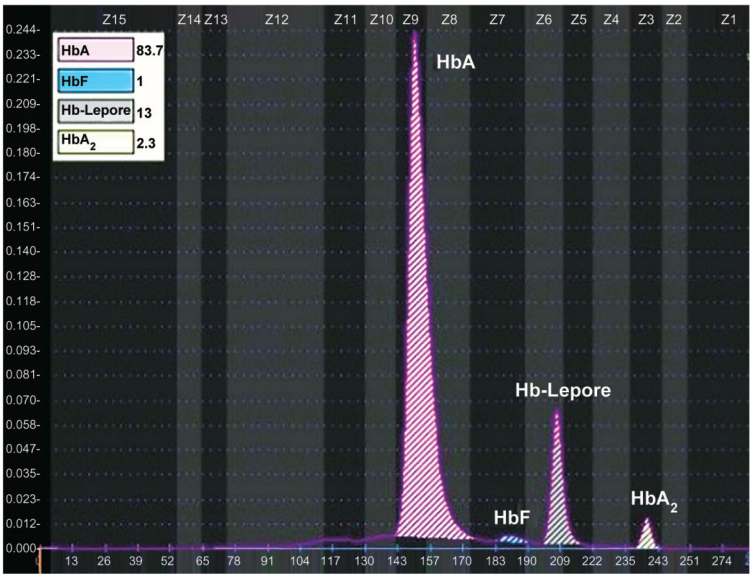


Fig. 16.30: HbE and HbA_2 comparison between HPLC and CE





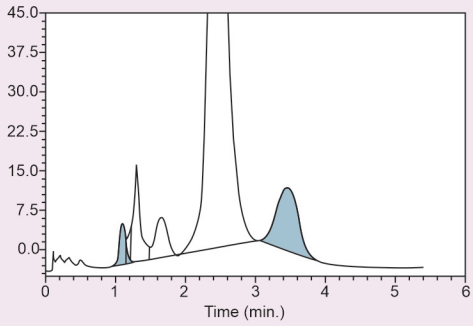
Peak name	Calibrated area	Area	Retention time (min)	Peak area
F	2.1*	---	1.09	48843
Unknown	---	1.1	1.22	24554
P2	---	5.0	1.29	116727
P3	---	3.0	1.65	88159
A_0	---	76.9	2.38	1790212
A_2	12.0*	---	3.44	259237

Total Area: 2, 327, 734

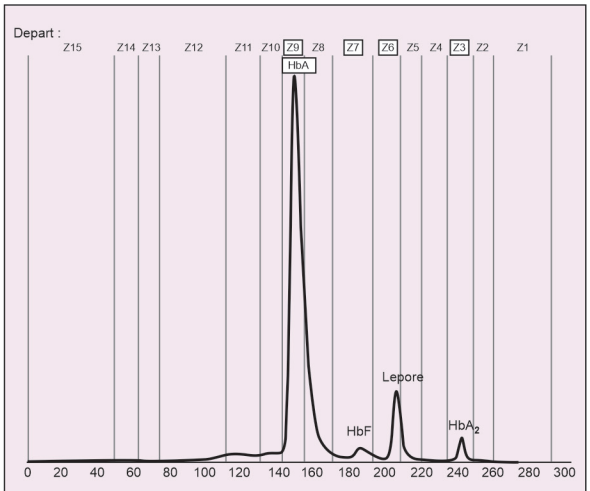
F Concentration = 2.1*
A_2 Concentration = 12.1*

* Values outside of expected ranges

Analysis comments:



Bio-Rad Variant II

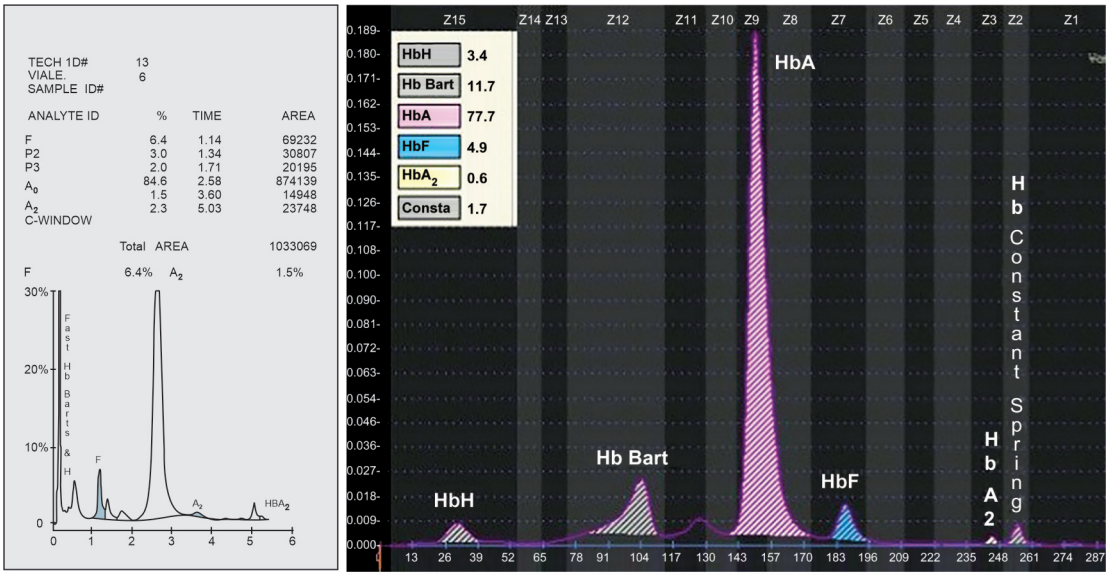


Haemoglobin Electrophoresis

Name	%	Normal values %
HbA	85.7	
HbF	2.4	
Lepore	9.5	
HbA_2	2.4	

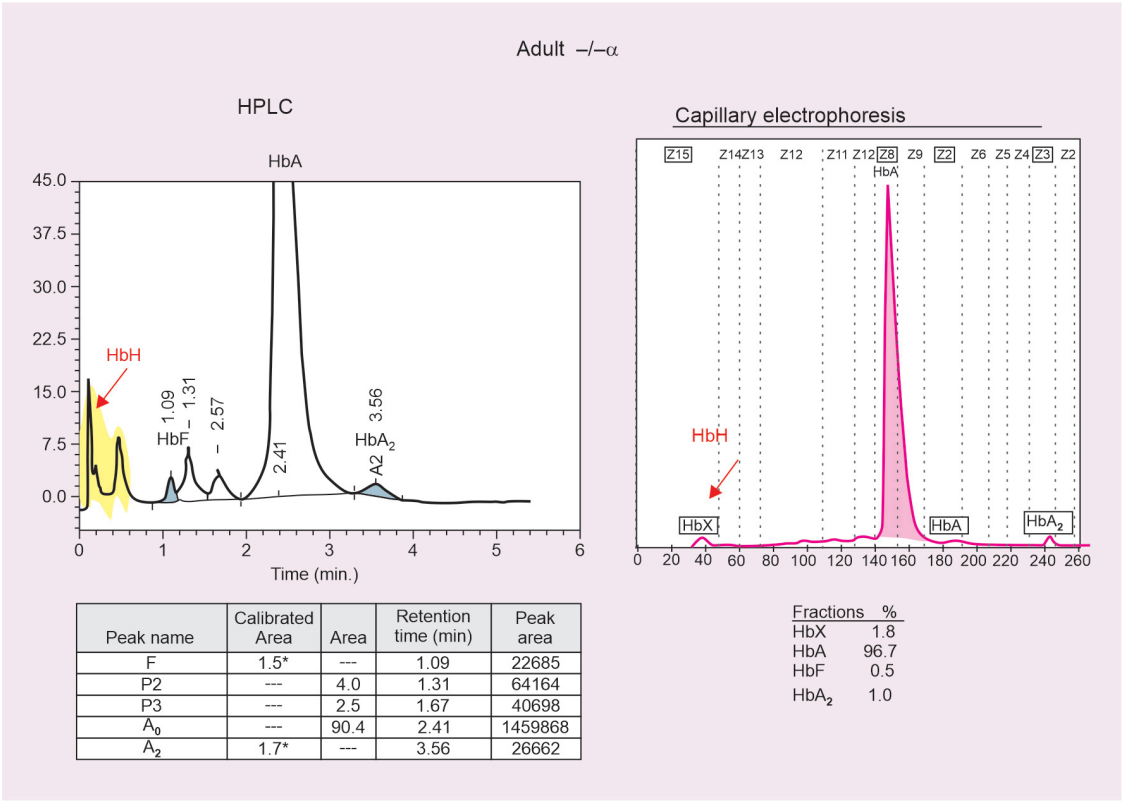
Sebia Capillary 2

Fig. 16.31: Hb-Lepore and HbA_2, comparison between HPLC and CE



HPLC: Bio-Rad Variant 1 Sebia Capillary 2

Fig. 16.32: HbH and Hb Bart, comparison between HPLC and CE



Peak name	Calibrated Area	Area	Retention time (min)	Peak area
F	1.5*	---	1.09	22685
P2	---	4.0	1.31	64164
P3	---	2.5	1.67	40698
A_0	---	90.4	2.41	1459868
A_2	1.7*	---	3.56	26662

Fig. 16.33: Thalassaemia and HbH disease. Correlation of HPLC with capillary electrophoresis (CE)

Table 16.6: Comparison of HPLC and capillary electrophoresis (CE)

Parameter	HPLC	CE
• Injected volume	1–100 µl	1–100 nl
• Flow rate of the liquid phase	1–10 nl/min	1–100 nl/min
• Flow profile	Parabolic	Plug
• Number of peaks separated	20–30	20–100
• Analysis time	10–60 minute	1–20 minutes
• Separation efficiency	>10,000 plates	>1,00,000 plates
• Separation technique	High pressure with complicated pumping system	Electrical field with stable high voltage source, no back pressure
• Solvents	Different solvents for different columns	Different solvents in the same column
• Level of developments	Mature technique	Newer technique, developing fast

Table 16.7: Advantages and disadvantages of capillary electrophoresis

Advantages	Disadvantages
• Small sample size (nl), compared to µl in other separation technique • Significantly greater speed than HPLC (voltage applied in CE versus pressure applied in HPLC) • High resolution due to the nature of the flow profile (electroosmotic vs. hydrodynamic in HPLC). • Virtually no band broadening • Can separate haemoglobin in 15 zones (zone 1 to zone 15). So, most of the abnormal Hb is easily separated and is diagnosed • Can diagnose both α-thalassaemia as well as β-thalassaemia. • Can separate HbA_2 and HbE unlike HPLC (HPLC elute in same region and cannot be separated) • Low cost per test • It flows uniformly through narrow column electroosmotically rather than laminar in HPLC. In laminar flow central fluid moves fast compared to peripheral fluid	• If HbA is absent, a second analysis is needed to obtain appropriate zones for verifying the identity of Hb. This is an additional step not required in HPLC. • Separation suffers if injection volume exceeds 1% of the column volume. • Stacking of the sample. Large volume sample stacking involves using reverse polarity, but the electrophoretic current must be monitored carefully or analyte will be lost. In pH stacking, if too much analyte is loaded the separation efficiency is reduced. The concentration of the samples must be dramatically increased to obtain the same signal-to-noise ratio. • HbA is separated to its component fractions of A_0 and A_1 (subdivides into several peaks). • Retention time of glycosylated and other derivatives of HbS can be same as HbA_0 and A_2.

Detection of HbA1C or Glycated Haemoglobin

Glycated haemoglobin or HbA1C is defined by International Federation of Clinical Chemistry (IFCC) working group as haemoglobin that is irreversibly glycated at one or both N-terminal valines of beta chains. It is formed from irreversibly, slow, non-enzymatic addition of a sugar residue to haemoglobin and the rate of production is directly proportional to the ambient glucose concentration. The long lifespan of erythrocytes (mean 120 days) enables HbA1C to be used as an index of glycaemic control over

the breathing 2–3 months and as the adequacy of treatment in diabetic patients.

Variuos factors may affect the accuracy of HbA1C measurement according to the assay method used, of which haemoglobin variants have been identified with many of them being clinically silent. HbA1C deviation of 1% reflects a change of 1.4–1.9 mmol/L in average blood glucose concentration. Therefore, a falsely high or low HbA1C value caused by the presence of a clinically silent haemoglobin variant may lead to over or under-treatment of diabetic patients.

HbA1C detection by HPLC method (Bio-Rad Variant 2, haemoglobin A1C program): It uses cation exchange HPLC.

- Sample type: EDTA blood
- Sample volume: 23 µl
- Assay principle: Cation exchange HPLC with detection by absorbance at 415 nm wavelength.
- Interfering factors:
  i. Bilirubin (if more than 20 mg/dl).
  ii. Lipids (triglycerides) → No interference up to 6000 mg/dl.
  iii. EDTA → No interference up to 11 times EDTA

Result

Table 16.7

HbA1C level and diabetic control	Blood glucose level; EAG (estimated average glucose)
HbA1C <4%: Excellent control	90–120 mg/dl
HbA1C 4–6%: Good control	120–150 mg/dl
HbA1C 6–7%: Fair control	150–180 mg/dl
HbA1C 7–8%: Action suggested	181–210 mg/dl
HbA1C >8%: Poor control	>211 mg/dl

Note

1. For patients with variant diseases there may be lowering of HbA1C due to low HbA synthesis.
2. EAG is a value calculated from HbA1C and indicates average glucose level over past three months.

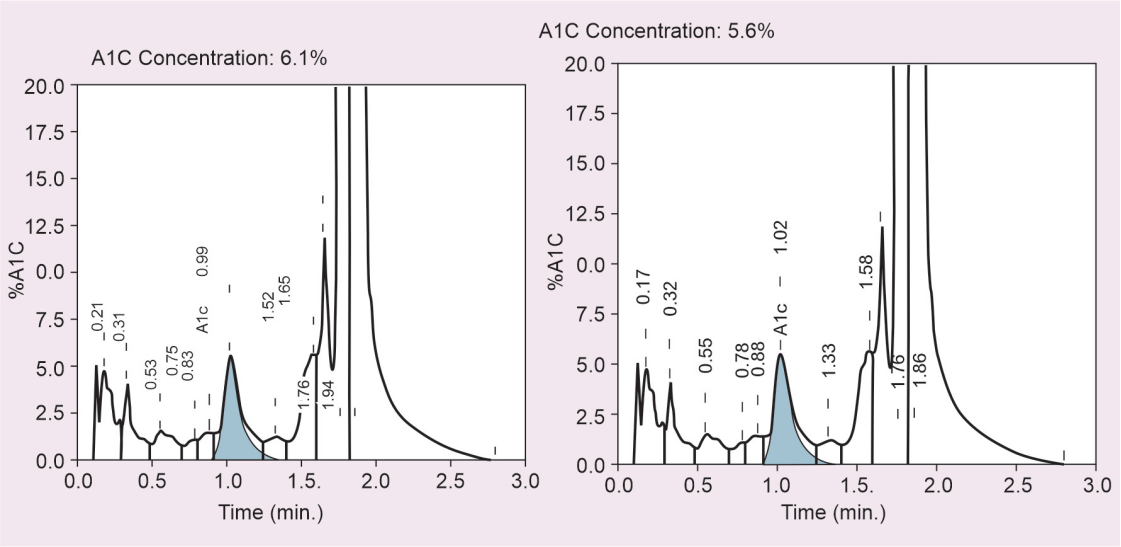
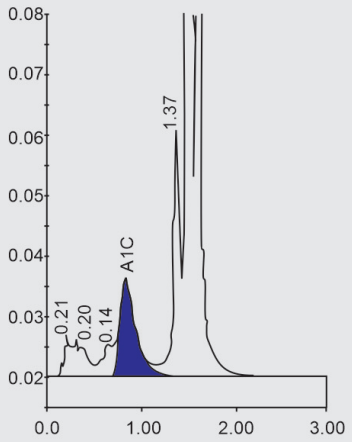


Fig. 16.34: HPLC (Bio-Rad Vaiant II) shows HbA1C concentration of 6.1% and 5.6% respectively on two samples

Patient report

Bio-Rad	DATE: 04/11/2018
D-10	TIME: 12:48 PM
SN:=D/7C059101	Software version: 4.20-2
Sample ID:	3640
Injection date	01/05/2018 10:17 AM
Injection #: 9	Method: HbA1c
Rack #: ---	Rack position: 2



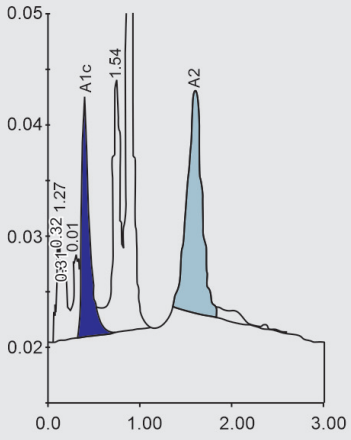
Peak table - ID: 3640

Peak	R time	Height	Area	Area%
Ala	0.20	7137	30382	1.3
Alb	0.28	8100	26104	1.1
Unknown	0.35	5015	35603	1.6
LA1C/CHb-1	0.64	5312	44842	2.0
A1C	0.82	16333	186851	14.1
P3	1.37	40382	219528	9.7
A_0	1.48	373769	1163510	51.2
Variant-Window	1.60	198765	566749	24.9
Total Area:	2273579			

Concentration:	%
A1C	14.1

Patient report

Bio-Rad	DATE: 04/11/2018
D-10	TIME: 12:48 PM
SN:#D/7C059101	Software version: 4.20-2
Sample ID:	3843
Injection date	01/05/2018 10:17 AM
Injection #: 3	Method: HbA2/F
Rack #: ---	Rack position: 3



Peak table - ID: 3843

Peak	R time	Height	Area	Area%
Ala	0.20	9355	40300	1.5
Unknown	0.27	10779	31090	1.2
Alb	0.34	6551	46810	1.8
LA1C/CHb-1	0.61	7488	59541	2.3
Alc	0.80	20920	236772	13.2
P3	1.50	22486	273867	10.4
A_0	1.74	296568	1482567	56.3
A_2	1.18	19942	460799	20.9*
Total Area:	2631747			

Concentration:	%
A1C	13.2
A_2	20.9*

Fig. 16.35: In HPLC method, HbA1C concentration is 14.1%, and 13.2% respectively
HPLC , D-10 Dual Program Reorder Pack (Bio-Rad)

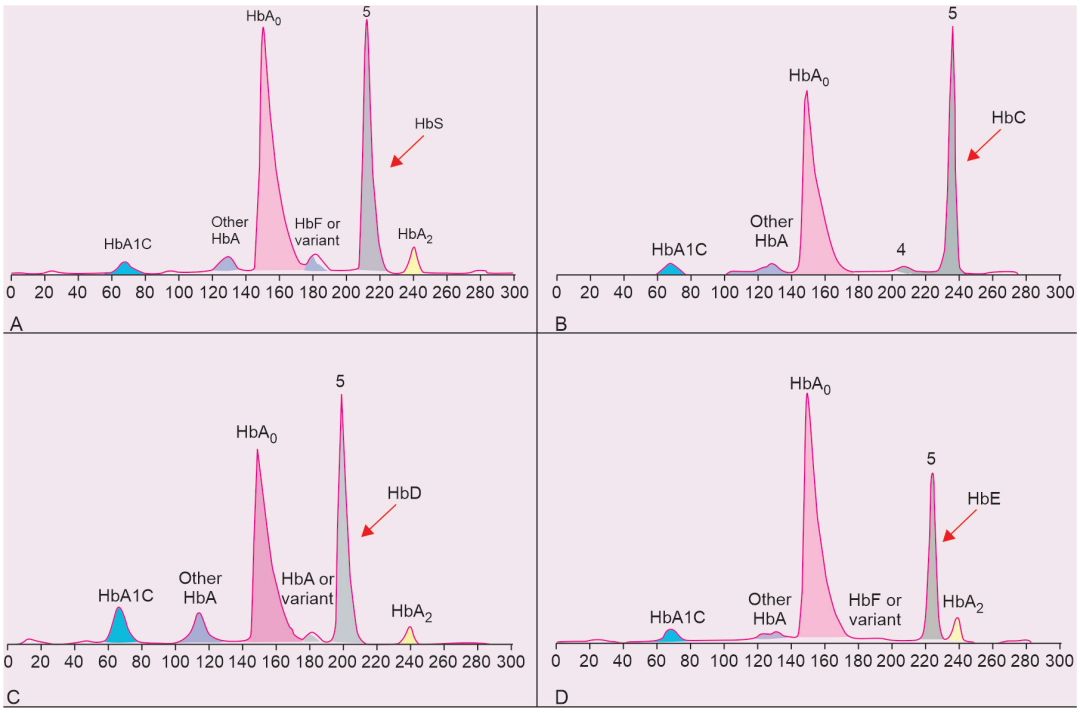


Fig. 16.36: HbA1C and other haemoglobins in capillary electrophoresis

Quiz on HPLC

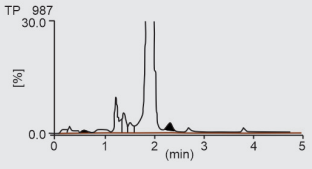
Look at the following chromatogram and give your diagnosis:

