COLLEGE NAME & CODE : Periyar Arts College, Cuddalore-01 & 105
COURSE NAME & CODE : II B.Sc., Microbiology & U26
SEMESTER : III
SUBJECT TITLE & CODE : HEMATOLOGY AND BLOOD BANKING & BSMB 33

UNIT-IV

Examination of Blood smear: Peripheral smear report: Size/colour/shapes/inclusions

Preparations of stains & Staining techniques:

Wright stain,	Leishmans stain
Gienisa's stain,	Jaswanth singh and Bhattarcharji stain
Fields stain,	Peroxidase stain

Blood parasites: Malarial parasite/Microfilaria.

EXAMINATION OF BLOOD SMEAR

(Ref: Medical Lab Technology-Methods & Interpretation (Volume 1, 6th Edition, JayPee Brothers) Ramnik Sood)

BLOOD FILM EXAMINATION

Preparation of a Thin Blood Film

A thin blood film is made by spreading a drop of blood evenly across a clean grease-free slide, using a smooth edged spreader.

Making of Spreaders (Fig. 9.9)

- Select a slide which has smooth edges
- Using a glass cutter and a ruler, mark off 4 equal divisions, each measuring 19 mm
- Break off at each division to give 4 spreaders
- Ready made spreaders are available.

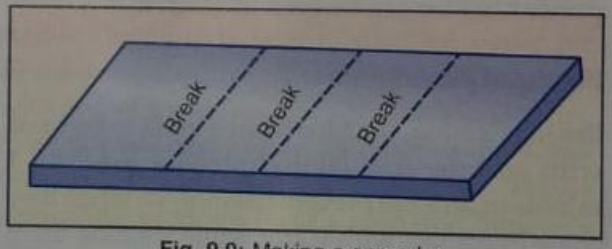

Fig. 9.9: Making a spreader

For anaemic blood, a rapid smearing is needed; whereas for thick concentrated blood, smearing should be done slowly. A well-spread smear shows no lines extending across or downwards through the film and the smear should be tongue shaped (Fig. 9.10).

Making Thick Smears

While the thin smears are used for describing blood cells, the thick smears are used for detecting malarial parasites and microfilariae. A

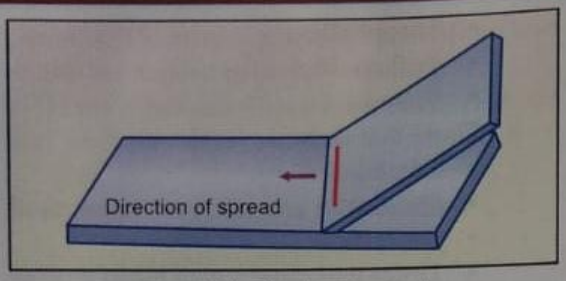

Fig. 9.10A: Direction of spread

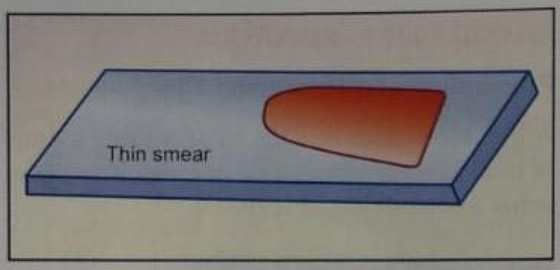

Fig. 9.10B: A thin peripheral blood smear

large drop of blood is taken on the centre of a slide and with the aid of a needle or slide corner spread the drop over ½ an inch square area. When dry, the thickness should be such that printed matter can be seen through it.

Fixing of Blood Films

Before staining, the blood films need to be fixed with acetone-free methyl alcohol for ½ to 1 minute in order to prevent haemolysis when they come in contact with water while staining them with aqueous (water-based) stains or when water has to be added subsequently. Alcohol denatures the proteins and hardens the cell contents. For Wright's stain and Leishman's stain, no prefixation is required as these contain acetone-free methyl alcohol; but for Giemsa's stain, prefixation is a must because the alcohol content is only 5% in the ready-to-use stain.

