Hematology and coagulation

Basic blood test

Complete blood count (Complete blood picture)
A hemogram consist of a red blood cells (RBC count), white blood cells (WBC),
The total amount of hemoglobin, hematocrit, red blood indices and a platelet count.
Complete blood count (CBC) consist of hemogram plus a differential WBC.

Complete blood count (CBC):
The CBC is a basic screening test and is a one of the most frequently ordered
laboratory procedures. The finding in the CBC give a valuable diagnostic
information about hematology and other body system, prognosis, response to
treatment and recovery.

White blood cell count

A sample of whole blood is mixed with a weak acid solution that lyses
nonnucleated red blood cells. Following adequate mixing, the specimen is
introduced into a counting chamber where the white blood cells (leukocytes) in a
diluted volume are counted.

Procedure.

1. 0.02 ml (20 µl) of blood obtained by (finger prick or EDTA tube blood sample)
is added to 0.38 ml (cc) of WBC solution into a clean & dry glass or plastic tube,
mix well.

2. Leave it for (2-5) minutes.

3. Take a drop of mixture and place it in a neubauer chamber by a pasteur pipet,
then count at 10X eye piece.
Figure 5-1 Hemacytometer counting chamber (WBCs). Areas marked A, B, C, and D are used to count white blood cells.

**WBC solution** consist of (2% glacial acetic acid+ few drops of gention violet or crystal violet).

1. 2% glacial acetic acid → hydrolysis of RBC.
2. Gention violet or crystal violet → stain the nuclei of WBC

**Neubauer chamber**

Neubauer chamber is thick piece of glass contains graduate area divided into 9 large squares by three adjacent lines, the outer 4 squares are used for WBC's count, each of which is divided into 16 squares, while the central square is used for platelets and RBCs count, this square is divided into 25 squares.

Ideally, we must count all the outer 4 squares, the obtained count then multiplied by 50 or 2 squares multiplied by 100.

**Calculation:**

WBC count in 4 squares × 50 = N × 10³/ mm³
Or
WBC count in 2 squares × 100 = N × 10³/ mm³

**Normal range of WBC count** (4-10) × 10³/ L
Differential white blood cell

**Differential count**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>55 - 70</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>20 - 40</td>
</tr>
<tr>
<td>Monocyte</td>
<td>2 - 8</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>1 - 4</td>
</tr>
<tr>
<td>Basophil</td>
<td>0.5 – 1</td>
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</tbody>
</table>

Leukocytosis: WBC > 11000/ mm³
It is caused by an increase of only one type of leukocytes, and it is given the name of the type of cell that shows the main increase:

- **Neutrophilic** Leukocytosis or **Neutrophilia**.
- **Lymphocytic** Leukocytosis or **Lymphocytosis**.
- **Monocytic** Leukocytosis or **Monocytosis**.
- **Eosinophilic** Leukocytosis or **Eosinophilia**.
- **Basophilic** Leukocytosis or **Basophilia**.

An increase in circulating leukocytes is rarely caused by a proportional increase in leukocytes of all types. When this does occur, it is usually a result of hemoconcentration. In certain diseases (e.g., measles, sepsis), the increase of leukocytes is so great that the blood picture suggests leukemia. Leukocytosis of a temporary nature (leukemoid reaction) must be distinguished from leukemia. In leukemia, the leukocytosis is permanent and progressive. Leukocytosis occur in acute infection, in which the degree of increase of leukocytes depends on severity of infection, patient’s age, and marrow efficiency and reserve. Other causes of leukocytosis include: leukemia, Myeloproliferative disorder, trauma or tissue injury, malignant neoplasms, especially bronchogenic carcinoma, toxins, uremia, coma, eclampsia, thyroid storm acute hemolysis, hemorrhage (acute), after splenectomy, polycythemia vera, tissue necrosis.

Drugs: I, ether, chloroform, quinine, epinephrine, corticotropin (adenocorticotropic hormone ACTH), when given to healthy person.

**Physiological leukocytosis** is found when there is no evidence of clinical disease such findings suggests the presence of:
- Sunlight, ultraviolet irradiation.
- Stress, exercise, pain, cold or heat, anesthesia.
- Nausea, vomiting, seizures.
Leukopenia: WBC < 4000/mm³
Causes of neutropenia (leukopenia)
Racial: congenital Kostman's syndrome, Cyclical neutropenia, Marrow aplasia
Marrow infiltration, Megaloblastic anaemia.

Some acute infections eg: Typhoid, milliary TB, viral hepatitis.