HLC-723G11 BTHAL VO3.04 879007 2019/12/30 09:43:38
ID 32812
Sample No. 2019123023050058 SL 0006 – 02
Patient ID
Name
Comment

CALIB F Y =1.0027X – 0.2204
A_2 Y =1.5350X + 0.3130

Name	%	Time	Area
F	0.3	0.60	10.89
A_0	82.6	1.93	2207.08
A_2	2.8	2.31	43.69
E+			
D+			
S+			
C+			

Total Area 2672.46
HbF 0.3 % **HbA_2 2.8 %**



[Unknown Peak]

Name	%	Time	Area
P00	0.6	0.21	15.99
P01	1.0	0.31	27.58
P02	0.0	0.71	0.92
P03	1.4	0.88	38.49
P04	5.2	1.23	138.16
P05	2.7	1.39	71.38
P06	1.8	1.51	47.18

HPLC-Quiz 1

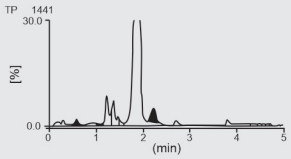
Chromatogram Report

HLC-723G11 BTHAL VO3.04 879007 2019/12/23 22:02:54
ID 32224
Sample No. 2019122321500060 SL 0001 – 04
Patient ID
Name
Comment

CALIB F Y =1.0027X – 0.2204
A_2 Y =1.5350X + 0.3130

Name	%	Time	Area
F	0.8	0.58	12.08
A_0	79.5	1.87	1059.13
A_2	5.2	2.22	42.72
E+			
D+			
S+			
C+			

Total Area 1332.26
HbF 0.8 % **HbA_2 5.2 %**



[Unknown Peak]

Name	%	Time	Area
P00	0.9	0.16	11.86
P01	0.9	0.30	11.73
P02	0.0	0.51	0.37
P03	0.1	0.66	1.46
P04	1.3	0.89	17.25
P05	4.5	1.22	59.76
P06	3.7	1.36	49.24

HPLC-Quiz 2

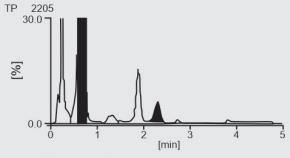
Chromatogram Report

HLC-723G11 BTHAL VO3.03 719503 2019-12-14 19:02:44
ID 31235
Sample No. 2019121419070018 SL 0002 – 05
Patient ID
Name
Comment

CALIB F Y =0.9937X – 0.1646
A_2 Y =1.5935X + 0.0980

Name	%	Time	Area
F	60.5	0.69	797.63
A_0	9.3	1.87	121.49
A_2	6.3	2.28	51.27
E+			
D+			
S+			
C+			

Total Area 1311.68
HbF 60.5 % **HbA_2 6.3 %**



[Unknown Peak]

Name	%	Time	Area
P00	14.4	0.24	189.31
P01	4.0	0.43	52.71
P02	1.6	0.77	20.72
P03	1.9	131	25.10
P04	0.6	1.48	7.30
P05	0.9	2.71	12.19
P07	1.3	3.78	17.22

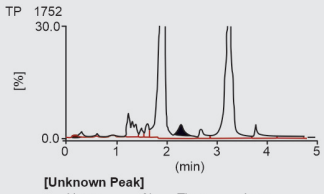
HPLC-Quiz 3

HLC-723G11 BTHAL VO3. 04 879007 2019/12/24 22:56:39
ID 32403
Sample No. 2019122422440068 SL 0002 – 03
Patient ID
Name
Comment

CALIB F Y =1.0027X – 0.2204
A2 Y =1.5350X + 0.3130

Name	%	Time	Area
F	0.4	0.61	6.97
A_0	48.5	1.92	623.59
A_2	3.5	2.28	27.89
E+			
D+			
S+	33.4	3.28	429.53
C+			

Total Area 1286.60
HbF 0.4 % **HbA_2 3.5 %**



[Unknown Peak]

Name	%	Time	Area
P00	0.5	0.17	6.78
P01	1.0	1.0	12.56
P02	0.0	0.0	0.63
P03	1.0	1.0	13.27
P04	5.2	5.2	66.73
P05	1.1	1.1	14.52
P06	2.2	2.2	28.74

HPLC-Quiz 4

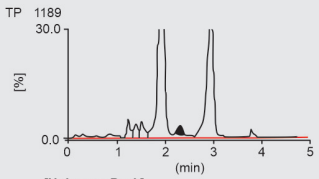
Chromatogram Report

HLC-723G11 BTHAL VO3. 04 879007 2019/12/28 16:56:31
ID 32592
Sample No. 2019122816440033 SL 0003 – 02
Patient ID
Name
Comment

CALIB F Y =1.0027X – 0.2204
A2 Y =1.5350X + 0.3130

Name	%	Time	Area
F	0.2	0.61	6.97
A_0	49.5	1.92	623.59
A_2	3.3	2.28	27.89
E+			
D+	36.7	2.94	1043.06
S+			
C+			

Total Area 2842.64
HbF 0.2 % **HbA_2 3.3 %**



[Unknown Peak]

Name	%	Time	Area
P00	0.5	0.17	13.21
P01	0.6	0.31	17.73
P02	1.1	0.86	31.18
P03	2.7	1.23	76.71
P04	2.1	1.39	60.00
P05	2.7	1.51	77.76
P06	1.4	3.78	40.80

HPLC-Quiz 5

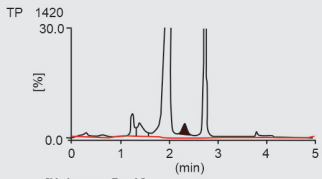
Chromatogram Report

HLC-723G11 BTHAL Vo3. 04 879007 2019/12/26 22:15:10
ID 32426
Sample No. 2019122622020056 SL 0004 – 06
Patient ID
Name
Comment

CALIB F Y =1.0027X – 0.2204
A2 Y =1.5350X + 0.3130

Name	%	Time	Area
F	0.6	0.61	10.13
A_0	61.6	1.95	845.61
A_2	3.7	2.31	31.98
E+	22.3	2.71	306.52
D+			
S+			
C+			

Total Area 1372.08
HbF 0.2 % **HbA_2 3.3 %**



[Unknown Peak]

Name	%	Time	Area
P00	0.6	0.17	8.50
P01	1.0	0.31	14.00
P02	0.0	0.79	0.44
P03	0.8	1.08	11.35
P04	3.9	1.23	53.59
P05	4.0	1.39	55.20
P08	1.3	4.08	17.44

HPLC-Quiz 6

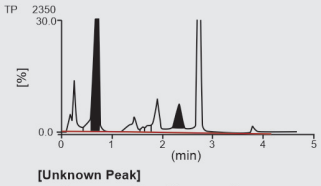
Chromatogram Report

HLC-723G11 BTHAL VO3. 04 879007 2019/12/27 14:26:44
ID 32377
Sample No. 2019122713240005 SL 0001 – 05
Patient ID
Name
Comment

CALIB F Y =1.0027X – 0.2204
A2 Y =1.5350X + 0.3130

Name	%	Time	Area
F	25.9	0.69	329.70
A_0	7.8	1.89	99.04
A_2	7.7	2.32	60.50
E+	44.0	2.71	558.75
D+			
S+			
C+			

Total Area 1270.10
HbF 25.9 % **HbA_2 7.7 %**



[Unknown Peak]

Name	%	Time	Area
P00	6.6	0.24	84.25
P01	1.8	0.43	23.24
P02	1.1	0.75	13.52
P03	3.7	1.43	47.31
P04	0.6	1.61	7.67
P05	1.2	1.73	15.50
P06	2.1	3.78	26.64

HPLC-Quiz 7

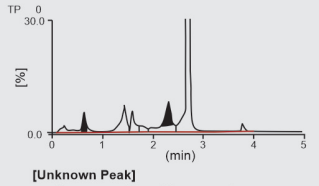
Chromatogram Report

HLC-723G11 BTHAL VO3. 04 879007 2019/12/26 15:28:13
ID 32311
Sample No. 2019122615250003 SL 0001 – 03
Patient ID
Name
Comment

CALIB F Y =1.0027X – 0.2204
A2 Y =1.5350X + 0.3130

Name	%	Time	Area
F	2.7	0.63	78.64
A_0	1.5	1.77	40.77
A_2	7.9	2.31	136.78
E+	75.3	2.69	2108.56
D+			
S+			
C+			

Total Area 2798.37
HbF 25.9 % **HbA_2 7.7 %**



[Unknown Peak]

Name	%	Time	Area
P00	1.1	0.23	29.66
P01	0.2	0.37	5.61
P02	0.3	0.43	8.47
P03	0.4	0.72	12.07
P04	0.1	1.05	2.93
P05	4.9	1.43	138.11
P07	3.9	0.02	108.11

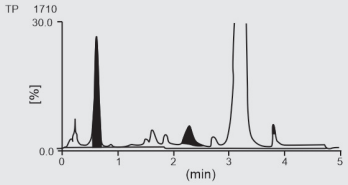
HPLC-Quiz 8

HLC-723G11 BTHAL VO3. 04 879007 2019/12/24 17:43:51
ID 32278
Sample No. 2019122615250003 SL 0001 – 03
Patient ID
Name
Comment

CALIB F Y =1.0027X – 0.2204
A2 Y =1.5350X + 0.3130

Name	%	Time	Area
F	9.9	0.61	117.54
A_0	3.4	1.84	39.81
A_2	5.4	2.25	39.16
E+			
D+			
S+	70.5	3.20	828.97
C+			

Total Area 1176.02
HbF 9.9 % **HbA_2 5.4 %**



[Unknown Peak]

Name	%	Time	Area
P00	2.6	0.23	30.04
P01	0.6	0.42	7.32
P02	0.5	0.69	5.42
P03	0.4	0.86	4.40
P04	0.6	1.31	7.07
P05	0.9	1.49	10.02
P08	2.9	3.78	33.73

HPLC-Quiz 9

ANSWERS TO HPLC QUIZ

HPLC-Quiz 1: **Ans: Normal pattern**.

Here, HbF is 0.3%, HbA_2 is 2.8% and HbA (adult Hb) or HbA_0 is 82.6% and there are some unknown peaks (without any clinical significance) which is seen normal persons.

HPLC-Quiz 2: **Ans: β-thalassaemia minor or heterozygous β-thalassaemia**.

Here, HbF is 0.8%, HbA_2 is 5.2% and HbA0 is 79.5%. Normal value of HbA_2 in adults is 1.5 to 3.7. So, value of HbA_2 is slightly raised while HbF and HbA_0 is almost normal which is typically seen in β thalassaemia minor or heterozygous β thalassaemia (value of HbA_2 is 4–9%).

HPLC-Quiz 3: **Ans: β-thalassaemia major or Homozygous β-thalassaemia**.

Here, HbF is 60.5%, HbA_2 is 6.3% and HbA_0 is 9.3%. Normal value of HbF, HbA_2 and HbA_0 is 0.0 to 2%, 1.5 to to 3.7%, and 94.3 to 98.5%. respectively. So, in this case, HbF is highly raised while HbA_2 is slightly raised which is typically seen in β-thalassaemia major or homozygous β-thalassaemia.

HPLC-Quiz 4: **Ans: Sickle cell trait or heterozygous sickle cell anaemia**.

Here, HbF is 0.4%, HbA_2 is 3.5% and HbA_0 is 48.5%. But HbS or sickle Hb is 33.4% (<50%).

This type of chromatogram is seen in sickle cell trait or heterozygous sickle cell anaemia (remember in sickle cell disease or homozygous sickle cell anaemia, HbS exceeds 50% and often >70%).

HPLC-Quiz 5: **Ans: HbD trait (HbD-Punjab)**.

Here, HbF is 0.2%, HbA_2 is 3.3% and HbA_0 is 49.5%. But HbD is 36.7% (<50%).

HPLC-Quiz 6: **Ans: HbE trait**.

Here, HbF is 0.6%, HbA_2 is 3.7% and HbA_0 is 61.6%. But HbE is 22.3% (<50%). Remember in HPLC, HbE and HbA_2 elute in the same region and they give peak in the same area. So, in HPLC, HbE and HbA_2 cannot be differentiated. But in thalassaemia minor or trait HbA_2 value is usually 4–9% (does not exceed 10%). So, a high value in HPLC (>10%) in this region is suggestive of HbE trait or disease (HbE >50%).

HPLC-Quiz 7: **Ans: HbE β-thalassaemia (double heterozygous)**.

Here, HbF is 25.9%, HbA_2 is 7.7% and HbA_0 is 7.8%. But HbE is 44%. So, in this case both the HbF and HbE are highly raised while adult haemoglobin (HbA_0) is very much reduced.

HPLC-Quiz 8: **Ans: HbE disease**.

Here, HbF is 2.7%, HbA_2 is 7.9% and HbA_0 is 1.5%. But HbE is 75.3% (> 50%) and HbE value is highly raised, while adult haemoglobin (HbA_0) is reduced to 1.5%.

HPLC-Quiz 9: **Ans: Sickle cell disease/ sickle β anaemia (double heterozygous)**.

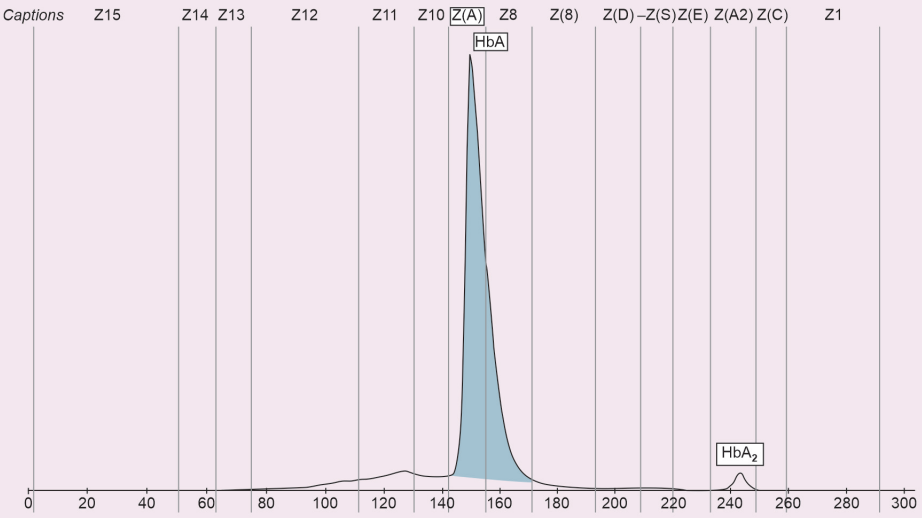
Here, HbF is 9.9%, HbA_2 is 5.4% and HbA_0 is 3.4%. But HbS or sickle Hb is 70.5% (>50%). So, both HbF and HbS are raised but HbS is raised to a great extent.

In this particular case, it could be sickle cell disease or it could be a heterozygous having both sickle cell disease as well as thalassaemia, known as sickle β-thalassaemia **(double heterozygous)**.

Advice: HPLC study of the parents and/or capillary electrophoresis for confirmatory diagnosis.

Quiz on Capillary Electrophoresis (CE)

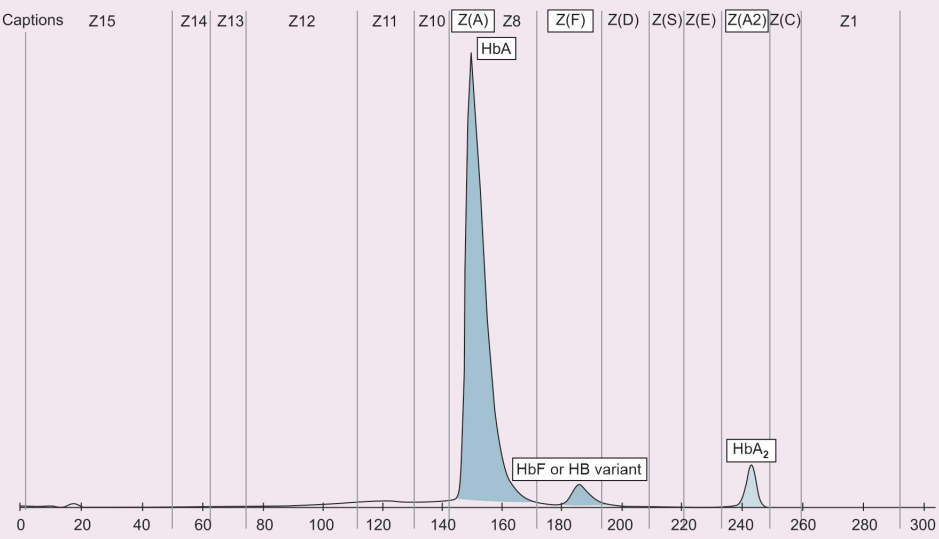
Look at the following chromatogram and give your diagnosis:



Haemoglobin electrophoresis

Name	%
HbA	97.8
HbA_2	2.2

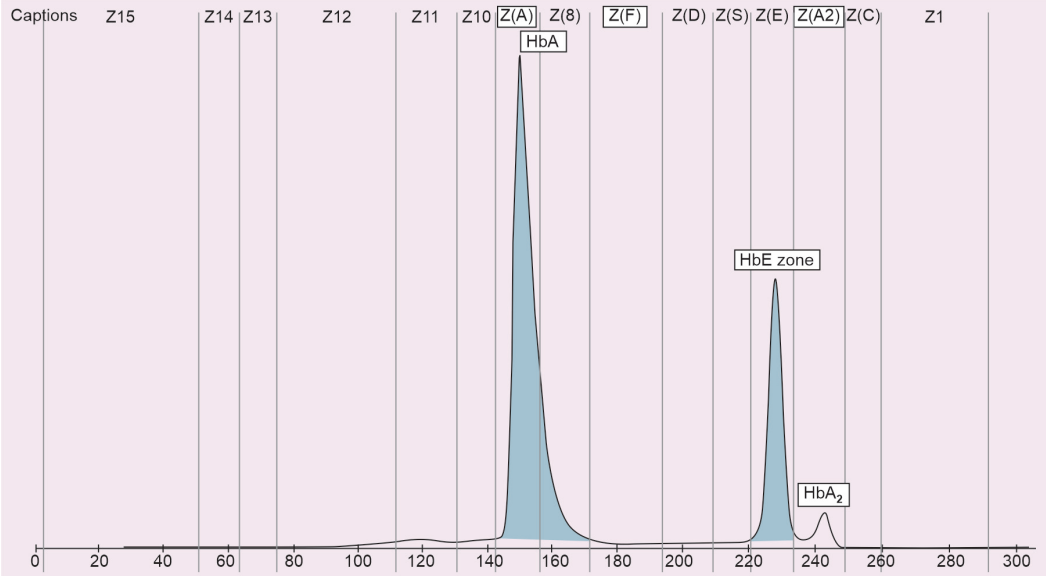
CE-Quiz 1



Haemoglobin electrophoresis

Name	%
HbA	90.7
HbF or Hb variant	4.4
HbA_2	4.9

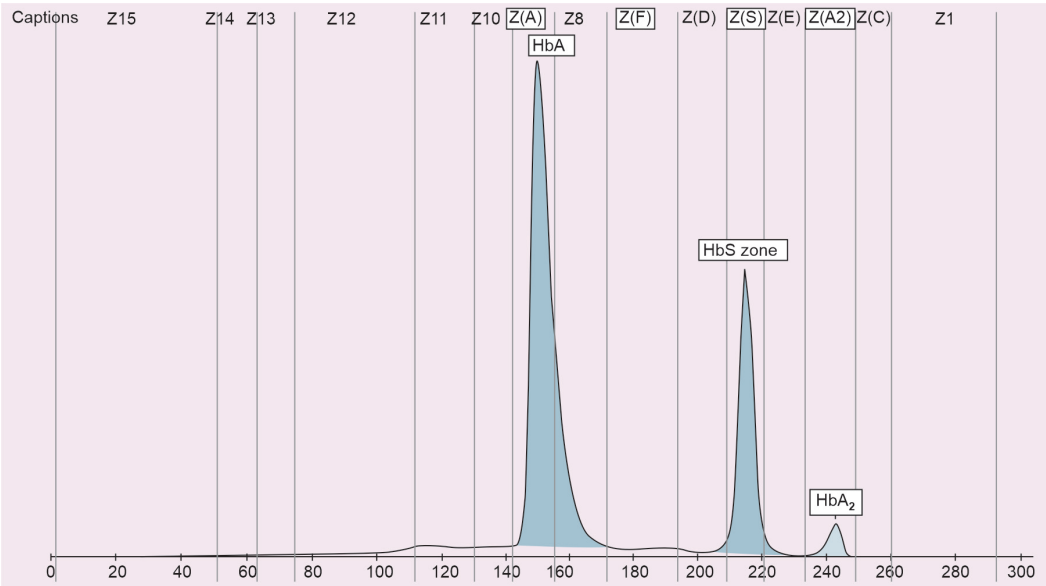
CE-Quiz 2



Haemoglobin electrophoresis

Name	%
HbA	73.1
HbE zone	24.2
HbA_2	2.7

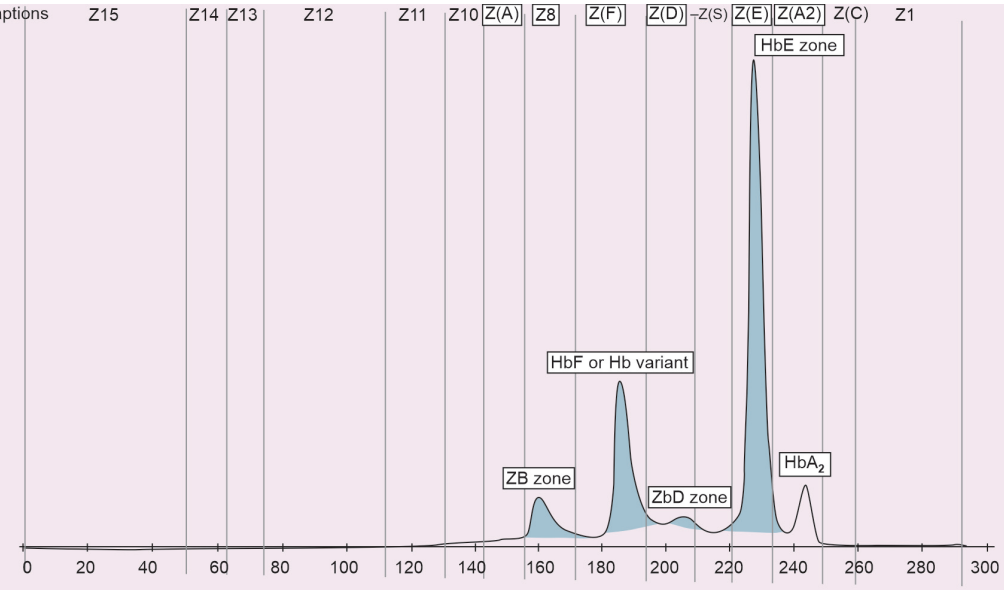
CE-Quiz 3



Haemoglobin electrophoresis

Name	%
HbA	68.8
HbE zone	28.2
HbA_2	3.0

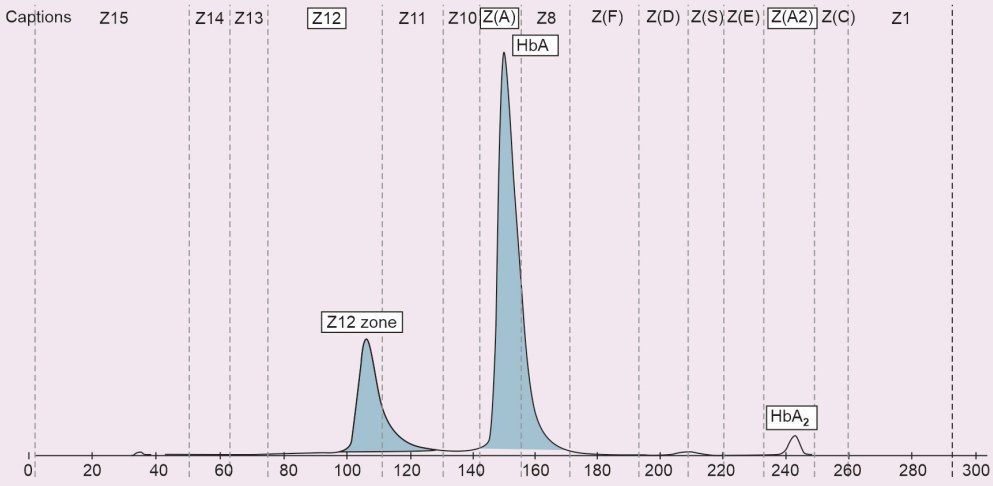
CE-Quiz 4



Haemoglobin electrophoresis

Name	%
Z8 zone	7.7
HbF or Hb variant	23.4
HbD zone	1.9
HbE zone	60.9
HbA_2	6.1

CE-Quiz 5



Haemoglobin electrophoresis

Name	%
Z12 zone	24.2
HbA	73.8
HbA_2	2.0

CE-Quiz 6

ANSWERS TO CAPILLARY ELECTROPHORESIS

CE-Quiz 1: **Ans: Normal pattern**.

In this capillary electrophoresis (CE), HbA or adult haemoglobin (in zone 9) is 97.8%, HbA_2 (in zone 3) is 2.2% which indicates normal pattern.

CE-Quiz 2: **Ans: β-Thalassaemia minor or trait**.

In this capillary electrophoresis (CE), HbA or adult haemoglobin (in zone 9) is 90.7%, HbA_2 (in zone 3) is 4.9% and HbF (in zone 7) is 4.9 %. So, HbA_2 is raised but <10% which indicates β-thalassaemia minor or trait.

CE-Quiz 3: **Ans: HbE trait or heterozygous**.

In this capillary electrophoresis (CE), HbA or adult haemoglobin (in zone 9) is 73.1%, HbA_2 (in zone 3) is 2.7% and HbE (in zone 4) is 24.2 %. So, HbE is raised but <50% which indicates HbE trait or heterozygous.

CE-Quiz 4: **Ans: Scikle cell trait (HbS trait) or heterozygous sickle cell anaemia.**

In this capillary electrophoresis (CE), HbA or adult haemoglobin (in zone 9) is 68.8%, HbA_2 (in zone 3) is 3% and HbS (in zone 5) is 28.2 %. So, HbS is raised but <50% which indicates HbS trait or heterozygous sickle cell anaemia.

CE-Quiz 5: **Ans: HbE β-thallasemia (double heterozygous).**

In this capillary electrophoresis (CE), HbA or adult haemoglobin (in zone 9) is nil, HbA_2 (in zone 3) is 6.1%, HbE (in zone 4) is 60.9% (highly raised) and HbF (in zone 7) is 23.4%. So, in this case both HbE and HbF are raised which indicate HbE beta-thalassaemia (double heterozygous).

CE-Quiz 6: **Ans: Hb Bart (γ4) or α-thalassaemia**.

In this capillary electrophoresis (CE), haemoglobin Bart or γ_4 (in zone 12 or z12) is 24.2% which highly raised and reduced level of HbA (adult Hb). This is suggestive of α-thalassaemia.

Advice: Genetic work up that requires assessment of deletions and duplications of genes.

Chapter 17

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 of 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. 17.1).

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

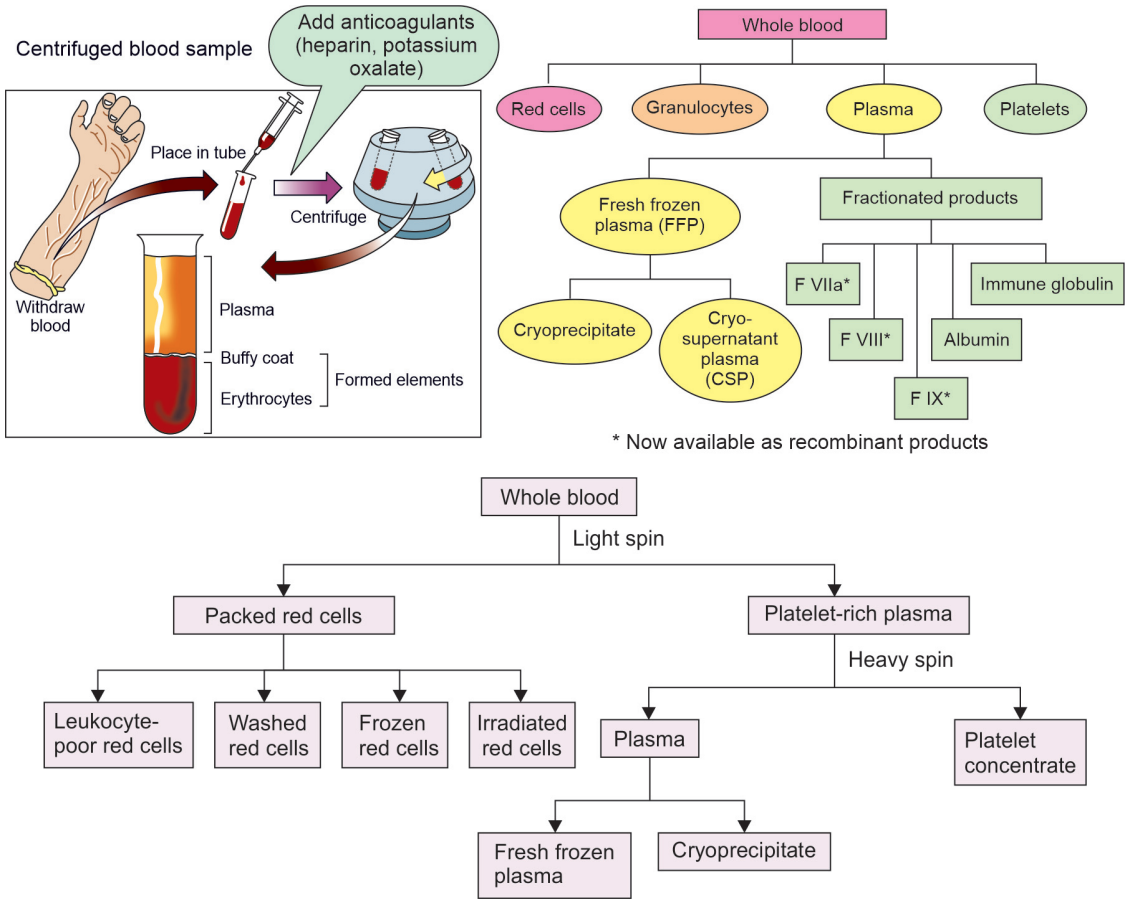


Fig. 17.1: Blood component preparation from whole blood (within 6 hours of collection)

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

i. **Citrate:** Anticoagulation by binding of calcium in plasma.
ii. **Phosphate:** Acts as a buffer to minimize the effects of decreasing pH in blood.
iii. **Dextrose:** Maintenance of red cell membrane and metabolism. Also needed for ATP generation for viability of red cells.
iv. **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 adennine-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. 17.14)

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, ant-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 17.1 and 17.2).

Table 17.1: 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 17.2: Different techniques of major cross-match

Types of cross-match	Antibody detection in recipient's serum
• Saline cross-match	IgM
• Antiglobulin test or Coombs test	IgG
• Albumin technique	IgG
• Enzyme technique	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. 17.2).
- Add one drop of 5% saline suspension of donor's red cells to the above test tube.

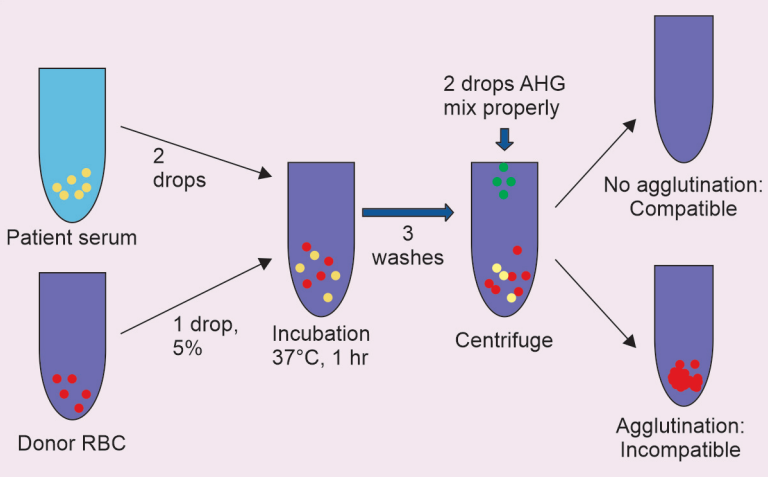


Fig. 17.2: Major cross-match

- Mix them and incubate at 37% 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 mean 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. 17.3).

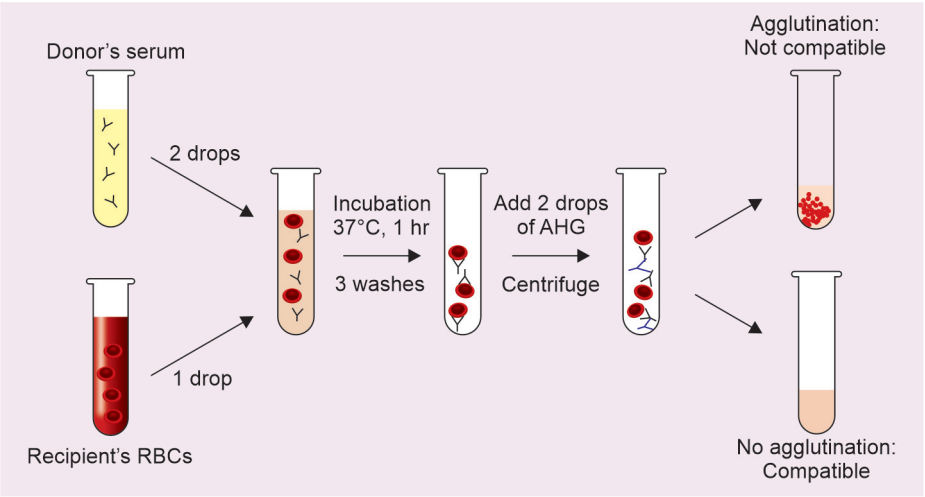


Fig. 17.3: Minor cross-match

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. 17.4).

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.

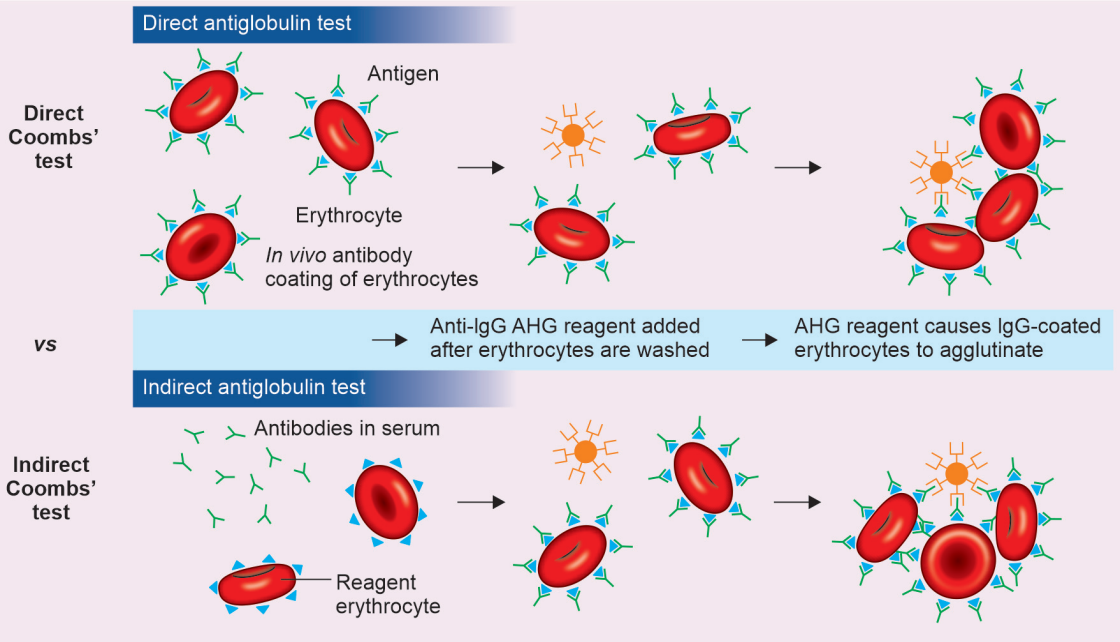


Fig. 17.4: Direct Coombs' test *vs* indirect Coombs' test

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 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 ≥3 × 10^{11} platelets.
- **Leukapheresis:** Not commonly used. For collection of large number of leukocytes; drugs or sedimenting materials need to be administrated 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. 17.5).

WHOLE BLOOD (Fig. 17.6)

- 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.

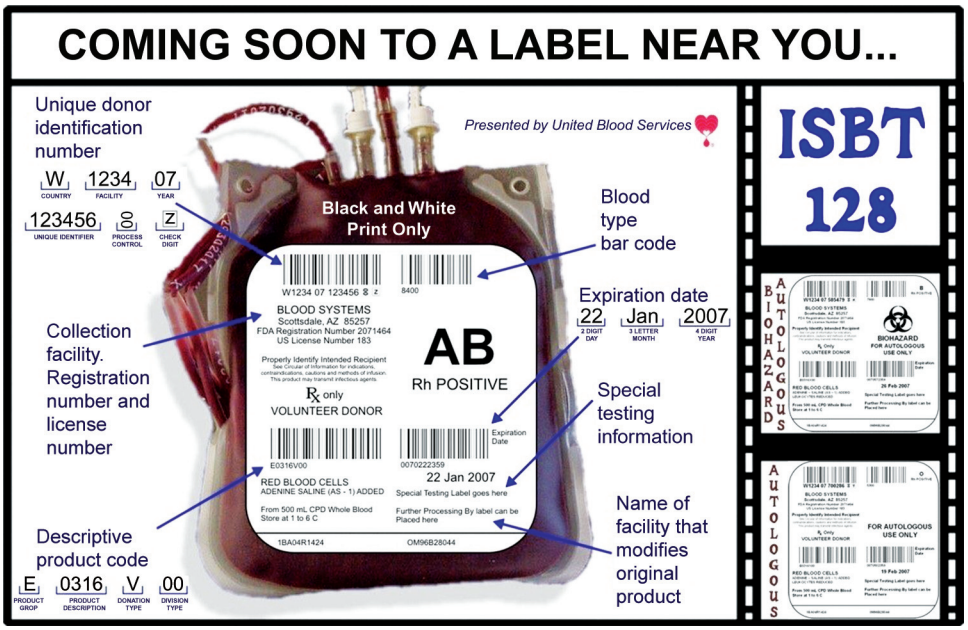


Fig. 17.5: Identification of different points of one unit of blood/blood product

- 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-phophate-dextrose (CPD)
  – CPDA-1 (adenine): 35 days
  – AS-1, AS-3, AS-5–42 days
- Administer through standard blood filter (150–280 micron)
- 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. 17.7).

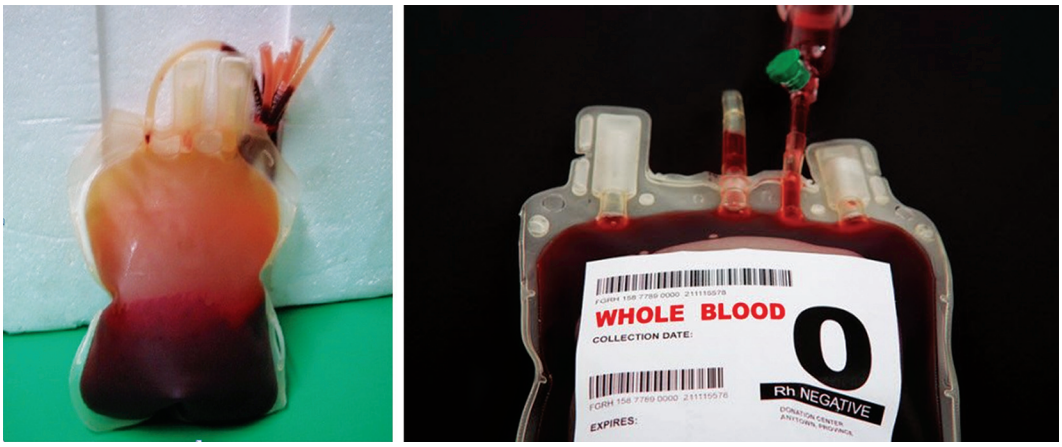


Fig. 17.6: Whole blood

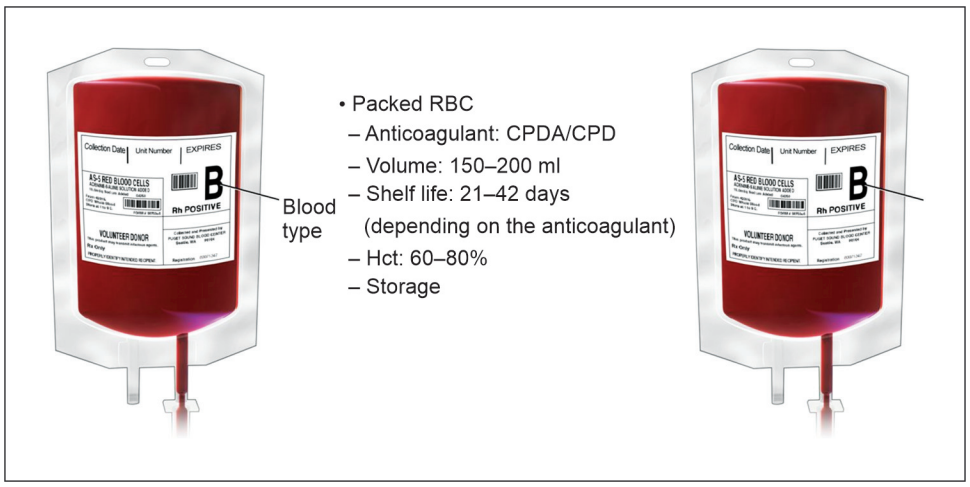


Fig. 17.7: Packed red blood cells

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°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-threating transfusion associated graft-versus-host disease (GVH) in at risk patients.
- Gamma-irradiation (250 c Gy) of red cells inactivates T lymphocytes and prevents graft versus host disease. This component can be stored at 4–6°C up to original expiry date or 28 days from irradiation.