Staining of Blood Films

Blood cells have structures that are acidophilic and some basophilic structures, so they vary in their reaction (pH). The nuclei are basophilic and stain blue. The highly basophilic (acidic) basophil granules also stain blue. Haemoglobin (being basic) stains acidophilic or red.

Stains that are made up of combinations of acid and basic dyes are called Romanowsky stain and various modifications are available, e.g. Wright's, Leishman's, Giemsa's, and Jenner's stains. Most use methylene blue as the basic stain, though toluidine blue is used in some. Most use eosin as the acid stain, though Azure I and Azure II are also used.

The dried film can stay for a couple of days in hot dry weather, but gets bad if they are not fixed in hot and humid climate that exists in India.

It is best to use neutral distilled water for diluting the stain. Stale distilled water becomes acidic after absorbing CO_2 from atmosphere. If the distilled water is alkaline RBCs stain a dirty bluish green colour, the parts of WBC which should stain blue will be slightly purplish, the granules of eosinophils bluish or greenish instead of pink and granules of neutrophils overstained. If the water is acidic RBCs stain bright orange and nuclei of the white cells a very pale colour.

The ideal pH is 6.8 and in order to maintain this buffered distilled water is used. Buffer water is a solution which tends to keep its original pH even on addition of small amount of alkali or acid. (Buffer tablets ready for use, to be dissolved in distilled water).

Buffer Solution used in the Laboratory

Solution No. I

NaOH (sodium hydroxide) 8 gm.

Distilled water 1000 cc.

Solution No. II

KH_2PO_4 (Potassium dihydrogen phosphate) 27.2 gm.

Distilled water 1000 cc.

PREPARATION OF STAINS
&
STAINING TECHNIQUES

(Ref: Medical Lab Technology-Methods & Interpretation (Volume 1, 6th Edition, JayPee Brothers) Ramnik Sood)

Take 23.7 cc of solution I, add to it 50cc of solution II, add 20 cc of the above mixed solution to 1000 cc of distilled water. This has a pH of 6.8.

Stain Preparation and Staining

Wright's Stain

Wright's stain (powder) 0.2 gm.
Acetone free methyl alcohol 100cc
Let stand this solution for a few days.
If the WBC granules do not stand out clearly, try out a 0.25 or 0.3% solution.

Method

Cover the slide with stain for 1-2 minutes taking care that it does not dry on the slide. Now dilute this with equal amount of buffer water (if the stain is ripe, a scum or film with a metallic sheen will form on the surface of the diluted stains on the slide). The diluted stain is allowed to act for 3-5 minutes and then flooded off with buffer or tap water. The stain should never be poured off or a precipitate of the stain will be deposited on the slide. Should this occur, it can sometimes be removed by flooding the slide with undiluted stain for 10-15 seconds and then washing it off again by flooding the slide once more with buffered water.

Leishman's Stain

Powdered Leishman's stain 0.15 gm.
Acetone-free methyl alcohol 133 ml.
All the stain should be dissolved (better if the stain crystals are well ground before), keep the stain in a glass stoppered bottle. Do not filter.

Method

Like that for Wright's stain but with double dilution of the buffer water; (i) Pour few drops (about 8) on the slide. Wait for 2 minutes, (ii) Add double the amount (16 drops) of buffered water. Mix by rocking and not by blowing and wait for 7-10 minutes, (iii) The stain is flooded off with distilled water and this should be complete in 2-3 seconds. Longer washing will remove stain, and (iv) Stand in a rack to drain and air dry. A fan will expedite the process.

Giemsa's Stain

Giemsa powder 0.3 gm
Glycerine 25.0 ml
Acetone free methyl alcohol 25.0 ml.

This makes stock solution and before use it has to be diluted by adding 1 ml (stain) to 9 ml of buffered distilled water.