Drugs: Antiaryrrhythmic drugs (arniodarone), Anticoagulant (phenindione),
Antihistamines (H2) (cimetidfine), Anti-inflammatory drugs (aminopyrine,
phenylbutazone), Antimicrobial drugs (chloramphenicol, sulphonamides)
Antipsychotics (chlorpromazine, clozapine), Antithyroids (carbimazole)
Hypoglycaemic (tolbutamide),

Irradiation exposure (wide field, high dose)
Immune disorders: HIV infection, Feltys syndrome, systemic lupus
erythematosus, Hypersplenism.

Red cell count:
Red cell count can be automated (electronic count) or manual (visual count), the
latter being less accurate. The manual manner uses counting chambers /
hemocytometer.

Reagents
We use isotonic solution for dilution to avoid RBCs rupture due to osmotic
pressure.
In case of whole blood sample (blood with anticoagulant) such as blood withdrawn
on vacuum tube containing EDTA we use physiological saline (85%).
In case with blood without anticoagulant such as blood obtained directly from a
lancet puncture from the finger we use:

Hayem's solution: isotonic solution containing mercuric chloride, sodium sulfate
and sodium chloride.

Procedure:
1) In clean and dry tube put 2ml (2000µl) of the isotonic solution.
2) Remove 10 µl from the isotonic solution from the test tube to get 1990 µl of the
isotonic solution.
3) Add 10 µl of blood sample in the tube and wipe the pipette of to avoid errors.
Now we get the dilution 10:2000 which means 1:200
4) Put cover on the opening of the test tube and hold it with your thumb and
mixing well and gently for 1-2 min by inverting the tube and upright it again.
5) Then with the pipette add the sample at the 2 sites for applying the sample but
avoid air bubbles. No pressure is required to fill the chamber, capillary action is
quite sufficient.
6) Place the loaded hemacytometer into a petri dish with a piece of wiped paper to keep the sample from drying out. This is to allow 10-15 minutes for the cells to settle.

8) Adjust the first small square at the center of the field then magnify by the 40X lens. Count the cells in all the 16 small squares in a zigzag manner.

9) Then count in the other 5 squares.

Calculation:

\[ N \text{ (of the RBCs in original sample)} = N\text{(counted by hemacytometer)} \times 10000 \]

RBCs/µl or RBCs/mm³

Normal values:

Men 4.7–6.1×10⁶/µL
Women 4.2–5.4×10⁶/µL

Decrease RBC value occur in:

Anemia, a common condition in which is a reduction in the number of circulating erythrocyte, the amount of Hb, or the volume of packed cells (Hct), anemia is associated with cell destruction, or dietary insufficiency of iron or of certain vitamins that are essential in the production of RBCs. Disorders such as: Hodgkin’s disease and other lymphomas, multiple myeloma, myeloproliferative disorders, leukemia, acute and chronic hemorrhage, lupus erythematosus, Addison’s disease, rheumatic fever, subacute endocarditis, chronic infection.

Increase RBC value (erythrocytosis) occur in:

1-Primary erythrocytosis
Polycythemia vera (myeloproliferative disorders).
Erythremic erythrocytosis (increased RBCs production in bone marrow).

2- Secondary erythrocytosis
Renal disease, External tumors, high altitude, pulmonary disease, cardiovascular disease, alveolar hypoventilation, hemoglobinopathy, tobacco, carboxyhemoglobin, decrease in plasma volume (dehydration): vomiting, diarrhoea.

**Hematocrit (Hct) : Packed cell volume (PCV)**

PCV is the ratio of the volume occupied by red blood cells to the volume of the whole blood.

**Advantages:**

1. Simple screening test for anemia.
2. In calculation of red blood indices
3. Calibration of automated blood count systems.

**Low PCV occurs in** anemia.

**High PCV occurs in:**

1. Severe dehydration (burns, diarrhea, excessive use of diuretics).
2. Polycythemia.
3. Excessive intake of exogenous erythropoietin.

**Method :**

1. Use PCV tube 75mm long, 1mm width.
2. Allow the blood to enter by capillarity leaving about 15 mm unfilled.
3. Seal the tube by heating by Bunsen burner or by plastic seal.
4. After centrifugation for 5 minutes, measure the PCV using a reading device.

**PCV is increased in :**

1. Macrocytic anemia.
2. Spherocytosis.
3. Thalassemia.
4. Hypochromic macrocytic anemia.
5. Sickle cell anemia.

In the above conditions, we have an accurate PCV. That is why PCV is an indicator of HB only in normochromic normocytic morphology.