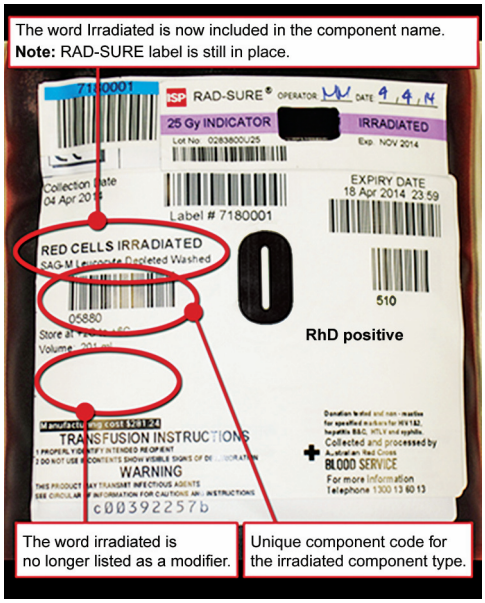
Indications

i. It is indicated for prevention of GVHD in susceptible individuals like immunodeficient individuals and patients receiving blood from first degree relatives.

ii. It is used for intrauterine or premature neonate transfusions (Fig. 17.8).

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°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. 17.8: Identification of irradiated blood

- **Indications**
  i. 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
  i. Bleeding due to thrombocytopenia (immune mediated or secondary), where platelet count is <20,000/mm^3
  ii. In case of abnormal platelet function or platelet function defects
  iii. 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**
  i. 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.

CCI = 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°C for about 30–45 minutes. Then it is temporarily stored at 2–6°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 grozen 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 proems 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 ≤18°C. Then thawed it slowly at 4–6°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 ≤–18°C and can be stored for 1 year. When required for transfusion, cryoprecipitate was thawed at 30–37°C and can be stored at 2–6°C temporarily for 6 hours. This unit can be transferred to patient. Each unit of cryoprecipitate must contains ≥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. 17.9).

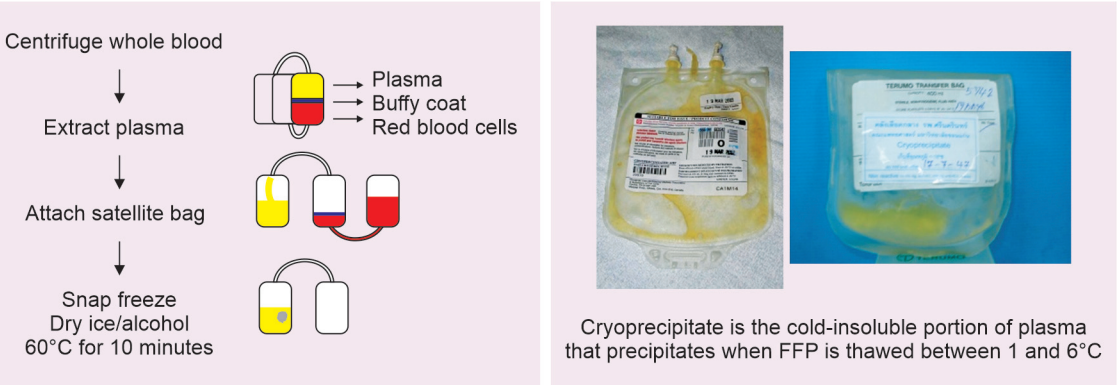


Fig. 17.9: Cryoprecipitate (CRYO)

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 17.3.

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 17.4).

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 17.3: 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 hypoproteinaemia.
• 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. 17.10 and Table 17.5).

Table 17.4: 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

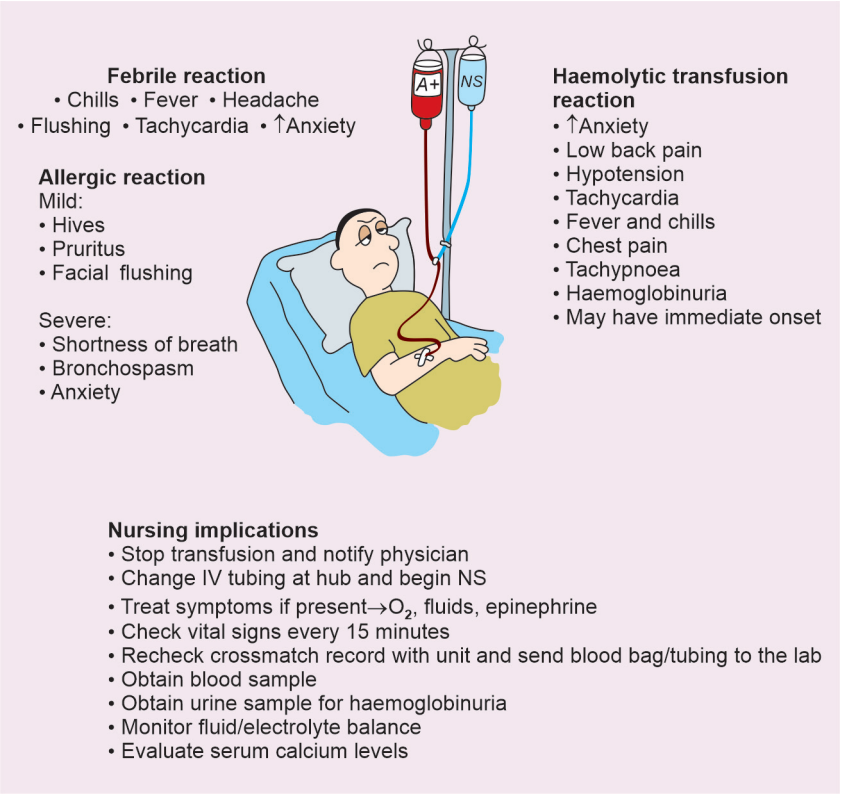


Fig. 17.10: Blood transfusion reaction

Table 17.5: Adverse effects of blood transfusion

Immediate (<24 hours)	Delayed (≥24 hours)
Non-immunological causes • 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) *Immunological causes* • 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 compliment activation)	*Non-immunological causes* • 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) *Immunological causes* • 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.

ANAPHYLAXIS

- 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 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)

- 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.

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. 17.11).
- **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.

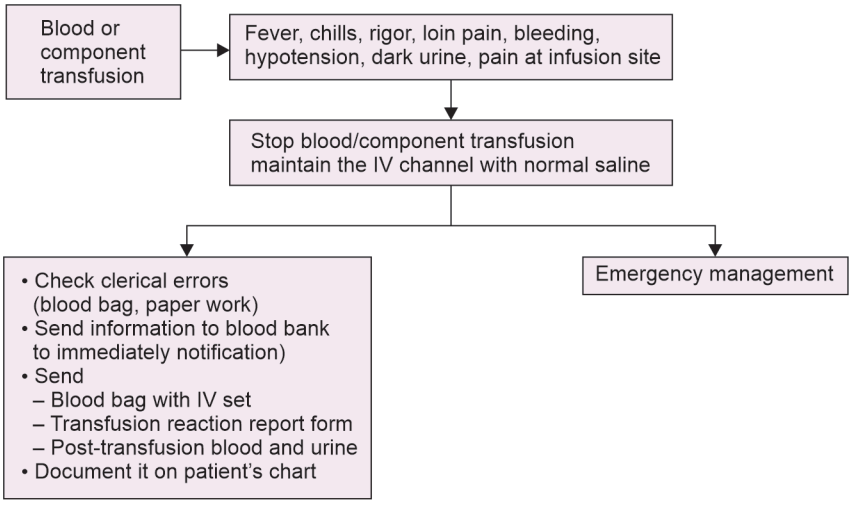


Fig. 17.11: Management protocol after a suspected transfusion reaction

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. The three attached bags are recentrifuged using a **heavy spin** to produce

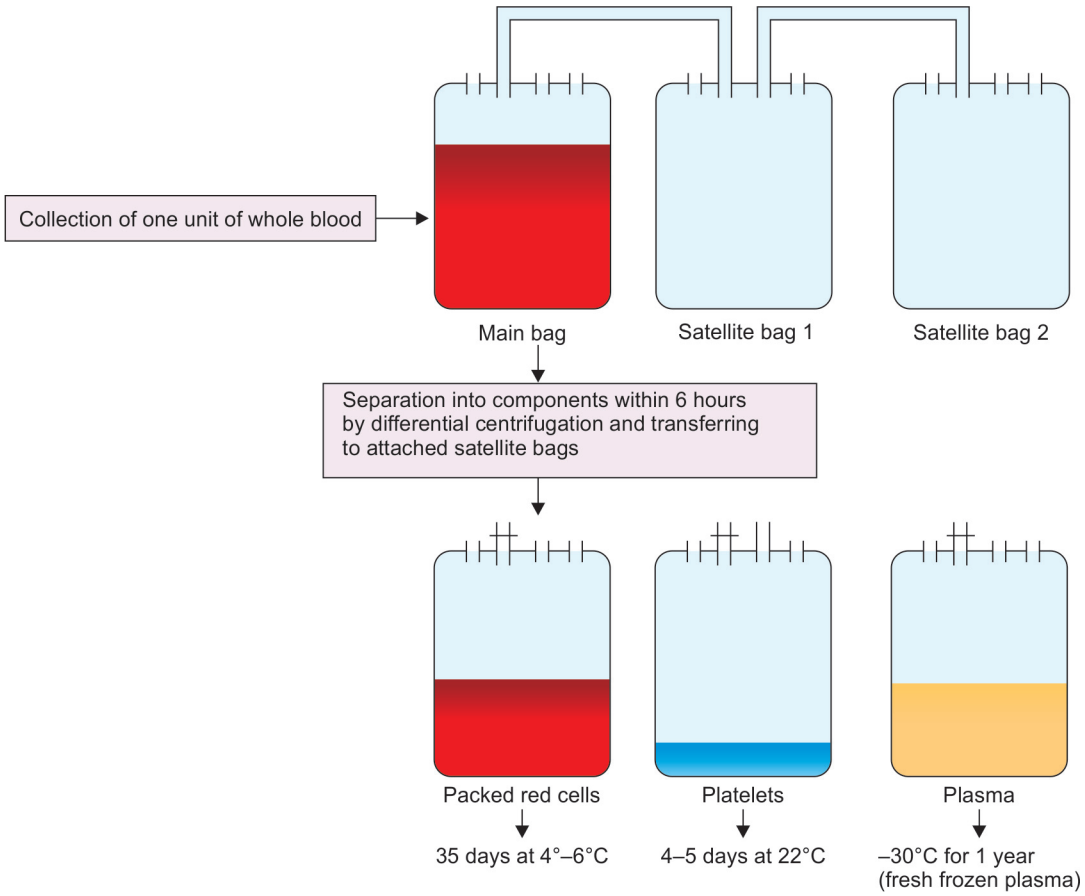


Fig. 17.12: Separation of blood components from whole blood

Table 17.6: 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/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/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 ≤–18°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°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°C.	Most useful for hypovolaemic shock or hypoproteinaemia

Contd.

Table 17.6: 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

an aggregated platelet button from PRP. About 200 ml plasma (platelet-poor) removed, leaving 50 ml pf platelet-poor plasma with platelet button. From this 200 ml **platelet-poor plasma FFP** is prepared.

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 17.7).

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 17.7: 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 17.8).

Table 17.8: 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)

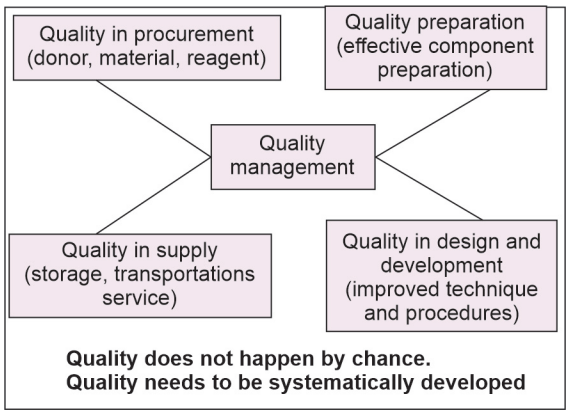
INTERNAL QUALITY CONTROL IN BLOOD BANK

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. 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. 17.13).



The quality requirements involve

- Quality control and proficiency testing
- Internal and external audits
- Personnel and organization
- Premises, equipment and materials
- Blood pocessing
- Complaints and component recall
- Investigation of errors and accidents

Fig. 17.13: 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 17.9: 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.

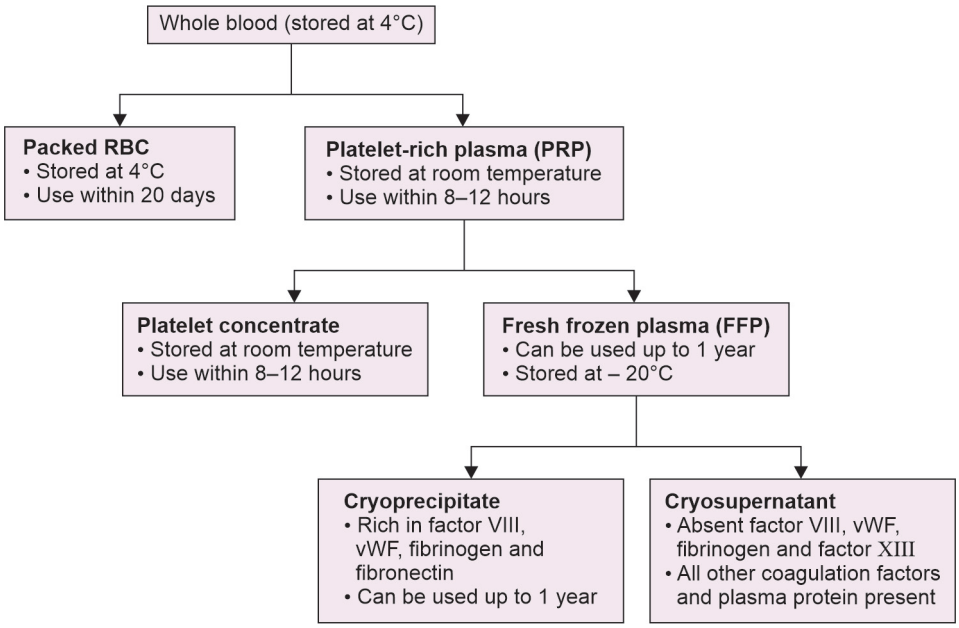


Fig. 17.14: Blood and different components and their use and storage

Chapter

18

Quality Control in Haematology

ISO definition of quality: "The degree to which a set of inherent characteristics fulfils requirements " (ISO 2000).

Features (definition) of quality: A measure of excellence or,

- a state of being free from defects, deficiencies, and significant variations,
- brought about by strict and consistent adherence to measurable and verifiable standards,
- to archive uniformity of output,
- that satisfies specific user requirements.

All laboratory tests are susceptible to error. It is virtually impossible ever to have a 0% error rate. To have value for clinical decision making, an individual laboratory test result must have a total error small enough to reflect the biological condition being evaluated.

For example, the methodology used for estimating the haemoglobin of a patient should yield consistent and accurate results, howsoever many times we perform the test on the same sample.

Quality control: It describes the steps taken by a laboratory to ensure that the tests are performed correctly. In simple words, this refers to 'minimization of errors'.

Quality control activities span the testing process from the moment of specimen collection until the time the physician receives the report. The primary purpose of quality control is statistically sample the measurement process to verify that the method continues to perform within the specifications consistent with acceptable systematic bias and imprecision.

Quality assurance: It is essentially similar to quality control. To ensure reliable test results with the necessary degree of precision and accuracy. However, it also includes steps taken by the laboratory to ensure that its quality control is working. So, quality assurance describes all the steps taken, both within and outside the laboratory to achieve reliable results.

Quality assurance (QA) in the haematology laboratory is intended to ensure reliable diagnostic test results with the necessary degree of accuracy and precision. There are some key definitions in QA which are fundamental to an understanding the process of QA.

- **Accuracy:** The closeness of agreement between the true value and the observed value.
- **Precision:** The closeness of agreement among a series of measurement of a single sample. A test result may be precise without being accurate.
- **Specificity:** Measures only the analyte of interest.

- **Limits:** The upper and lower limits of detection of the assay.
- **Range:** The interval between the upper and lower limits of detection.
- **Robustness:** A measure of how much a test or assay is affected by small variations in methodology.
- **Material standards or reference preparations:** These are used to calibrate analysers and to assign values to calibrators.
- **Reference method:** This is a precisely defined technique which, in association with a reference preparation, provides sufficient precise and accurate data for scientific purposes and for assessing the validity of other methods.
- **Selected method:** This is one which is directly comparable with and traceable to the international reference method. It serves as an alternative to the reference method when an international reference material is not available.
- **Working (or recommended) method:** This is intended for use in routine practice taking account of economy of labour and materials and ease of performance and having been down by a validation study with a reference method to be sufficiently reliable for its intended purpose.
- **Accreditation:** This is a process by which an authorised national body or organisation gives formal recognition that a laboratory is competent to carry out specific tasks, as mentioned against published standards. As for example, NABL (National Accreditation Board for Testing and Calibration Laboratories) gives accreditation to laboratories in India.

REFERENCE PREPARATIONS AND CONTROL MATERIALS

The main international authority concerned with reference preparations or reference reagents for laboratory medicine is **WHO (World Health Organisation).** Other international authorities who also supply reference materials are: **ICSH (International Council for Standardisation in Haematology), IRMM (Institute for Reference Materials and Measurements),** etc.

International standards (reference materials) are not intended for routine use but serve stable standards for assigning values to commercial (or laboratory produced) **'secondary standards or calibrators'** (Table 18.1)

Table 18.1: Reference standards and materials available internationally

Organisation	Standards and materials	Source
• WHO	• Blood products including haemoglobin A_2 (HbA_2) and haemoglobin • Blood safety • Coagulation factors • Fibrinolytic agents, protease inhibitors, anticoagulants • Human sera and immunoglobulins • Platelet-specific proteins • Haematinic	Most products are kept at the National Institute for Biological Standards and Controls (www. nibsc.org). The catalogue lists other custodian laboratories where appropriate (www.who.int/blood products/ catalogue).
• IRMM	• Leukaemia monitoring • Thromboplastin (rabbit and bovine) • Clinical chemistry • Haemoglobin cyanide • Human serum proteins	https: // ec. europe. eu/jrc/en/ reference – materials
• ICSH	• Haemoglobin reference standard	www.eurotrol.com

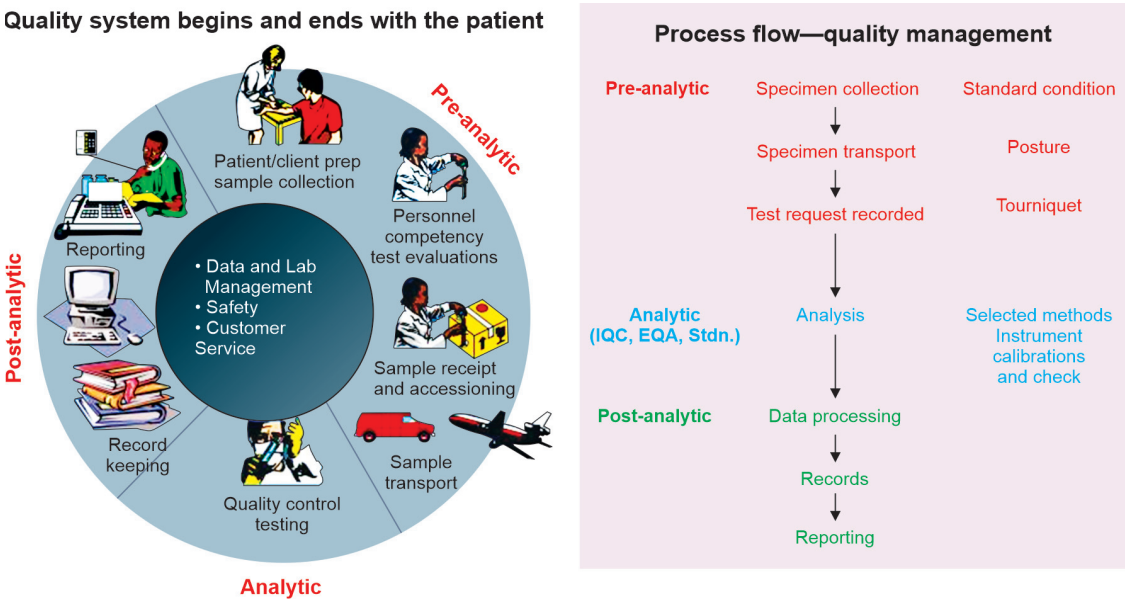
QUALITY CONTROL PREPARATIONS

Controls or quality controls are preparations which are used for either **internal quality control (IQC)** or **external quality assessment (EQA).** Some control preparations have assigned values but they should not be used as standards because the assigned values are usually only approximations and the preparations may be stable for a limited period.