Method

The blood film is fixed with methyl alcohol for 3-5 minutes and dried. Pour on diluted stain and keep for 15 minutes or longer. Wash off with tap water or neutral distilled water and dry.

Staining of Thick Films

Thick films have to be dehaemoglobinised before staining with one of the previously mentioned stains. The slide is kept in distilled water for 10 minutes, then taken out, dried and stained with any of the stains already mentioned. They must not be fixed before staining, or the water will not haemolyse the cells. The stains commonly used are Field's stain and Simeon's stain.

Field's Stain

Field's stain A
Methylene blue 0.8 gm
Azure I 0.5 gm
Disodium hydrogen
phosphate (anhydrous) 5.0 gm
Potassium dihydrogen
phosphate anhydrous 6.25 gm
Disttiled water 500 ml

Field's stain B

Eosin (yellow eosin, water soluble) 1.0 gm
Disodium hydrogen phosphate (anhydrous) 5.0 gm
Potassium dihydrogen phosphate (anhydrous) 6.25 gm
Distilled water 500 ml.

Grind all solids well and dissolve in the said solvent, keep the stains for 4 hours for ripening and filter before use. Keep the stains in covered jars. The depth of the solution should be about 3 inches, the level should be maintained by adding more of the stain solution.

Method

1. Dip the film for one second in solution A.
2. Remove from solution A and immediately rinse by waving very gently in clean water for a few seconds, until the stain ceases to flow from the film and the glass of the slide is free from stain.
3. Dip for one second in solution B.
4. Rinse by waving gently for 2-3 seconds in clean water.
5. Place vertically in a rack to drain and dry.

Simeon's Modification of Boye's and Sterenal's Method

This stain can be used instead of Leishman's or Wright's stain when methyl alcohol is not available to prepare them.

Solution I

Eosin pure 1 gm
Distilled water 1000 ml

Solution II

a. Medicinal methylene blue 1 gm, Distlilled water 75 ml } dissolve completely

b. Potassium permanganate 1.5 gm, Distilled water 75 ml — dissolve completely

1. Mix (a) and (b) in a flask. A massive precipitate is formed.
2. The flask is kept in a water bath at boiling point for half an hour during which time the precipitate re-dissolves.
3. Filter. The stain is now ready for use, it needs no further dilution.

Method for Staining Thin Films

1. Fix the smear by immersion into rectified spirit—1 minute.
2. Rinse with tap water—4 seconds.
3. Immerse into solution I—10 seconds.
4. Rinse with tap water—4 seconds.
5. Immerse into solution II—15 seconds.
6. Rinse with tap water—4 seconds.
7. Immerse again into solution I—5 seconds.
8. Rinse with tap water—4 seconds.
9. Allow to dry in an upright position.

Procedure for Staining Thick Smears

1. Dehaemoglobinise by immersion into tap water, if necessary.
2. Immerse in Sterenel's blue (solution II)—6 seconds.
3. Wash in tap water.
4. Immerse in eosin solution (solution I)—12 seconds.
5. Wash in tap water, allow it to dry in air. Examine under microscope.

The stains are useful for screening purposes.

Mounting and Preservation of Films

Unstained films cannot be preserved well. Due to hardening of plasma, they do not stain well after some time. Stained films if left unmounted tend to fade away rapidly. Canada balsum should not be used as it decolourises the smear. Gurr's neutral mounting medium is quite satisfactory. Use only thin coverslips for mounting.

BLOOD PARASITES

(Ref: Textbook of Haematoogy, Dr.Tejindar Singh, 3rd Edition, Arya Publications, 2018)

Chapter 29

Haemoparasites

Malaria
- P. vivax
- P. falciparum

Filariasis

Leishmaniasis

Parasites are frequently detected in peripheral blood in Indian patients. Indian subcontinent is endemic for both malaria and filariasis. Both the diseases can be diagnosed by careful examination of a well made and stained peripheral smear.

MALARIA

Malaria has a world wide distribution, mainly in tropical and temperate regions.