**False reading can be produced by:**

1. Failure to mix the blood sample adequately.
2. EDTA anticoagulant in excess of 1.5 mg/ml (cell shrinkage → falsely low PCV).
3. Variation of the pore of the tube.
4. Heating difficulty for sealing.
5. Continuous use of centrifuge for several hours, especially in hot climates, will result in it becoming overheated causing the sample either to lyse or the plasma to evaporate.
6. Storing blood beyond 6-8 hours results in an factual increase in PCV especially in hot climates.

**Normal values for PCV**

Men: $0.45 \pm 0.05\%$

Women: $0.41 \pm 0.05\%$

**Hemoglobin Hb**

**Hemoglobin**: it is a red polypeptide complex present inside the red cells which carries oxygen from lungs to the tissues and transporting carbon dioxide from the tissues to the lungs.

**Composition:**

Hb is composed of two main portions:

1. The hem part: It is composed of four porphyrine (pyrole) rings and there is an iron atom in its center. The iron atom also binds to oxygen molecules and to globin chain.
2. The globin part: Each hemoglobin molecules contain four globin chains (2 pairs of globin) either 2α or 2β in case of adult hemoglobin (HbA).

Low Hb occurs in:

1. Anaemia.
2. Overhydration: excessive fluid intake or in pregnancy (haemodilution).

High Hb occurs in:

1. Polycythemia.
2. Severe dehydration (burns , diarrhea, excessive use of diuretics).

Methods:

Hemoglobin can be measured by one of the following methods:

1. The cyanmethaemoglobin method.
2. The oxyhaemoglobin method.
3. The alkaline – haematin method.

The best method is the cyanmethaemoglobin method because the color of the cyanmethaemoglobin complex is stable and can be read easily, and we can do proper control of the test because of the required standards are available .

The cyanmethaemoglobin method:

The basis of this method is to dilute blood in a solution containing potassium cyanide and potassium ferricyanide (Drabkin's solution).

1. 0.02 ml or 20 µl of blood is added to 5 ml of Drabkin's solution in a clean and dry tube, mix well.
2. Leave to stand for 15 minutes (to ensure complete conversion of haemoglobin, carboxyhaemoglobin and methaemoglobin except sulfahaemoglobin to cyanmethaemoglobin).
3. Measure the absorbance of the solution I a photo electric colorimeter (spectrophotometer) at a wave length of 540nm using the Drabkin as blank (for zeroing).
4. Make a standard curve or table relating optical density readings to haemoglobin concentration in g/100 ml of blood.
Normal range:
Men          150 ± 20g/L.
Women     135 ± 15g/L

Red cell indices

Calculated using special formula

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

Mean cell haemoglobin (MCH)
Men and women                   92.5 ±2.5 pg

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

RBC count: now used computerized system for research purposes.

Platelet count

Manual count are used routinely in under resourced laboratories

Platelet counts are best performed on ethylenediaminetetra acetic acid (EDTA) anticoagulated blood that has been obtained by clean venipuncture. They can also be carried out on blood obtained by skin prick, but the results are less satisfactory than those on venous blood. Manual platelet counts are performed by visual examination of diluted, lysed whole blood using a Neubauer counting chamber as for total white blood cell counts.

Method

The diluent consist of 1% aqueous ammonium oxalate in which the red cells are lysed. This method is recommended in preference to the using formal-citrate as diluent, which leaves the red cells intact and is more likely to give incorrect results, when the platelet count is low.
Before diluting the blood sample, examine it carefully for the presence of blood clots. If these are present, a fresh specimen should be requested because clots will cause the platelet count to be artificially low.

1. Make a 1 in 20 dilution of well-mixed blood in the diluent by adding 0.1 ml of blood to 1.9 ml of ammonium oxalate diluent (10 g/l). Not more than 500 ml of diluent should be made at a time, using scrupulously clean glassware and fresh glass – distilled or deionized water. If possible, the solution should be filtered through a micropore filter (0.22 µm) and kept at 4 Cº. For use, a small part of the stock is refiltered and dispensed in 1.9 ml volumes in 75×12 mm tubes.

2. Mix the suspension on a mechanical mixer for 10-15 min.

3. Fill a neubauer counting chamber with the suspension, using a stout glass capillary or Pasteur pipette.

4. Place the counting chamber in a moist Petri dish and leave untouched for at least 20 min to give time for platelets to settle.