Control may be available commercially and they can also be made locally. But these local made controls are difficult to make. Also, difficulties arise during validation of such homemade materials (Fig. 18.1).

Calibrators and Calibration

Multi-parameter haematology analysers require routine calibration checks and/or adjustment in order to produce accurate results.



Pre-analytical	Analytical	Post-analytical
Right specimen	Laboratory professionals	Recording
Right collection	Reagents	Interpretation
Right labeling	Equipment	Turnaround time
Right quantity	Selection of test SOP	Report to right user
Righ transport	Records	
Right storage	Biosafety	

Fig. 18.1: Factors influencing quality

Clinical Laboratory Standards Institute (CLSI) defines **calibration** as the process of testing and adjusting the instrument or test system readout to establish a correlation between the instrument's measurement of the substance being tested and the actual concentration of the substance.

Calibration verification is the testing of materials of known concentration in the same manner as patient specimens to assure the system in accurately measuring samples throughout the reportable range.

Calibration can be accomplished with the use of fresh whole blood samples which have been assayed by reference methods. **Streck calibrators** are stable suspensions of erythrocytes, leukocytes, and platelets. Assigned values are derived from replicate analysis on whole blood—calibrated haematology analysers.

When to Calibrate?

Calibration should take place in accordance with the recommendations outlined in the instrument operator manual, typically once every 6 months or whenever any of the following occur:

- All of the reagents used for a test procedure are changed to new lot numbers, unless the laboratory can demonstrate that changing reagent lot numbers does not affect the range used to report patient test results, and control values are not adversely affected by reagent lot number changes.
- Following a major preventive maintenance or repair/replacement of critical parts that may influence the test's performance. This includes sending a test system to the manufacturer for repairs.
- Control materials reflect an unusual trend or shift, or are outside of the laboratory's acceptable limits, and other troubleshooting means of correcting the unacceptable control values which fail to identify and resolve the problem.
- The laboratory has determined that the test system's reportable range for patient test results should be checked frequently. Calibration must be considered last step in a troubleshooting sequence. Frequent unnecessary recalibration can mask an underlying problem with the instrument's performance.

Calibration Process/Procedure

- Remove vials calibrator from refrigeration and warm to room temperature (18–30°C) for 15 minutes before use.
- Refer to mixing procedure by the supplied company.
- Prime the instrument by aspiring 2 normal blood samples. Disregard results.
- Follow the instruction for calibration in the instrument operator manual. Each instrument has specific requirements for the file to use and number of runs to be performed.
- Calculate the mean of each parameter.
- Compare the results from your instrument to the assay targets:
  a. If the recovered mean values are within the system specific assayed tolerance limits, and your control data does not indicate a change is warranted, the instrument does not require calibration. It may be helpful to correlate your laboratory control means with a peer group for this assessment.
  b. If the recovered mean values are close to the system specific tolerance limits and your control data indicates an adjustment is warranted, proceed with accepting the new calibration factors.
- Calibration of the specific parameter(s) should be done in accordance with the procedure in your instrument manual. Calibration may not be required for all parameters.
- Once calibration is complete, verify calibration by analysing the calibrator 3 times in the patient mode. Compare the

means of the results to the ranges on the calibrator assay sheet to verify the adjustment is appropriate.
- Print the new calibration factors and retain with your instrument records as documentation along the verification runs.

Quality control verification: Following calibration, it is recommended to run controls and verify results are within range prior to performing patient testing. Print the post-QC (quality control) results for your records.

Precision check: Prior to calibration, it is prudent to ensure proper instrument performance through a precision check (Fig. 18.2). It is recommended to use a normal patient sample less than 4 hours old for the purpose of this check.

Precision Procedure

- Run the sample consecutively in a replicate file, at least for 11 times.
- Discard the first run and obtain a mean, SD and CV for all parameters that can be calibrated.
- Evaluate the results. The CV % should fall within the acceptable limits of your specific analyzer, limits can be reference in the performance characteristics/specifications section of the instrument operator manual.
- If any parameter's CV % exceeds the limits, refer to the troubleshooting or diagnostic section of the operator's manual as necessary. Repeat the precision check.
- **Do not with calibration if the precision study does not pass:** If troubleshooting does not bring the CV % to within acceptable limits, you may need to contact service for your analyser.

Quality Assurance (QA) Programme

It includes

- Internal quality control (IQC)
- External quality assessment (EQA)/proficiency testing (PT)
- Standardisation

What is Quality Assurance?

- Simply it is all as about those activities and measures through which the laboratory can make sure that all generated results are reliable, i.e. accurate and precise.
- It is comprehensive that covers all aspects of laboratory, i.e. pre-analytical, analytical and post-analytical phase of testing.

What is Accuracy?

- Closeness of measured value to assigned **target value**. Any deviation in readings is attributed to systematic errors. It can easily be assessed by comparing the current findings to the **mean of true value** (Fig. 18.3).

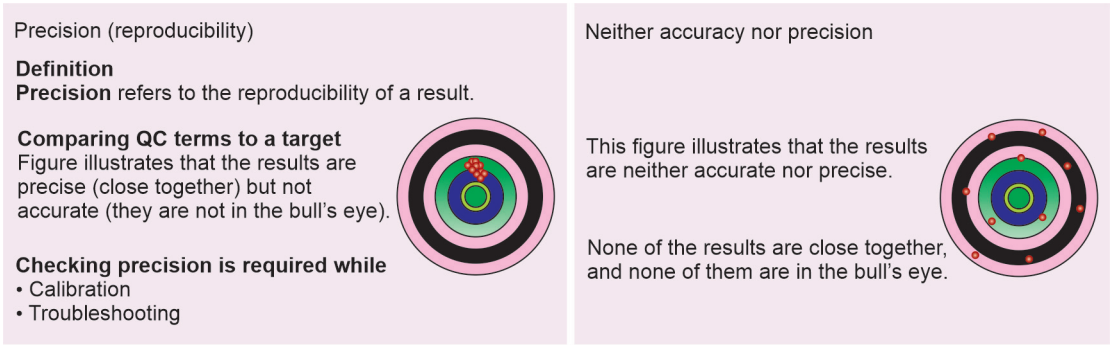


Fig. 18.2: Precision and not precision

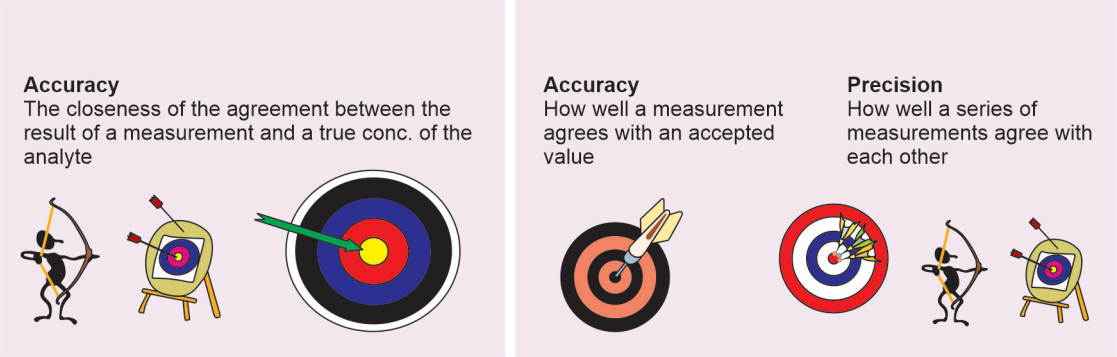


Fig. 18.3: Accuracy and precision

What is Precision?

- Closeness of measured value to each other (reproducibility or repeatability).
- Any noticeable deviation is attributed to random errors and it can easily be observed by checking SD and CV% .

What is Outcome of Compliance to QA?

- Enhancement of patient safety
- Trustfulness of laboratory results
- Customer satisfaction (customers may be patients, clinicians or clinical departments).
- Fulfilling the organization goals
- Reaching excellency (Fig. 18.4 and Table 18.2)

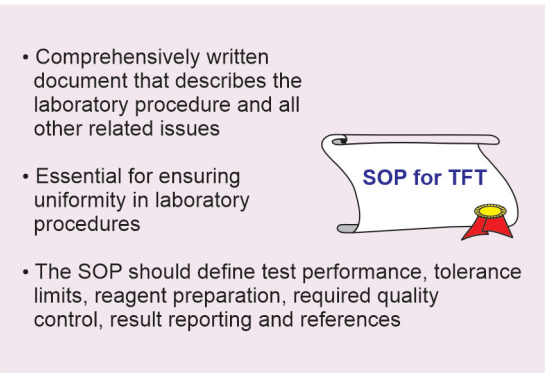


Fig. 18.4: Standard operating procedures (SOPs)

Table 18.2: Schedule for quality assurance procedures

1. Analysis of patient's results
Daily to check constantly of mean MCV, MCH, MCHC

2. Control chart with control material
- Daily, with each batch of specimens or at regular intervals in a continuous process
- Duplicate test on two or three patient's samples: If control chart or **delta check** shows discrepancies

3. Calibration with reference standards
- Diluting systems: Initially and at 1–2 weeks interval
- *Instruments:* 6-month intervals or more frequently if control chart or EQA indicates bias or fluctuation in results and after any repair/service
- Pipettes, balances: 6–12 months interval

4. Correlation assessment of test report
- Cumulative results: Following previous tests and if changes in clinical state.
- Blood smear examination if unusual test results and/or instrument flags appear

Components of QA in Haematology Laboratory

- Pre-analytical stage
- Analytical stage
- Post-analytical stage

Pre-analytical Stage

- Mainly concerned with specimens integrity. As per international survey (IOM), most of laboratory errors occur in pre-analytical stage followed by post-analytical stage, simply because of great human interventions at this phase.
- Anticoagulants must be EDTA for CBC, FBC, Hb electrophoresis, Citrated for ESR and trisodium citrate for coagulation tests.
- Order of blood draw: Coagulation first.
- Stability of blood must be ensured which is 4 hours for coagulation at room temperature. Specimen of special coagulation should be separated, double centrifugation and frozen.
- Specimens for haematology tests must be free from clots, properly filled and correctly labelled.
- Lipemic samples of CBC will result in erroneous readings, namely a high Hb, MCV and MCH. Therefore, lipaemic samples must be pretreated to obtain a correct result. A simple way to do so is to centrifuge it, remove the plasma and replace the same volume you extract with **isotone**. Do this for 2–3 times and re-run the sample.
- Proper mixing on rotator is crucial for reliable FBC test. Improper mixing will result in false flags, namely M0 blast, basophilia or any others and eventually unnecessary hold of differential counts.
- Diluted specimens must be suspected from CBC report (if visually not detected). Do proper correlation through delta check and match with biochemistry.
- Cold agglutination must be suspected from CBC report; obviously it will result in Hb/RBCs matching failure (Rule of 3) in addition to high MCH, MCV. So, sample should be incubated for 30–60 minutes before testing as well it is recommended to run it on **manual mode** since it is faster than **automatic mode**.
- Lastly remember as good is your specimen's integrity as reliable is your final report.

Analytical Phase (Stage)

Quality control of analytical phase is composed of:

- Infernal quality control (IQC)
- External quality assessment (EQA) or proficiency testing (PT)

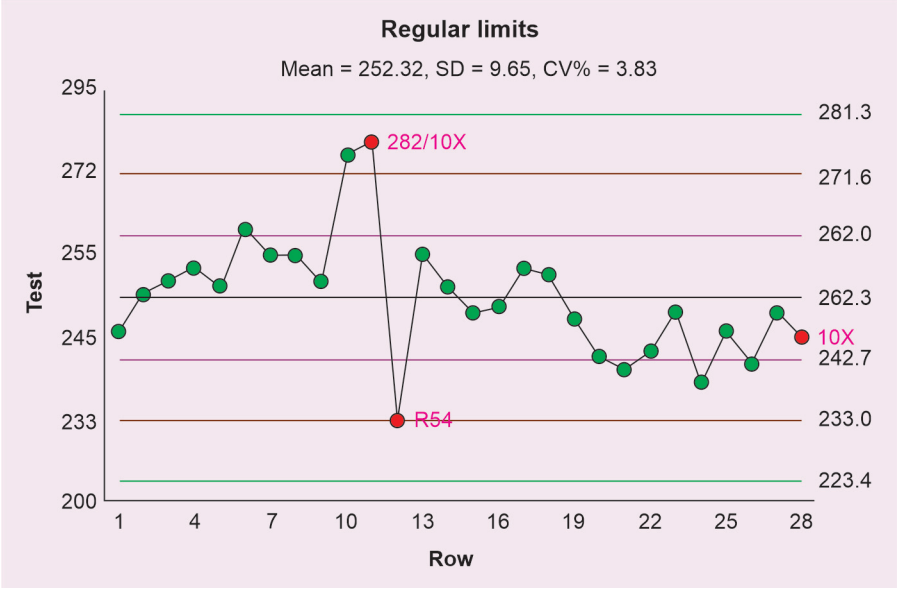
INTERNAL QUALITY CONTROL (IQC)

IQC monitors the performance of the test procedure in laboratory on daily or batch-to-batch basis. This includes measurements on specially prepared control materials, repeated measurements on patient's specimens and statistical analyses of patient's test data. It ensures continual validation of the reliability of the results produced by the laboratory, before reports are released.

Control charts: These were first applied in clinical chemistry by **Levey-Jennings** as a means to monitor laboratory performance using control materials. Now, this is widely utilised for both automated and manual procedures used in haematological laboratory. These charts are known as **Levey-Jennings charts or popularly LJ charts** (Fig. 18.5).

Controls with low (level 1 or L1), normal (level 2 or L2) and high (level 3 or L3) should be used to monitor the method, across the range of its linearity (Fig. 18.6). It is advisable to run at least one control sample per batch. The result obtained with the control samples can be plotted manually on a LJ chart as described below, or it can be plotted automatically by the instruments, which may give alert signal, if the analysis falls outside the preset, acceptable limits of performance.

When plotting a control chart manually, the mean value and standard deviation (SD) of the control specimen is first established. Then using arithmetic graph paper, draw a horizontal line to represent the mean (as a



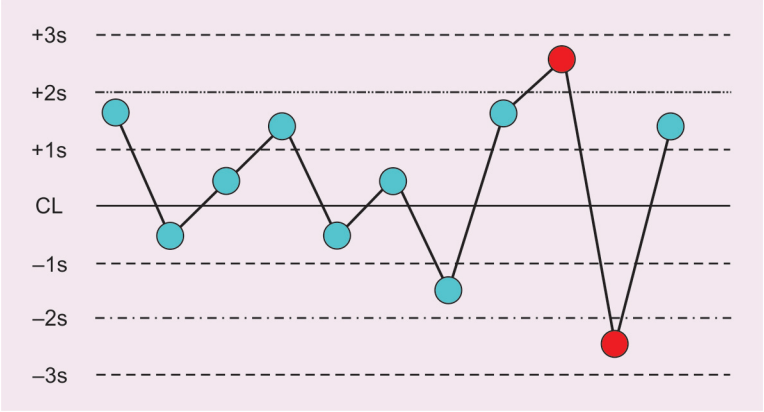


Fig. 18.5: Levey-Jennings charts or popularly LJ charts

Fig. 18.6: Quality controls (L1, L2 and L3)

base) and on an appropriate scale of quantity and unit, draw lines representing +2SD and –2SD above and below the mean. Plot the results of successive control sample measurements. If the test results are within +2SD or –2SD, then results are satisfactory. If ≥±2 SD but ≤± 3SD, then there is caution. If result is >3SD, then it is unacceptable (gross error or blunder). Laboratory shall use 2 levels of controls at least once a day. The 24 × 7 laboratory shall use these controls every 12 hours interval.

i. Duplicate Test on Patient's Specimens

This is the easiest procedure to carry out. If it is possible, every specimen should be tested in duplicate. For this calculate the standard deviation; SD = $\sqrt{\left(\frac{\Sigma \text{ of } d^2}{2n}\right)}$,

where d^2 = difference between duplicates squared,

n = number of specimens tested in duplicate

Interpretation: None of the duplicate tests should differ from each other by more than 2 SD as calculated. This is a method to identify random errors. If the test is always badly done the SD will be wide and will not be sensitive to individual errors.

Example: Suppose 10 specimens have been selected for WBC count (Table 18.3).

Table 18.3: WBC count or TLC (× 10^9/L)

Specimen	1st count	2nd count	d	d^2
1.	5.4	5.8	0.4	0.16
2.	8.3	10.5	2.2	4.84
3.	17.2	18.0	0.8	0.64
4.	5.4	5.4	0	0
5.	12.2	11.8	0.4	0.16
6.	14.3	13.8	0.5	0.25
7.	6.2	6.4	0.2	0.04
8.	8.2	8.6	0.4	0.16
9.	7.3	7.5	0.2	0.04
10.	5.4	5.9	0.5	0.25

So, $\Sigma d^2 = 6.54$ and $\frac{d^2}{2n} = \frac{6.54}{20} = 0.327$

Hence, SD = $\sqrt{\frac{\Sigma d^2}{2n}} = \sqrt{0.327}$

$= 0.5718 \simeq 0.57$

Therefore, SD = 0.57 and 2 SD = 1.14

Conclusion: Test on specimen 2 is unsatisfactory, and must be repeated.

ii. Check Tests

These are similar to duplicate tests but they use specimens which have been measured originally in an earlier batch.

Interpretation: The tests should agree with each other within ±2SD. This procedure will detect deterioration of apparatus and reagents which may develop between tests if it is certain that there has been no deterioration in the specimens on storage. Thus, this test is suitable for Hb and RBC, less so for WBC and platelets, and it is unsuitable for PCV, if there is a delay of six hours on longer between the two tests.

It is useful the same specimens for check tests and duplicate tests. The SD can be established on the basis of technical competence from duplicate tests. If the SD for the check test is greater, this is a clear indication of deteriorating apparatus or reagents **provided that the specimens have not altered.**

iii. Delta Test

This is a **comparison of the current result with a recent previous result** on the same patient. It is not appropriate when patients are being actively treated or when the clinical circumstances may result in a change in the blood count. As account must also be taken of diurnal variations, only relatively large differences in the count should be considered to indicate the likelihood of a fault:

Hb: 2 g/dl

PCV: 0.5

MCV: >6 fl

MCH: >5 pg

WBC: Normal to abnormal or 20–25% change

Platelets: Reduced or increased by more than 50%

The blood count on any patient should not differ from counts obtained in the previous 2–3 weeks by more than an amount which takes into account both the test CV and physiological variation, providing that the patient's clinical condition has not altered significantly.

The test can, of course, also be carried out on the blood of healthy individuals whose blood count remains virtually constant on a day-to-day basis, subject only to physiological change.

iv. Replicate Tests on Control Specimens

Repeated measurements on a single specimen will define the error of reproducibility (precision) and it is a method for evaluating technical excellence and/or an instrument which is unstable. As all the measurements are carried out with the same pipettes and reagents, it will not detect faults in these.

This test can be performed on any suitable blood sample but it is useful to use control material when the SD of the repeat instruments has been obtained, as described below, this can be used for the control chart, For Hb, use the lysate or preserved blood. For replicate testing do eleven identical tests in one sample.

Calculate mean

Calculate the difference from mean for each measurement.