It is caused by different species of Plasmodium.
- Plasmodium vivax
- Plasmodium ovale
- Plasmodium malariae and
- Plasmodium falciparum.

INDIAN SCENARIO

In India P. vivax and P. falciparum are quite rampant. In the red cells the parasites pass from ring stage to amoeboid form and then schizont. Male and female sexual forms—gametocytes are seen in the peripheral blood in 2nd and subsequent cycles. Malaria is rampant throughout the year but large number of cases are observed following monsoons in India.

Gold standard of malaria diagnosis is demonstration of the parasite in the peripheral blood.

CLINICAL FEATURES

Malaria is clinically characterized by high fever with chills and rigors; these paroxysms of fever last for 4–16 hours followed by afebrile period lasting 1–2 days depending upon the species of malarial parasite. Febrile paroxysms coincide with the erythrocytic schizogony of the malarial parasite. Hyperplasia of the reticuloendothelial system results in splenomegaly, which is slate grey to black in colour because of deposition of haemozoin pigment in the reticuloendothelial cells.

COLLECTION OF BLOOD SMEARS

Peripheral blood smears should be made 2–6 hours after the peak of the febrile paroxysms, as the number of parasites is more during this period.

Both thin and thick smears made from a finger prick are stained with Giemsa/Leishman stain. Thin smear is examined first and if malarial parasites are not found, then thick smears should be evaluated.

PLASMODIUM VIVAX

In the peripheral blood, trophozoites, schizonts and gametocytic stages are present.

TROPHOZOITE

In the ring form, a blue ring with a red nucleus is present, while in the growing form, ring becomes irregular in a large RBC with development of Schuffner's dots (Fig. 29.1).

SCHIZONT

It is larger (9–10 µ size) and almost fills an enlarged RBC. There are reddish Schuffner's dots with the parasite being blue, with a red nucleus (Fig. 29.1).

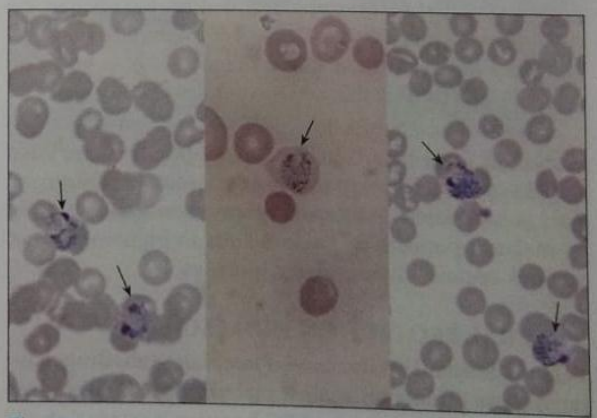

Fig. 29.1 • Plasmodium vivax. Trophozoite stage. Parasite is blue with a red nucleus. Red cell demonstrates Schuffner's dots.

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GAMETOCYTE

Gametocyte is much larger than a schizont; the female gametocyte has eccentric red nucleus (Fig. 29.2) and is blue in colour with Schuffner's dots while the male has pale blue cytoplasm with large and diffuse pale red nucleus and plenty of Schuffner's dots.

PLASMODIUM FALCIPARUM

In the peripheral blood only ring stage and gametocytes are present. Schizonts are not present in blood since schizogony takes place in tissues.

TROPHOZOITE STAGE

Rings are 1–1.5µ in size, blue in colour with a red nucleus. The rings have a regular outline. Some of the RBCs demonstrate multiple rings and some rings have 2 nuclei (Fig. 29.3).

GAMETOCYTE STAGE

Gametocytes are crescentric (Fig. 29.4) and only a thin red cell border is recognised. Female gametocyte is blue with a central, compact red nucleus and malarial pigment in cytoplasm. Male gametocyte is paler in colour and has a diffuse lightly stained nuclear material in the central zone.