Examine the preparation with the 10× and 40× eyepieces. The platelets appear under ordinary illumination as small (but not minute) highly refractile particles if viewed with the condenser racked down: they are usually well – separated, and clumps are rare if the blood sample has been skillfully collected. To avoid introducing into the chamber dirt particles, which might be mistaken for platelets, all equipment must be scrupulously clean.

Platelets are more easily seen with the phase – contrast microscope. A special thin bottomed (1µm) counting chamber is best for optimal phase – contrast effect. The number of platelets in one or more areas of 1mm² should be counted.

Calculation:

The number of platelets per liter of blood are: N × 200 × 10⁶/L

N is the number of platelets counted in an area of 1mm².

Errors in manual cell count:

The errors associated with manual are technical and inherent.

Technical errors can be minimized by avoiding the following:
1. Poor technique in obtaining the blood specimen.
2. Insufficient mixing of the blood specimen.
3. Inaccurate pipetting and the use of badly calibrated pipettes or counting chambers.
4. Inadequate mixing of the cell suspension.
5. Faulty filling of the counting chamber.
6. Careless counting of cells within the chamber.

**Bleeding time**

*Bleeding time* is a test used to determine how quickly your blood clots to stop you from bleeding. The test involves making small, superficial cuts on your skin similar to light scratches.

The test is a basic assessment of how well your blood platelets work to form clots.

**Method**

Place a sphygmomanometer cuff around the patient’s arm above the elbow, inflate to 40mm Hg and keep it at this pressure throughout the test. Clean the area with 70% ethanol and allow to dry. Choose an area of skin on the volar surface of the forearm that is devoid of visible superficial veins. Use a commercial template device to make one or two standard longitudinal incisions deep enough to cause slight bleeding.

With the edge of a filter paper, at 15 sec intervals blot off the blood exuding from the cut. Avoid contact with the wound during this procedure because this may disturb the formation of the platelet plug. When bleeding has ceased, carefully oppose the edges of the incision and apply an adhesive strip to reduce the risk of keloid formation and an unsightly scar.

**Normal range**

Normal range is 2-7 min. An upper limit of 4 min has been reported in one study on men and women who had not used aspirin or other relevant drugs in the ten days before the test. Ideally, every laboratory should determine its own normal range and if possible ensure that the test is performed by the same operator.
**Blood typing**

By using anti A, anti B, anti D against washed RBC and observe the agglutination after 1 minutes incubation at 37 C.

**Coombs test**

The antiglobulin test (also called Coombs test) is based on the principle that antihuman globulins (AHGs) obtained from immunized nonhuman species bind to human globulin such as IgG or complement, either free in serum or attached to antigens on red blood cells (RBC).

There are two major types blood group antibodies, IgM and IgG. Because of their large pentamer structure, IgM antibodies bind to corresponding antigen and directly agglutinate RBCs suspended in saline. IgG antibodies are termed non agglutinating because their monomer structure is too small to agglutinate sensitized RBCs directly. The addition of AHG containing anti – IgG to RBCS sensitized with IgG antibodies allows for hemagglutination of these sensitized cells .Some blood group antibodies have the ability to bind complement to the RBC membrane. Antiglobulin test detect IgG and or complement sensitized RBCs.

**Direct antiglobulin test (DAT)**

**Principle and application of the DAT**

The DAT detect in vivo sensitization of RBCs with IgG and or complement components. Clinical conditions that can result in in-vivo coating of RBCs with antibody and /or complement are:

1. Hemolytic disease of the newborn (HDN).
2. Hemolytic transfusion reaction (HTR).
3. Autoimmune and drug- induced autoimmune hemolytic anemia (AIHA).

**Indirect antiglobulin test (IAT)**

**Principle and application of the IAT**
The IAT is performed to determine in–vitro sensitization of RBCs and is used in the following situation:

1. Detection of incomplete (nonagglutinating) antibodies to potential donor RBCs (compatibility testing) or to screening cells (antibody screen) in serum.
2. Determination of RBC phenotype using known antisera (e.g., Kell typing, weak D testing).
3. Titration of incomplete antibody.

**Manual antiglobulin test techniques**

I. DAT

A. Procedure
   1. Label 10 or 12 ×75 mm glass test tube. Test and control, respectively, and add 1 drop of a 3% v/v suspension of test cells to each.
   2. Wash the cells a minimum of three times with saline, and ensure that all saline is completely decanted after the last wash.
   3. To the tube labeled “test” add 1 to 2 drops of AHG as recommended by the manufacturer and mix.
   4. To the control tube, add 1 to 2 drops of 3% v/v bovine albumin in saline and mix.
   5. Centrifuge both tubes at 500 RCF for 15 to 20 seconds.
   6. Following centrifugation, completely resuspend the cell pellet by gently tapping and rolling the tube. Read and score agglutination macroscopically with the aid of a background light source and low power magnification.
   7. Incubate the tubes for another 5 minutes at room temperature and repeat steps 5 and 6.