Calculate SD = $\sqrt{\frac{\Sigma d^2}{n-1}}$, where Σd^2 = sum of square differences

Calculate CV% = $\frac{100\,SD}{mean}\%$, CV = coefficient Variation

Interpretation: The SD and CV give an index of precision. The desirable level of precision should be such that errors caused by the measurement procedure do not significantly affect clinical interpretation of the measurement. Thus, for example, if a clinician usually diagnoses that a haemorrhage has occurred when the haemoglobin falls by 10% of its previous level, it is necessary to be confident that the CV of the test is <5%. This means that the SD of measurement should not be greater than 4 g in 80 g/L or 8 g in 160 g/L.

With some tests, such as RBC by haemocytometry; it is not possible to achieve the desirable level of precision by the usual technique in the routine laboratory, as the CV is usually in the order of 10%. On the other hand, with automated cell counters one expects a CV of 2% for the RBC, as these instruments have a high level of precision.

Example: Consecutive measurements of Hb (g/L) are:

142, 141, 146, 144, 143, 140, 146, 150, 150, 143, 146

Mean $(\bar{x}) = \frac{1591}{11} = 144.6$

Sample No.	Hb level in g/L	$d = (x - \bar{x})$	$d^2 = (x - \bar{x})^2$
1.	142	2.6	6.76
2.	141	3.6	12.96
3.	146	1.4	1.96
4.	144	0.6	0.36
5.	143	1.6	2.56
6.	140	4.6	21.16
7.	146	1.4	1.96
8.	150	5.4	29.16
9.	150	5.4	29.16
10.	143	1.6	2.56
11.	146	1.4	1.96

So, $\sqrt{\frac{\Sigma d^2}{n-1}} = \sqrt{11.05}$

= 3'32 and SD = 3.3

Hence, $CV\% = \frac{3.32 \times 100}{144.6} = 2.30$ or 2.3%

$$[CV\% = \frac{SD \times 100}{mean}]$$

Conclusion: Although there was a 10 g/L difference between highest and lowest levels, overall haemoglobin measurement is being with an acceptable level or precision for clinical purposes.

v. Control Chart

This uses the mean and SD obtained on the control material (lysate or preserved blood) as its basis. Originally devised for industrial purposes by W. A. Shewart, it was applied to clinical laboratories by Levey-Jennings, known as **LJ chart**.

Interpretation: When the test is in control, all the measurements on successive samples will approximate to the established mean, with only minor deviations which will oscillate above and below the line of the mean. The chart will suggest that there is a **fault in the technique, instrument, pipette or reagents,** if one of the following occurs.

- One control value outside the mean ± 2SD → warning
- One control value outside the means 3SD reject; SE or RE
- Two consecutive controls exceed mean 2SD → reject: SE
- Four consecutive controls exceed mean +1 SD or mean – 1 SD → reject: SE
- Six consecutive controls on one side of mean → warning: SE

Where SE = systematic error and RE = random error.

But first check that the material itself has not become infected or in other ways has begun to deteriorate.

vi. Cumulative SUM (CUSUM) Control

This is another way to display the data obtained in the test for precision. The CUSUM is the running total of the differences between successive measurements and the mean which was established initially. The plus sign and minus sign must be taken into account (Fig. 18.7).

This is regarded as more sensitive indicator of faulty technique or equipment. It is especially useful for detecting a consistent change in performance due to drift, as there will be a progressing increase in the deviation (plus or minus). By contrast, when there are only random differences from the mean some will be positive and some will be negative, i.e. plus and minus, so that the CUSUM will oscillate around zero.

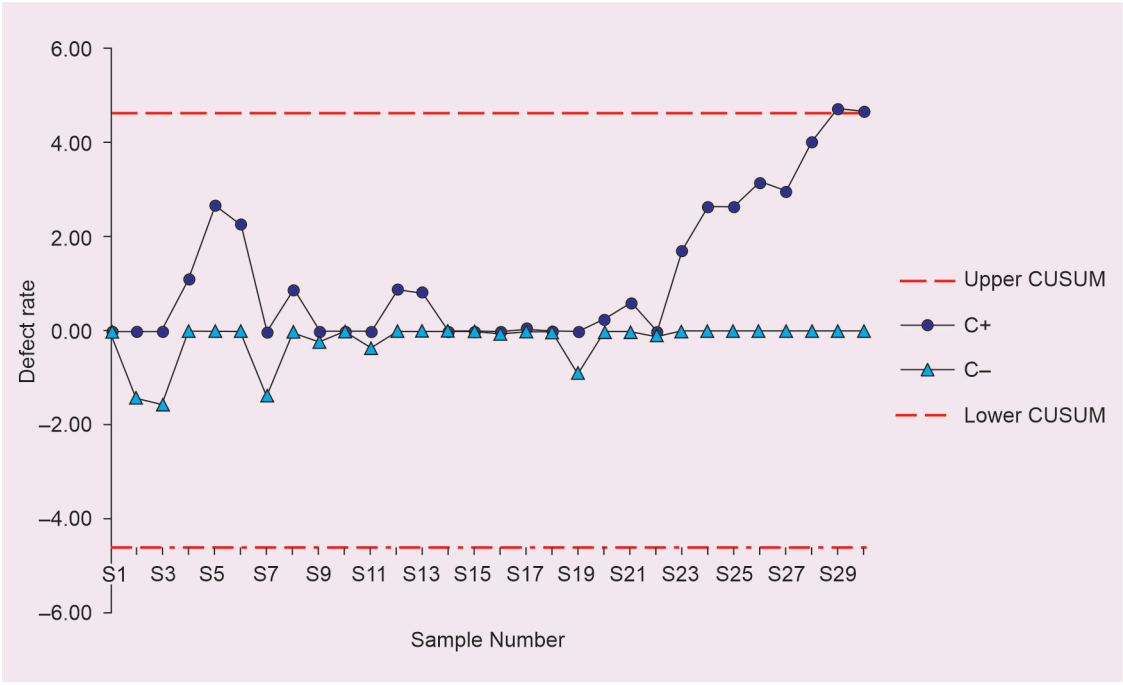
An advantage of CUSUM is that it can be incorporated into a computer programme so that the user is alerted when a problem occurs without the need to plot the data as long as control results are within prescribed limits. The CUSUM method is as follows:

- Decide on minimum significant change to be detected; d = usually 2SD.
- Calculate "K" = d/2
- Calculate upper reference value (URV) = mean + K

  Lower reference value (LRV) = mean – K
- Calculate the decision interval = 2.0 K

Method of CUSUM result recording

- Set CUSUM at zero.
- Do not start the CUSUM if the control value lies between URV and LRV
- Start CUSUM if control value (X) lies outside URV or LRV. The CUSUM figure is the difference between the control value and appropriate RV, i.e. either X-URV or LRV-X.
- To this difference add the next CUSUM difference and continue to add sequentially the difference between the control value and the same reference value, even when the control value falls with the reference values or outside the opposite reference value.
- If the CUSUM sign changes but the value is outside the opposite reference value,



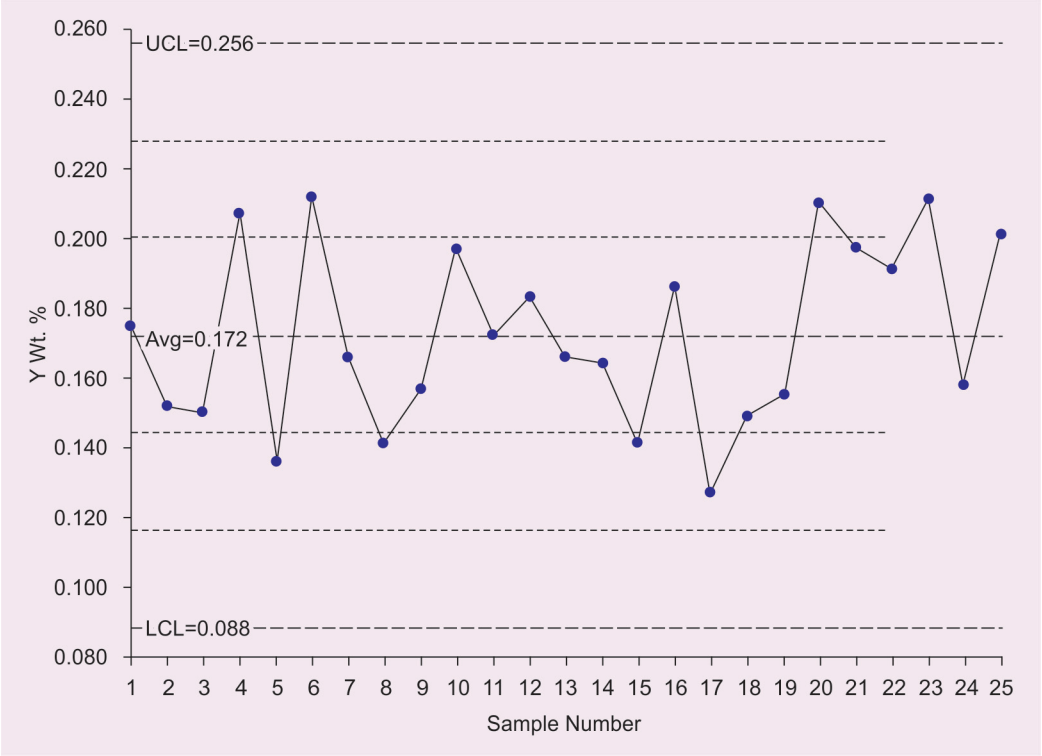


Fig. 18.7: QI Macros CUSUM chart

this indicates a possible abrupt shift in calibration.

- If the CUSUM equals or exceeds the decision interval, this suggests a significant change in accuracy: Check calibration, correct as necessary, set CUSUM to zero and start a new CUSUM.

vii. Correlation Check

This implies that any unexpected result of a test must be checked to see whether it can be explained on clinical grounds on it correlates with other tests. Thus, for example an unexpected higher or lower haemoglobin might be explained by a blood transfusion or a hemorrhage, respectively. A low MCHC should be confirmed by demonstrating hypochromic RBCs on a Romanowsky-stained blood film; a high MCV must correlate with macrocytosis. Similarly, the blood film should be examined to confirm a leukocytosis or leukopenia, a thrombocytosis or thrombocytopenia. But be careful as the blood film itself may be misleading if not correctly made and stained.

EXTERNAL QUALITY ASSESSMENT (EQA) SCHEME

A variety of external quality assessment tools are available in Western Europe and North America to assist laboratory management in identifying areas of concern in clinical testing and to meet external regulatory requirements. These are variously referred to as to survey or proficiency materials which may consist of plasma, whole blood, virtual microscopic images and photomicrographs. The materials are processed through laboratory workflow similarly to patient specimens. The results are scored and are returned to the hospital by authorised body who are sending proficiency materials as a report, often with a commentary written by experts in the field. Individual laboratories must have corrective policies to address substandard test results. This programme is known as **proficiency testing or PT programme**.

In North America, these services are largely provided by the College of American Pathologists (CAP, Northfield, IL, USA). Other prominent organisations that provide proficiency testing (PT) are North American Specialised Coagulation Laboratory Organization (NASCOLA). American Proficiency Institute (Lansing, MI, USA) and the United Kingdom National External Quality Assessment Scheme (NEPAS, Watford, UK). Although the US-based organisations are not affiliated with the government, Joint Commission guidelines and Medicare reimbursement rules stipulate that clinical laboratories in the United States participative in surveys.

In India, two major haematology EQA programs pun by two large medical institutions, All India Institute of Medical Sciences, New Delhi (AIIMS, New Delhi) and Christian Medical College, Vellore (CMC, Vellore). AIIMS runs Hemogram or CBC PT program, whereas CMC runs Hemostasis Module through Departments of Transfusion Medicine and Haematology.

The **Haemogram or CBC EQA Program** has more than 3000 participants. This program is based on WHO International Council for Standardisation in Haematology: 2008, ISO/IEC 17043: 2010 and ISO/IEC 13528; 2015 standard.

The **haemostasis module** was initially funded by a grant from the Katherine Dormandy Trust, partnered with UKNEQAS and targeted about 25 laboratories which performed testing for patients with haemophilia. After that in 2004, this EQA program was opened to all laboratories in the country along with production of stable homogeneous lyophilized material in India and data management at CMC, Vellore. The basic program of this haemostasis module provides testing for the screening tests and fibrinogen assays. The additional program offers assays of factor VIII: C, factor IX, von Willebrand antigen, ristocetin cofactor assays. This program also provides inhibitor assays. EQAS of CMC has also extended

support to some countries like Philippines (2006), Thailand (2007), Sri Lanka (2009) China (2010), and South Africa (2014-ongoing). CMC, Vellore also provides EQAS for coagulation.

In recent years, Tata Memorial Hospital, Mumbai has initiated a **flow cytometry, EQA Program** for diagnostic immunophenotyping, B-ALL, MRD, and stem cell enumeration.

ICSH (International Council for Standardisation in Haematology) has prepared guidelines for the organisation and management of EQA schemes, which are intended to help maintain a meaningful standard in the organisation of such schemes and to harmonise the way they all function. The following principles and technical criteria should be met in EQA programs:

i. Specimens should be sufficiently frequent to make sequential performance records meaningful and to identity, participants who are persistently unsatisfactory as soon as possible.
ii. There should be at least two specimens, for every test with values at diagnostically critical levels.
iii. The materials used in surveys must be stable, at least until the closing date of survey.
iv. Specimens for blood count and other high throughput tests should be distributed monthly and other tests every 2–3 months interval.
v. The survey specimens must be negative for HIV, hepatis B and C antigens.
vi. EQA schemes must be financially and operationally independent of commercial pressure.
vii. Data processing must be very rapid, and reports should be sent to the participants promptly.
viii. **Confidentiality of the participants,** as well as organiser must be maintained. Provision of information on an individual participant's results to a third party (e.g. a licencing authority or during NABL assessor visit in India) is the responsibility of the participant and not the EQA organiser.
ix. To ensure that EQA relates to practices, survey samples should simulate natural specimens as closely as feasible. The participating laboratories should be obliged to handle them in the same way as they handle routine specimens.
x. EQA provider organisations should work independently.

For some tests, participation in PT program is not a feasible option for one or more of the following reasons:

- Non-availability of a formal national PT program for analytes of interest.
- Only few laboratories (<20) performing/participating the test.
- The analyte to be measured is unstable, e.g. blood gases, ammonia, G6PD.
- Control material of the same matrix is not available.
- The sample is completely consumed during performance of the test (e.g. ESR).

Alternative Approaches

For those tests where a formal EQA is not available, the laboratory shall adopt alternative approaches to validate performance.

Such alternative approaches are

- Replicate testing
- Examination of split samples (within the laboratories by different personnel)
- Use of reference methods and materials, where available
- Exchange of samples with other accredited laboratories.

  When the laboratory exchanges samples with other laboratories as an alternative approach to EQA participation, following needs to be addressed:

  a. In the case comparison between 2 laboratories, one will function as the "reference laboratory" against which

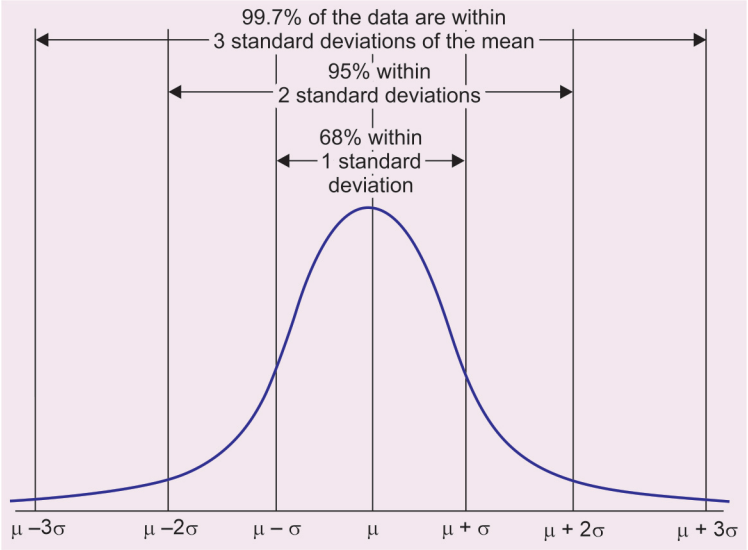
the other will be compared. This is to be documented as an MoU (memo of understanding).

b. When there are several laboratories, compare the result against the "reference laboratory".

Measurement of Uncertainty (MU) (Fig. 18.8)

Definition: Measurement of uncertainty is a parameter, associated with the result of measurement which defines the range of the values that could reasonably be attributed to the measured quantity.

Measure of uncertainty (MU) relates to the margin of doubt that exists for the result of any measurement, as well as how significant the doubt is. For example, a piece of string may measure 20 cm plus or minus 1 cm, at the 95% confidence level. As a result, this could be written as: 20 cm ±1 cm, with a confidence of 95%. Therefore, we are 95% sure that the piece of string is between 19 cm and 21 cm long.



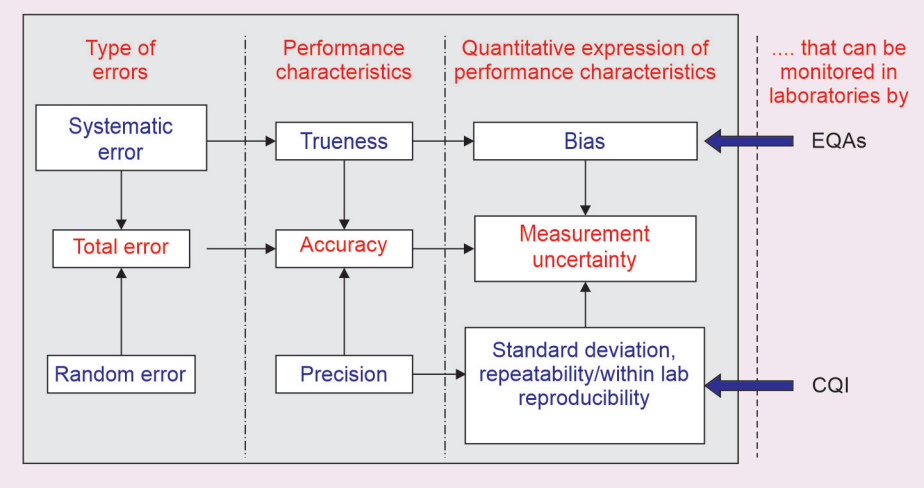


Fig. 18.8: Measurement of uncertainty (MU) and error measurement

How do we calculate measurement of uncertainty (MU) using quality control (QC) data?

Employing your QC data to calculate uncertainty makes several assumptions, your test system is under control, the patient samples are treated in the same manner as your controls and gross **outliers** have been removed. If you choose to use your QC data to calculate this, you should ensure that you use a commutable control with a matrix, similar to that of a patient sample, with analytes present at clinically relevant levels.

To calculate MU, laboratories must look at the intra-assay precision and inter-assay precision of their test.

Intra-assay precision: Sometimes known as **"within run" precision** is where 20 or more replicates of the same sample are run at the same time, under the same conditions (calculated from a single experiment). Intra-assay precision helps to **assess systematic uncertainties**.

Inter-assay precision: Sometimes known as a **"between run" precision**, is where 20 or more replicates are run at different times, e.g. 1, replicate every day, for 20 days (can be calculated from routine IQC data). Inter-assay precision can help **identify random uncertainties** within the test system.

The Australian Association of Clinical Biochemists (AACB) recommends that at least 6 months' worth of QC data are used when calculating the inter-assay precision.

Once the data is collected, you must calculate the standard error of the mean (SEM) of the intra-assay precision (A) and the SD of the inter-assay precision (B) in order to measure uncertainty. Once A and B have been calculated, they need to be squared, added together and the square root of the sum found:

$$u = \sqrt{A^2 + B^2}$$

As uncertainty is calculated as SD and 1 SD is equal to 68% confidence on a standard Gaussian curve, we can conclude that if we multiply using a coverage factor of 2, we can attain 2 SD confidence of 95%. This is known as the **expanded uncertainty (U):**

$$U = 2 \times u$$

What is the Advantage of Measurement of Uncertainty (MU) for a Lab?

Laboratories need to carry out MU as it is a requirement of ISO: 15189. It states as "The laboratory shall determine measurement uncertainty for each measurement procedure, in the examination phases used to report measured quantity values on patients' samples. The laboratory shall define the performance requirements for the measurement uncertainty of each measurement procedure and regularly review estimates of measurement uncertainty".

MU also helps determine whether the difference between two results is negligible due to uncertainty on significant due to a genuine change in condition of the patient, giving a greater confidence in reported results."

Z-SCORE

A Z-score is numerical measurement used in statistics of a value's relationship to the mean (average) of a group of values, measured in terms of standard deviations (SD) from the mean. If a Z-score is 0, it indicates that the data point's score is identical to the mean score. It is also called **standard score**.