THICK FILM FOR MP

RBC outlines are not visualised since dehaemoglobinisation is carried out before smear is examined. Malarial parasites (with distorted morphology) are made out. Thick film is a concentration method for malarial parasites and one microscopic field is equivalent to 40–50 microscopic fields of a thin smear. However, thin film is essential for morphology and to characterize the type of malarial parasite. Thick film method is preferred for mass surveys for a quick diagnosis. It is better to make both thin and thick films on the same slide.

Due to parasitemia of RBCs, red cell physiology is disturbed. Globin part of the haemoglobin is broken down and resynthesized into parasite protein and the unutilized part is haemozoin – a brown pigment which is present in malarial parasite and also results in pigmentation of various organs – liver, spleen, brain, etc.

NATURAL PROTECTION FROM MALARIA

Persons deficient in **Glucose-6-Phosphate dehydrogenase** have some protection against malaria since Plasmodium cannot survive inside the RBC because it needs G-6-PD enzyme for survival.

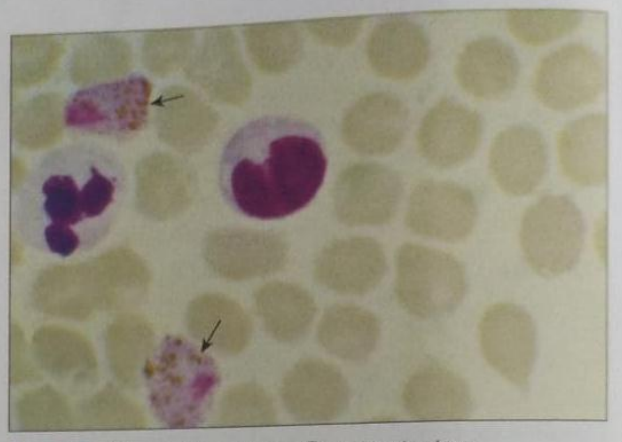

Fig. 29.2 • Plasmodium vivax. Gametocyte stage.

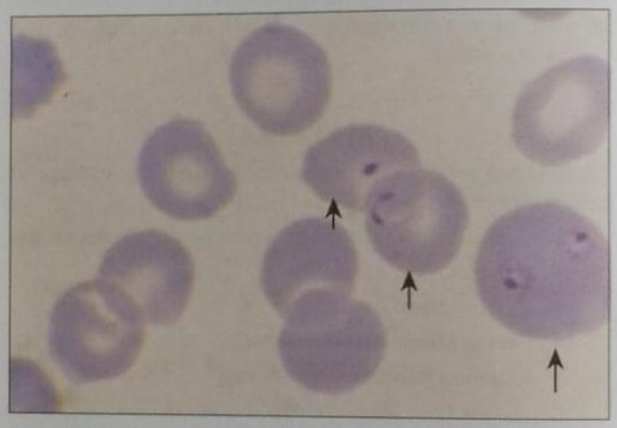

Fig. 29.3 • Plasmodium falciparum. Ring stage. One RBC demonstrates more than 1 ring.

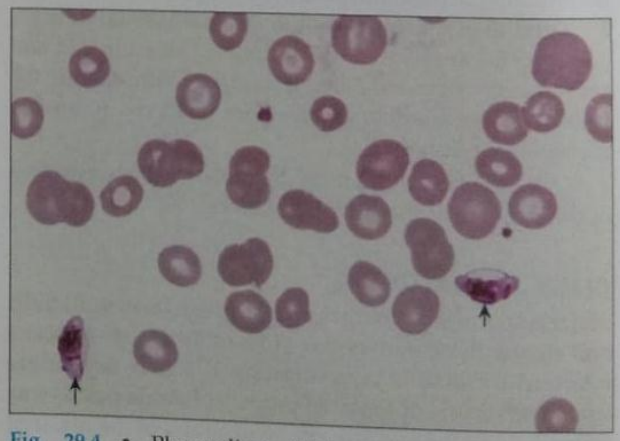

Fig. 29.4 • Plasmodium falciparum. Gametocyte stage. Gametocytes are crescenteric and contain a blob of brown hemozoin pigment.