Most manufacturers recommended this additional step because it has been shown that some negative or even weak reactions may increase in strength. These reactions have attributed to the presence of C3d and to a lesser extent, IgA on the cell surface.

Conversely, the reaction with some cells may weaken after the extra incubation, this has been attributed to detachment of IgG antibody when excess anti-IgG has been added.

B. Controls
   To all negative tubes add 1 drop of control cells weakly sensitized with IgG, mix the cells, and repeat steps 5&6.

II. IAT

A. Procedure
1. Into a laboratory glass add 10 or 12 ×75 mm glass test tube; place 2 to 4 drops of test serum and 1 drop of a washed 3% v/v suspension of RBCs.
2. Mix the suspension, and incubate for 30 minutes in a 37°C water bath.
3. Centrifuge the tube at 500 RCF for 15 to 20 seconds.
4. After centrifugation, completely resuspend the cell pellet by gently tapping and rolling the tube. Read and score agglutination macroscopically with the aid of a background light source and low power magnification.
5. Wash the cells at least three times with saline, and ensure that all saline is completely decanted following the final wash.
6. Add 1 to 3 drops of AHG as recommended by the manufacturer and mix.
7. Repeat steps 3 and 4.

**B. Controls**

To all negative tubes add 1 drop of control cells weakly sensitized with IgG, mix the cells, and repeat steps 3&4.

**Erythrocyte sedimentation rate (ESR)**

When anticoagulated whole blood is allowed to stand in narrow vertical tube for a period of time, the RBCs—under the influence of gravity—settle out from the plasma. The rate at which they settle is measured as the number of millimeters of clear plasma present at the top of the column after one hour (mm/hr).

**Westergren method:**
The Westergren method requires collecting 2 ml of venous blood into a tube containing 0.5 ml of sodium citrate. It should be stored no longer than 2 hours at room temperature or 6 hours at 4°C. The blood is drawn into a Westergren-Katz tube to the 200 mm mark. The tube is placed in a rack in a strictly vertical position for 1 hour at room temperature, at which time the distance from the lowest point of the surface meniscus to the upper limit of the red cell sediment is measured. The distance of fall of erythrocytes, expressed as millimeters in 1 hour, is the ESR.

**Mechanism of ESR:**

1. Depends upon the difference in specific gravity between red cells and plasma.
2. It is influenced very greatly by the extent to which the red cells form rouleaux, which sediment more rapidly than single cell sediments.
The all-important of rouleaux formation and red cell clumping are mainly controlled by the concentration of fibrinogen and other acute phase proteins, e.g., haptoglobulin, ceruloplasmin, α1 acid-glycoprotein, α1 antitrypsin and C reactive protein. Rouleaux formation is also enhanced by the immune globulins. It is retarded by albumin. Defibrinated blood normally sediments extremely slowly.

3. The ratio of red cells to plasma, i.e. the PCV, the viscosity, the verticality or otherwise of the sedimentation tube, the bore of the tube and the dilution of the blood.

Anemia, by altering the ratio of red cells to plasma encourages rouleaux formation and accelerate sedimentation. In anemia, too, cellular factors may affect sedimentation. Thus in iron deficiency anemia a reduction in intrinsic ability of the red cells to sediment may compensate for the accelerating effect of an increased proportion of plasma.

Sedimentation can be observed to take place in three stages:

1) Stage 1: Rouleaux formation - First 10 minutes.

2) Stage 2: Sedimentation or settling stage - 40 mins.

3) Stage 3: Packing stage - 10 minutes (sedimentation slows and cells start to pack at the bottom of the tube).

**Some interferences, which increase ESR:**

Increase level of fibrinogen, gamma globulins.

Technical factors: tilted ESR tubes, high room temperature.

**Some interferences, which decrease ESR:**

Abnormally shaped RBCs (sickle cells, spherocytosis)

Technical factors: short ESR tubes, low room temperature, delay in test performance, (> 2 hours), clotted blood sample, excess anticoagulant, bubbles in tube.

**Normal range:**

Healthy men are: <15mm/hr;
healthy females, they are somewhat higher: <20mm.

The values are slightly higher in old age, in both genders

Chronic inflammatory disease (collagen and vascular diseases) increase ESR.
Polycythemia decrease ESR.