So, Z-score is the difference between the value reported by the laboratory and the assigned value corrected for variability.

$$\text{Z-score} = \frac{\text{Reported value} - \text{Assigned value}}{\text{Standard deviation}}$$

$$\text{Or, Z-score} = \frac{x - \mu}{SD}$$

[Where, x = test value, μ = mean value and SD = standard deviation]

For example, let us say you have a test score of 190. The mean value is 150 and standard deviation 25. Then Z-score will be:

$$Z = \frac{x - \mu}{SD} = \frac{190 - 150}{25} = 1.6$$

The Z-score tells you how many standard deviations from the mean you score. In this example, you score is 1.6 standard deviations above the mean.

Interpretation: Z-score 0 to 2: Acceptable, Z-score 2 to 3:Warning signal, Z-score >3: Unacceptable [as per ISO/IEC13528: 2015 standard] (Fig. 18.9).

Note

i. Z-score value between 0 and ±2 is texted in green colour
Z-score value between ±2 and ±3 is texted in orange colour.
Z-score value > ±3 are texted in red colour.

ii. Z-score among lab (EQA) = your result sum of two values:
Consensus result sum of two values/(normalised IQR).
Z-score within lab (IQA) = your result difference of two values
– Consensus result difference of the values/(normalised IOR)

IQR (interquartile range): In descriptive statistics, the interquartile range or IQR, also called the midspread, middle 50%, or H-spread, is a measure of statistical dispersion, being equal to the difference between 75th and 25th percentiles, or between upper and lower quartiles,
IQR = $Q_3 - Q_1$
Normalised IQR = 0.7413 × IQR.
Use of IQR:
a. The IQR can be used to identify outliers.
b. Unlike total range, the IQR has a breakdown point of 25%, and is thus often preferred to the total range.

iii. Homogeneity and stability testing of PT sample are done as per ISO 13528: 2015 Standard. To pass homogeneity test, between sample SD (Ss) should be smaller than the check value (0.3* SDPA). To pass the stability test, average difference in measurement values of first and last day sample. $(\overline{X} - \overline{Y})$ should be smaller than the check value (0.3* SDPA).

So, Z-score = $\frac{\text{Your result} - \text{Comparator mean}}{\text{Comparator SD}}$

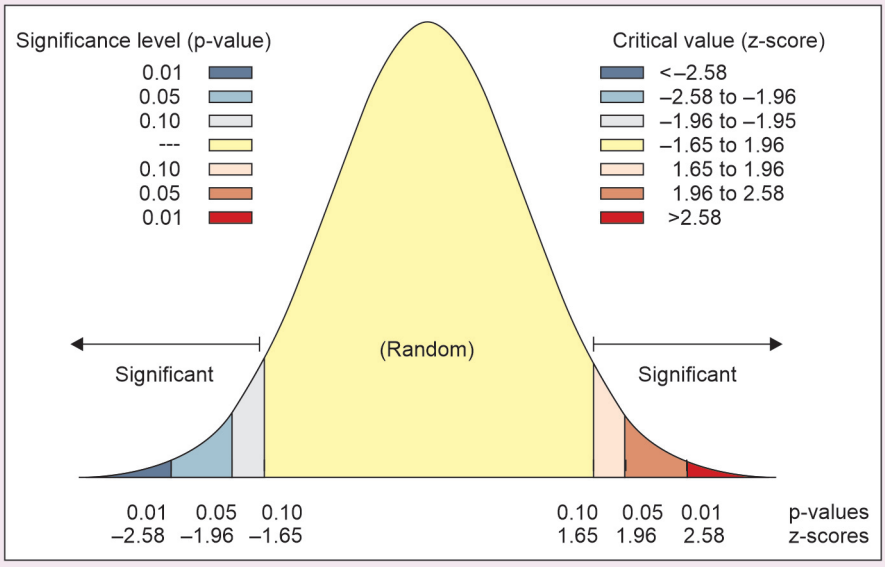


Fig. 18.9: Z-score

TYPES OF ANALYTICAL ERRORS

Analytical errors fall into two subcategories according to EURACHEM/CITAC Guide CG4 as follows:

i. Random errors (RE), and
ii. Systematic errors (SE)

Random errors (SE): Result of a measurement minus the mean that would result from an infinite number of measurements of the same measurand (test).

The mathematical definition is ΔRE-xi–$\bar{x}$ (where xi = a single measurement and $\bar{x}$ = the average of successive measurements).

In fact, random errors affect the precision of all measurements. Random errors are attributed to either undetermined reasons (inherent error) or well-defined causes. Their magnitude (ΔRE) is equal to the precision of a measurement and it is always greater than zero ($\Delta RE > 0$). ΔRE can be diminished by increasing the number of measurements (it influences the $\bar{x}$). Lange ΔRE increases the dispersion of the results of a true value.

Systematic errors (SE): Systematic error is defined as a component of error which, in the course of a number of analyses of the same measure and (test), remains constant or varies in a predictable way. Quite often is attributed as:

Mean that would result from an infinite number of measurements of the same measure and carried out under repeatability conditions minus a true value of the measure and is expressed mathematically as $\Delta SE = \bar{x} - \mu$ (where $\bar{x}$ = the average of successive measurements and μ= a true value).

Systematic errors can be attributed to certain reasons and therefore can be eliminated much easier than random errors. cannot be diminished by increasing the number of measurements. As opposed to random errors their magnitude can be zero ($\Delta SE \geq 0$).

Total analytical error: The mathematical definition of the total analytical error (TE) is:

Total analytical error = Random error + Systematic (TE = $\Delta RE + \Delta SE$)

Under ideal circumstances, total analytical error equals to zero, but this cannot be achieved in daily practice. Only ΔSE can be zero ($\Delta SE \geq 0$), whereas ΔRE is always greater than zero ($\Delta RE > 0$), because of the existence of inherent error.

Since TE > 0 is unavoidable, TE of every single determination must be lower than a specified limit. This limit is called "allowable total analytical error" (a TE) and it is different for each analyte being determined in a clinical laboratory.

EQAS AND EVALUATION

i. Consensus Method

From the results obtained by the participants, the centre (PT provider) calculates a mean or median and SD. If the mean is used, its SD is calculated in the usual way by the formula:

$$SD = \sqrt{\frac{(x - \bar{x})2}{n - 1}}$$, (where x = individual result, $\bar{X}$ = mean and n = total number of results.)

The data are then adjusted by excluding any which are >2SD from the mean. The mean and SD (now termed "weighted") are then recalculated. If the median is used its SD is calculated as central 50% ÷ 1.35. By this procedure, outlying results or outliers are automatically excluded in the calculation.

For these calculations of SD to be meaningful, there must be at least 20 participants in a set, and at least half the participants should have sufficiently good performance in comparison with each other to avoid having a (weighted) SD which is unhelpfully wide.

The SD may also be adjusted by one of the following methods

- Restrict analysis to a selected group of good performance

- Use a predetermined constant CV from which the SD of the specific sample is calculated.
- Use a predetermined SD based on clinical significance.
- Calculate a mean SD from pooled SDs for all samples in the previous six months, including the present survey.

If different methods are known to react in different ways, giving different measurement on the same analysis, it may be necessary to treat the participant results in each group separately.

A **"deviation index" (DI)** is then calculated for each individual participant. Deviation index (DI) is analogous to a 'Z-score'. The DI is the amount of deviation of the participant's result (xi) from the target value. (Xpt) relative to a unit of 1 SD calculated for use in the EQA or proficiency testing scheme (δ_{pt}). The DI is then calculated using this formula:

$$DI = \frac{xi - Xpt}{\delta pt}$$

$$Or \quad = \frac{Actual\ result - weighted\ mean\ or\ median\ test}{Adjusted\ SD}$$

The median should be used rather than the mean when there is a non-Gaussian distribution with a wide scatter of results.

Based on ISO 13528, a DI of less than 0.5 from the target mean or median denotes excellent performance.

<± 0.5: Excellent performance

<1: Satisfactory performance

1.0–2.0: Still acceptable but borderline

2.0–3.0: Requires review of techniques and check on calibration

>3.0: Defect requiring urgent investigation

ii. Assigned Value Method

When calibration and control materials are made, it is possible to assign values by, it is possible to assign values by using reliable methods, as far as possible internationally recommended reference methods together with certified reference materials. It is also possible to establish the reproducibility for each test as performed by one or more skilled worker in the national or regional centre. Thus, results from participants can be judged by the extent or the deviation from the assigned mean in terms of clinical decision-making significance and taking account of unavoidable imprecision of the method used, as well as normal diurnal variations. The following limits are adequate to meet these requirements:

- Hb, RBC (by electronic), and PCV: 4%
- MCV, MCH, MCHC: 5%
- WBC: 10%
- Platelet count: 15%
- Reticulocyte count by microscopy: 30%

This method is valid no matter how few participants there are. Their limitation is that there is no assurance to that the methods used in assigning values to the participants are themselves to free of bias. It is advisable for the centre to use several different techniques for each test, and for the tests to be performed in three different laboratories which have been designated as **reference centres** because of their expertise.

CALIBRATION

i. Calibration of Volumetric Pipettes

(Fig. 18.10)

The volumetric pipette is filled to the calibration mark with distilled water, which is then transferred to a weighed beaker in accordance with the normal usage of the pipette. The beaker is reweighed. The ambient temperature is noted. The volume of the pipette (in ml) is calculated by dividing the weight of water (in mg) by one of the following factors depending on temperature:

Temperature		Factor
18	⟶	0.9986
19	⟶	0.9984
20	⟶	0.9982
21	⟶	0.9980

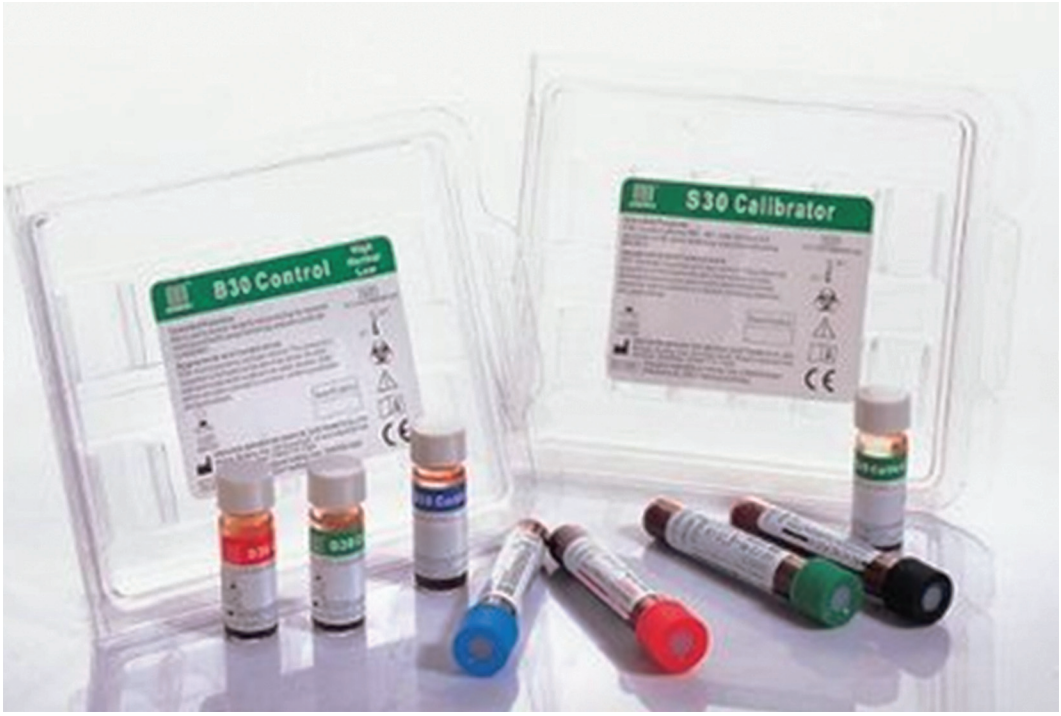


Fig. 18.10: Calibrators (control of controls)

22	⟶	0.9978
23	⟶	0.9976
24	⟶	0.9973
25	⟶	0.9971
26	⟶	0.9968
27	⟶	0.9965
28	⟶	0.9963
29	⟶	0.9960
30	⟶	0.9957

The calibration must be performed in duplicate for each pipette.

ii. Calibration of Micropipettes

The micropipettes commonly used in haematology have a capacity of 20 µl (0.02 ml). The procedure described in above method can be applied. However, as the weight of 20 µl of water is approximately 20 mg, to ensure a measurement error <2%, it is essential to use a precision balance with a sensitivity of 0.1 mg (0.0001 g). As this type of balance is not commonly available, the following modified method is recommended.

As a first step, calibrate an 0.2 ml pipette, a 5 ml pipette and a 50 ml volumetric flask by measuring the weight of water on an analytic balance as described in above method noting the ambient temperature. The true volume (in ml) is obtained by dividing the weight of water (in g) by the appropriate temperature dependent factor given above.

Subsequently, for calibration of the micropipette a 2–3 ml specimen of fresh whole blood in EDTA is well mixed, lysed (e.g. by adding few drops of sapiens solution), and then diluted 1/251 in haemoglobin cyanide reagent using the previously calibrated 0.2 ml pipette and the 50 ml flask. At the same time the blood is diluted (in duplicate) in haemoglobin cyanide reagent using the 20 µl micropipette and the previously calibrated 5 ml pipette.

The dilutions are carried out in duplicate, and the absorbance (A), read at 540 nm on a spectrophotometer.

The dilution obtained by the micropipette is:

$$\frac{A_1}{A_2} \times 251,$$

where, A_1 = Absorbance using the previously calibrated (0.2 ml) pipette

A_2 = Absorbance using the micropipette (20 µl)

This figure should be 251; if not, a correction factor must be applied to obtain the true dilution whenever the micropipette is used.

iii. Calibration of Autodiluters

A lysed sample of whole blood is diluted manually (in duplicate) in haemoglobin cyanide reagent, by means of calibrated pipette described above.

Absorbance is read at 540 nm. Duplicate samples are then diluted in haemoglobin-cyanide reagent by means of the autodiluter and absorbance at 540 nm is raised.

If absorbance, using manual dilution (A_1) differs from the absorbance using the autodiluter (A_2), a correction factor (f) must be applied:

$$f = \frac{A1}{A2}$$

Corrected A_{540} HiCN at 1/251 dilution = $A_2 \times f$.

TARGET VALUES

The absence of metrological stands in haematology (with the exception of Hb) makes it difficult to establish the 'true' value for a test in haematology EQA. The EQA target value for a quantitative haematology assay can usually be assumed to be the result obtained by best performance of selected participants in the survey by experts using reference methods or the consensus mean or median of participant's results after trimming to offer outliers.

Consensus values for quantitative tests should only be calculated when the number of participants' results available in sufficient to allow statistically meaningful analysis of results, and this generally will require a minimum of 20 participants per analysis group.

Before the consensus summary statistics (mean of median, SD and CV) are calculated, it is necessary to remove **outliers** using appropriate statistical methods. Major errors/blunders (e.g. resulting from transportation of the EQA test specimens or reporting in incorrect units) should be removed, either by the removal of any result that is more than 3SD from the mean or by the exclusion of the top and bottom 5% of participant's results. The resulting "trimmed" consensus mean and SD are then recalculated. If there is non-Gaussian distribution of data with a wide scatter of results is present, then the median is used. If the median is used, the estimated SD is calculated as:

Estimated SD = Central 50% spread of data 1.349 (interquartile range or IQR)

Quantitative Tests

Participants' results are compared to the target value through statistical analysis and deviation of the EQA result from the target is measured. Guidance on statistical analysis of EQA data has been given in ISO 17043, ISO 13528 and by IUPAC.

Bias: The bias (D %) expresses the percentage difference of the EQA result from the target value. It is calculated as follows, where xi is the test result and x_{pt} is the target or assigned value:

$$\text{Bias (D\%)} = \frac{xi - x_{pt}}{x_{pt}} \times 100$$

The pattern of bias in successive surveys indicates whether there is a constant calibration error or a progressive fault.

Youden Plot

A Youden plot is a type of **scatter plot** used primarily plotting results from multiple laboratories on the same graph. It is meant to uncover bias in measurement. Youden plots are used in medical analysis and quality control, and allow to see quickly and easily:

- If labs are equivalent
- Which particular labs are outliers?
- Whether inconsistencies are primarily between labs (reproducibility problems) or within a lab (repeatability problems).

The Youdon plot (xyplot) and the analysis it involves is also known as a **two-sample diagram**, two-sample plan, or two sample collaborative testing (Fig. 18.11).

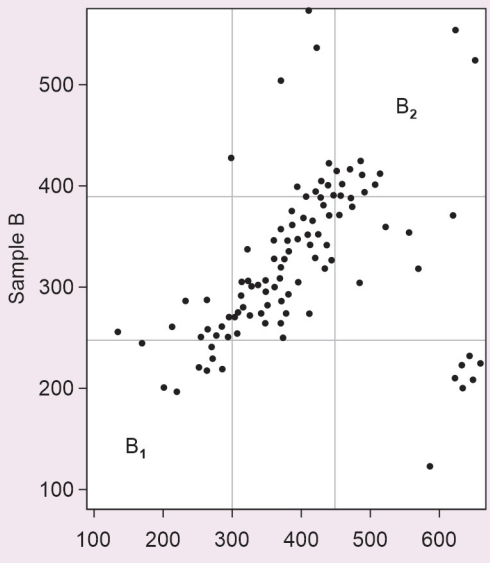
Setting up a Youden Plot

If Youden plot two-sample collaborative testing is to be performed, the same material is sent to all labs involved; typically, the labs will make one run on two different products, or two runs on the same product.

A basic xy graph is set up, with the x-axis any y-axis each representing one of the reported values of two samples. For example, if the laboratories each performed run 1 (sample A) and run 2 (sample B) on a single sample, the x-axis might be designated as run 1 (sample A) and the y-axis as run 2 (sample B).

When the setup is made, data from each lab are plotted on this graph as a scatter plot. Each point will represent one laboratory.

Two median lines are drawn, parallel to the x- and y-axis respectively. Excluding outliers, the horizontal line should have an equal number of points above and below it; the vertical line should have an equal number of points to the left and right. The point where these lines meet is called the **Manhattan median**. Then quadrants are assigned, based on this Manhattan median: Clockwise from upper left, these will be (– +), (++) (+ –) (– –). After this, a third 45° reference line can be drawn from the Manhattan median.



- Useful for relating measurements on two samples in a survey to provide a graphic display
- Distinguishes between a consistent bias and random error
  - The range of SDs calculated from the overall results with sample A and sample B, respectively, are drawn on the x-axis and the y-aixs
  - Results in the central square are satisfactory; those in B demonstrate a consistent bias with measurements that are too low (B_1) or too high (B_2), whereas results in other areas indicate random errors in the 2 sample

Fig. 18.11: Youden (xy) plot

So, in short result for the two samples are plotted on the horizontal (x) and the vertical (y) axis, respectively, and the standard deviations (2 SD or 3 SD) for the two sets are drawn and interpreted.

Clinical significance in performance assessment

In assessing performance, the use of limits based on the SD may be too rigid in some cases and too lenient in others. To ensure that results are clinically reliable, results should be within a certain percentage of the assigned value. This must take account of unavoidable imprecision of the method and diurnal variations. In practice, the following limits are adequate to meet these requirements for deciding whether there is a risk to patient care.

- Hb and RBC (by the cell counter): 3–4%
- Hct, MCV, MCH, MCHC: 4–5%
- Leukocyte count: 8–10%
- Platelet count: 10–15%
- Vitamin B_{12}, folate, iron, ferritin, haemoglobin A_2 and haemoglobin F quantitation: 5%

Quantitation Tests

Qualitative or categorical tests (e.g. blood film morphology, blood group or sickle solubility test results) are evaluated in comparison to the target value, which may be determined from a gold standard method.

When a consensus result is used, this should be reported by 80% or more of the participants. Even when gold standard method result is available, 75% or more of participants' results should be in agreement before it is wed for performance evaluation.

POINT OF CARE TESTING (POCT)

Point of care testing (POCT or bedside testing) is defined as medical testing at or near the point of care—that is, at the time and place of patient care. So, POCT refers to laboratory tests at the site of patient's care (as if lab is coming to patient), e.g. bedside, in the emergency department, clinics, nursing homes, operation theatres, health fares, intensive care units or in the home setting.

The basic aim of POCT is to provide rapid test results where immediate medical therapy of the patient is required. Tests performed during POCT are minimally invasive, easy to perform, convenient and require small amount of sample and also immediate results of these tests are obtained.