In **sickle cell disease**, the Plasmodium infects the RBC and induces quicker sickling and sickled RBCs with parasite inside them are sequestered by the reticuloendothelial cells of the spleen, thus providing protection against malaria.

BLOOD ALTERATIONS IN MALARIA

These include high reticulocyte count due to haemolytic component, more so in falciparum malaria, monocytosis and anaemia in chronic malaria. Anaemia progressively becomes moderately severe due to hypersplenism. Bone marrow in falciparum malaria demonstrates hyperplasia of the histiocytes. Many histiocytes demonstrate dark brown pigment. At times significant hemophagocytosis is seen and few cases with hemophagocytic syndrome with cytopenias are observed.

GEL CARD TEST FOR MALARIA

Plasmodium produces an enzyme Plasmodium LDH (pLDH). 10µl of fresh/frozen/dried whole blood is taken. RBCs are lysed and pLDH from parasitised RBCs is released. Presence of pLDH is detected using monoclonal antibodies against specific epitopes of pLDH in gel cards. Positive test indicates active Plasmodium infection. Test is quick, specific, sensitive and ideal for field conditions.

FILARIASIS

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 localise in the lymphatic vessels and lymph nodes causing obstruction to lymph flow resulting in lymphedema and elephantiasis. Embryos of W. bancrofti (microfilaria) pass through lymph nodes, lymphatics and reach circulating blood and demonstration of *microfilaria* (Fig. 29.5) in peripheral blood is diagnostic of filariasis. Filariasis induces eosinophilia. Any smear with significant eosinophilia should be examined for microfilaria.

Smear for demonstration of microfilaria should be made preferably between 10 p.m. and 2 a.m. or **midnight smears**.

Microfilaria of W.bancrofti shows the hyaline sheath which is longer than the larval body. Somatic cells appear as granules and extend from head to the tail end except the terminal tail sheath – a distinguishing feature of W.bancrofti microfilaria (Fig. 29.5).

TRYPANOSOMES

These affect mainly the African subcontinent. These are flagellate protozoa having a narrow body and are mobile and seen in the peripheral smear. Rare cases have been reported from India.

LEISHMANIA DONOVANI

Kala-azar is endemic in Bihar. Amastigote forms – L.D. bodies are observed in reticulum cells in the bone marrow and spleen (Fig. 29.6). In the bone marrow and spleen there is increase in plasma cells. There is associated increase in γ-globulins. L.D. body has a nucleus and a kinetoplast. Rarely L.D. bodies are seen free or in monocyte in peripheral blood when parasitemia is very high.

Patients of Kala-azar present with h/o prolonged irregular fever with moderate to massive splenomegaly. Bone marrow aspirate should be examined for increased plasma cells and L.D. bodies.

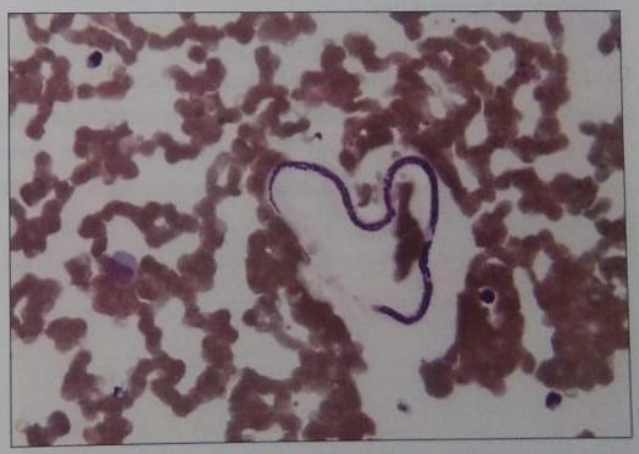

Fig. 29.5 • Wuchereria bancrofti, Microfilaria in the peripheral blood. Filariasis is usually associated with eosinophilia.