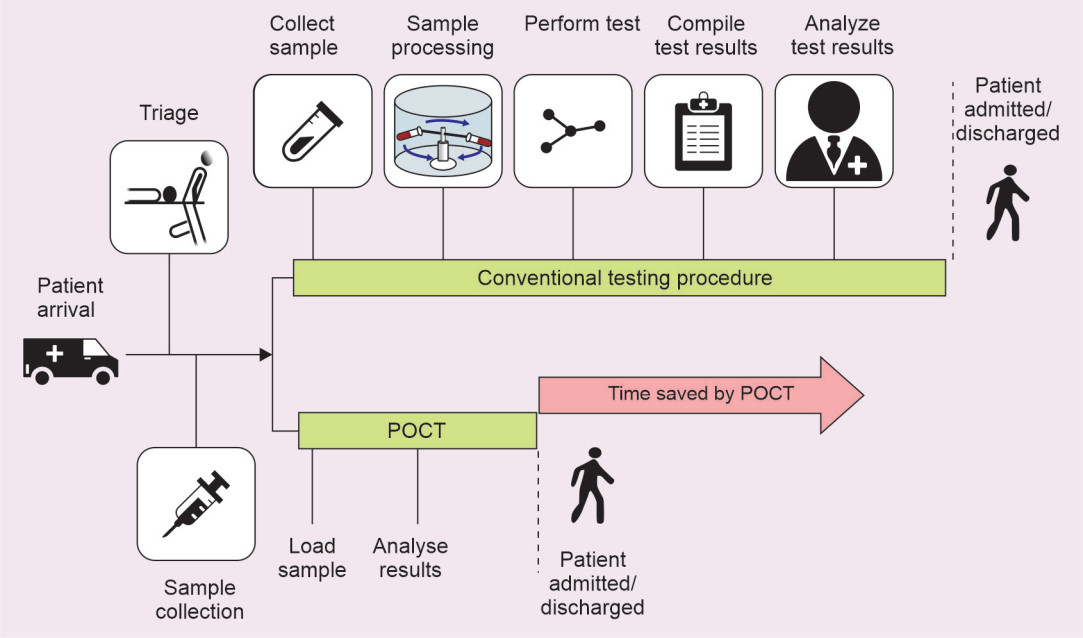
Common tests performed during POCT include: Blood glucose testing, blood gas and electrolytes analysis, rapid coagulation testing, rapid cardiac markers diagnostics, urine strip testing, pregnancy testing, focal occult blood analysis, haemoglobin estimation, platelet function testing, coagulation time, international normalized ratio (INR) and cholesterol screening.

Advantages of POCT

- Test results are shared instantly with health care providers.
- A reduction in morbity and mortality has been observed
- Frequent monitoring and test availability for 24 hours,
- Clinical trials have shown that individuals monitoring blood thinners (anticoagulants) like warfarin at home by POCT, had fewer major complications from the treatment.
- POCT is also used to measure coagulation time during open heart surgery and organ transplants.

Disadvantages of POCT

- Training of staff is required for handling the instrument.
- Calibration of the instrument may not be accurate (Fig. 18.12).



Efficiency	Tests performed at the point of care allow for more efficient workflow processes
Speed	Rapid test results enable expeditious decision-making and treatment.
Expanded capabilities	Testing is possible at a wide range of sites, including underserved populations, rural area, and disaster zones
Stability and handling	Reduced possibility of sample deterioration. In some cases a reduction in the specimen volume required is an advantage for patients requiring frequent testing
Shorter process	Fewer steps are necessary, including no transportation of samples and easier communication of results
Portability	Portable devices mean fewer space requirements and a flexibility to move the testing to either the physician lab or to the exam room
Patient outcomes	Immediate availability of results allows patients to be treated and on their ways to recovery

Fig. 18.12: Point of care testing (POCT or bedside testing)

WESTGARD RULES (MULTI-RULES)

"Multi-rule Quality Control" developed by Dr James O. Westgard based on statistical concept in 1981.

- Uses a combination of decision criteria or control rules.
- Allows determination of whether an analytical run is "in control" or "out of control".
- Six basic rules in the Westgard Scheme: 1_{2s}, 1_{3s}, 2_{2s}, R_{4s}, 10x and $4_{1s.}$
- These rules are used individually or in combination (multi-rule) to evaluate the quality of analytical runs.
- It ran detect random error (imprecision or CV) and systematic errors (bias or inaccuracy, shifts, trends) (Fig. 18.13).

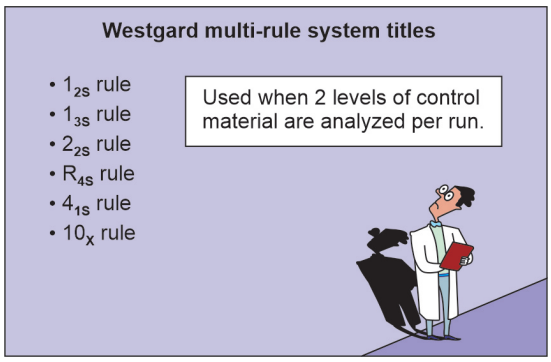


Fig. 18.13: Westgard rules (multi-rules)

1$_{2s}$ Rule or 'Warning' Rule

- A single on any one of 2 or 3 controls (low, normal, high or L1, L2, L3) results falls outside 2SD.
- The result may be on either side: +/–, i.e. +2SD or –2SD.
- Applicable across runs and across QC materials.
- Do not reject run (Fig. 18.14).

1$_{3s}$ Rule or 'Rejection' Rule

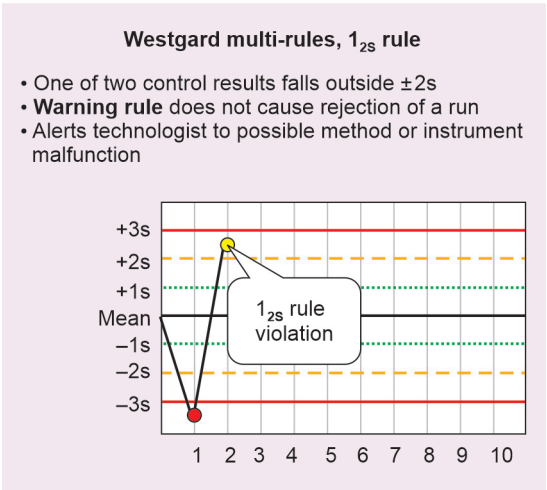
- A single or any one of 2 or 3 controls falls outside 3SD.
- Applicable across runs and across QC materials.
- The 1$_{3s}$ rule identifies unacceptable random error or possibly the beginning of a large systematic error (18.15).

2$_{2s}$ Rule, Rejection

- This rule detects systematic error
- Applicable across runs and across materials
- It is violated 'within run' when values of both levels of control materials or 2 out of 3 control materials exceed the 2SD limits in the same direction (i.e. either + or –), which means both control results are meant +2SD or mean –2SD.
- It is violated across runs when the previous value for the same control material level exceeds the mean +2SD or mean –2SD limit in the same direction (Fig. 18.16).

R$_{4s}$ Rule, Rejection

- This rule indicates only random error.
- It is applicable only across materials and within the run NOT for the same material and between runs.
- It is violated within the run when value of one of control materials exceed the mean +2SD or mean –2SD and the other control materials exceed the mean +2SD or mean –2SD but in the opposite direction. So, total deviation is 4SD (Fig. 18.17).



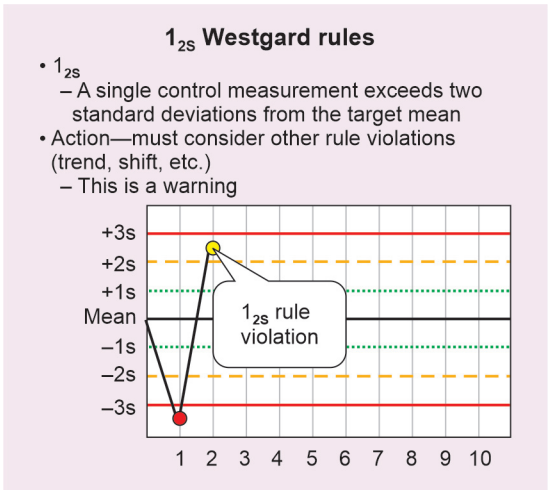


Fig. 18.14: Westgard 1$_{2s}$ rule or 'warning' rule

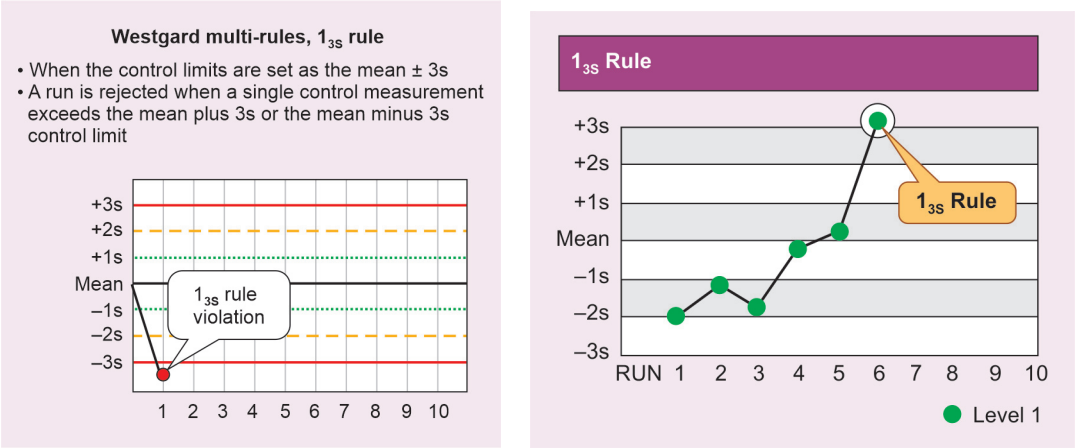


Fig. 18.15: Westgard 1_{3s} rule or 'rejection' rule

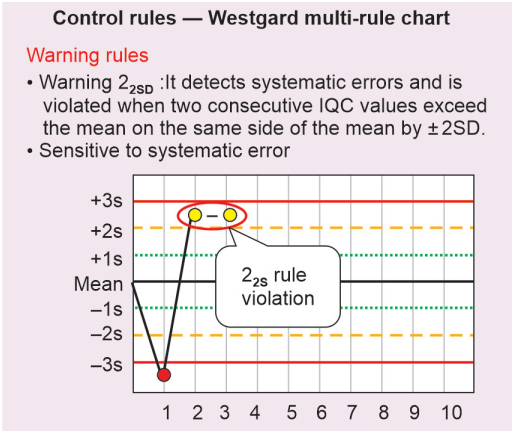


Fig. 18.16: Westgard 2_{2s} rule, rejection

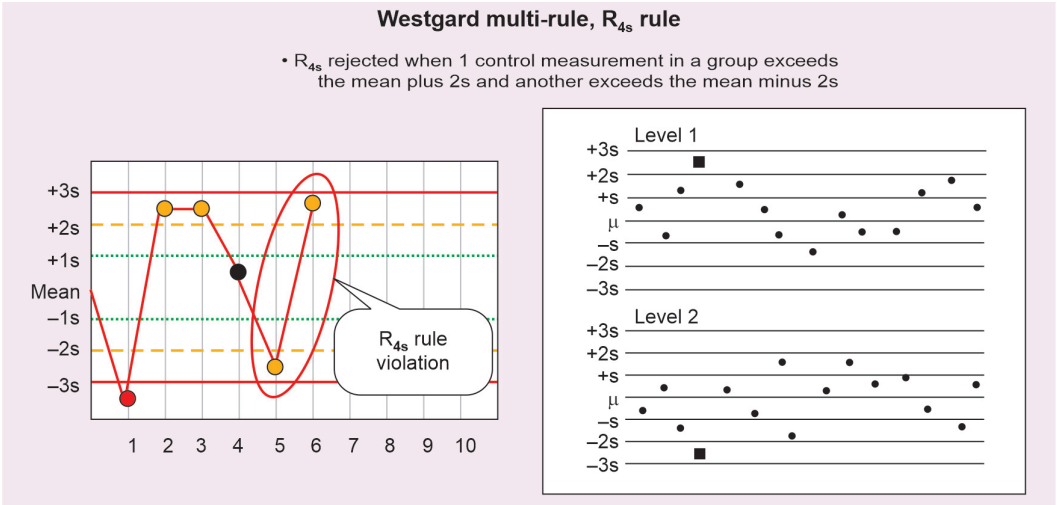


Fig. 18.17: Westgard R_{4s} rule, rejection

10_x Rule, Rejection

- It requires control data from previous runs.
- This rule detects systematic error or bias, and accuracy problem.
- It indicates a trend/shift.
- It is applicable to both 'across run' and 'across materials'.
- It is violated across control materials if the last 5 consecutive values are on the same side of the mean within 1SD (either direction), **regardless of control level**.
- The rule is violated **within the control materials** of the last 10 values for the same control level are on the same side of the mean within 1SD (+/– direction) (Fig. 18.18).

4_{1s} Rule, Rejection

- Requires control data from previous runs.
- Four consecutive IQC results for one level of control exceeds mean +/–1SD are outside ±1SD. (or)
- Both levels of control have a consecutive result that exceed mean 1SD are outside 1SD in the same direction (both +1SD/ – 1SD).
- Interpretation same as 10_x rule (Fig. 18.19).

Trend

7_T: Reject when seven control measurements trend in the same direction, i.e. progressively higher or progressively lower (Fig. 18.20).

What to do when a Westgard rule is violated?

- Warning rule: Use other rules to inspect the control points.
- Rejection rule. Out of control
  - Stop testing
  - Identify and correct the problems
  - Repeat test on patient samples and controls
  - Do not report results until problem solved and controls indicate performance.

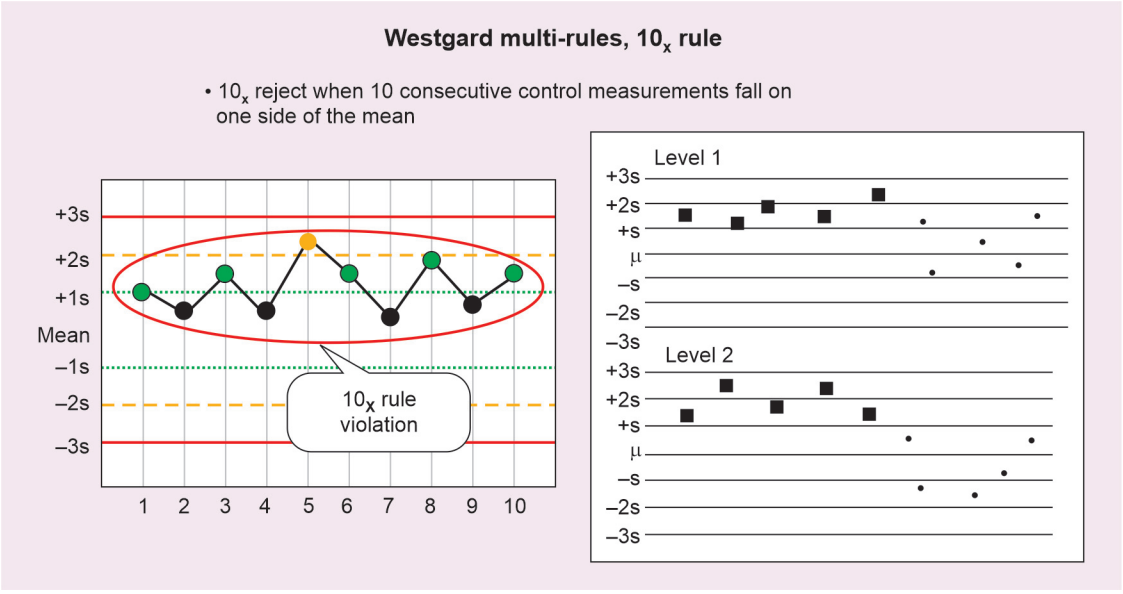


Fig. 18.18: Westgard 10_x rule, rejection

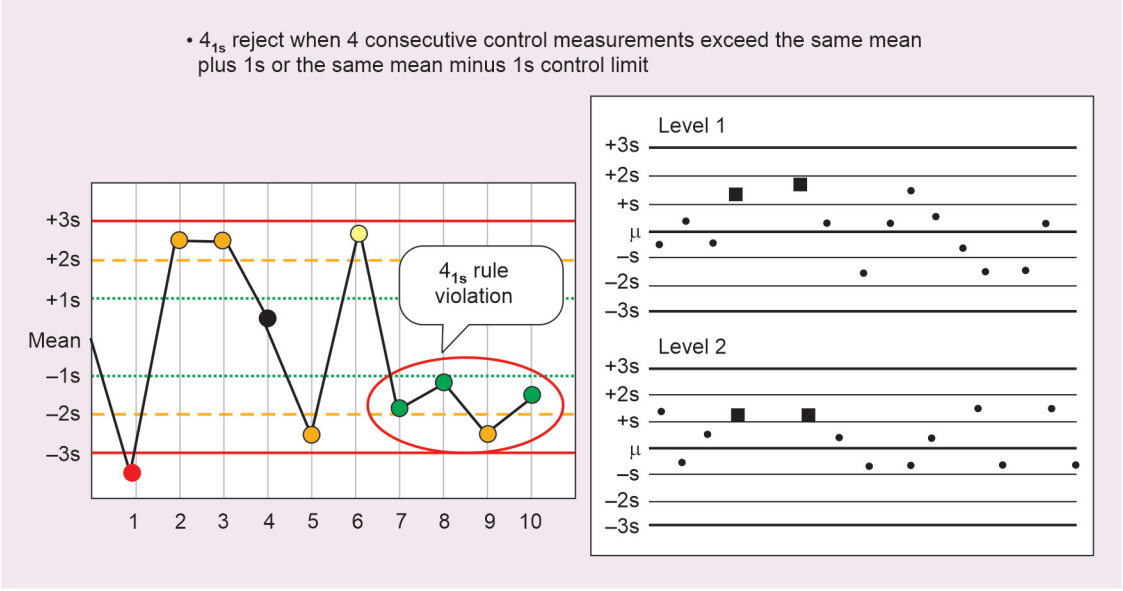


Fig. 18.19: Westgard 4_{1s} rule, rejection

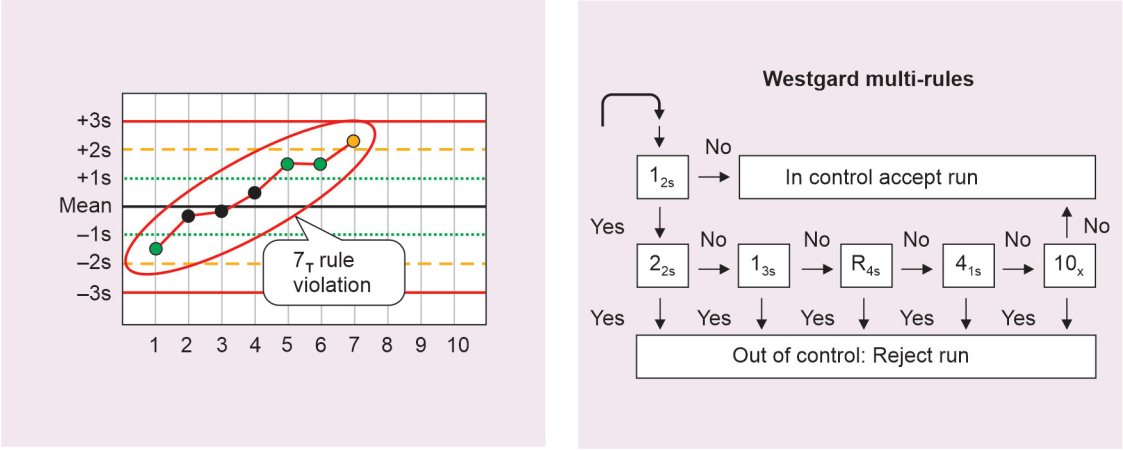


Fig. 18.20: This decision tree helps to decide whether to reject or accept a run. If you answer yes, it means that rule has been violated

Resolving QC Problems

- Take action in a sequential manner to identify a problem, not in a random manner
- Analyse QC materials
- Check if problem is resolved—if QC problem still persists take next step.
- Record the problem, action taken
- Technologists encouraged to perform corrective action steps by themselves.

Steps in Resolving QC Problems

1. Check whether QC is out of control for many analytes using same wavelength of analyser.
2. Check whether the QC is out-of-control for both levels and any one level for same analyte.
3. Check whether reagent has reached its on-board stability period/nearing it.

4. Check whether QC material has reached its open vial stability period/nearing it.
5. Check whether latest calibration was ok.

Solutions in Resolving QC Problems

1. Repeat assay on control specimen using fresh aliquot of QC pool.
2. Repeat assays on control specimen using a newly reconstituted set of controls.
3. Look for obvious problems: clots, blocks in probes, carryover reagent levels, and mechanical fault.
4. Recalibrate instrument for the analyte in question reassay all controls.
5. Install a new lot of reagent bottle (one or all), recalibrate and reassay all controls.
6. Perform machine maintenance, recalibrate and reassay all controls.