TECHNIQUE OF BLOOD EXAMINATION FOR MALARIA PARASITES

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Many practitioners, medical officers, ship surgeons, and laboratory workers formerly not familiar with malaria are now being confronted with its diagnostic problems. The technique involved in the blood examinations is simple enough, but it is not widely realized that a staining technique giving the best results for blood cells does not do so for malaria parasites. Faulty methods often lead to a failure to detect parasites present in small numbers, so the following brief notes may guide workers inexperienced in this field.

Materials and Reagents

A sharp cutting skin needle, gauze or wool swabs, and some spirit or ether are required for the skin puncture.

Slides.—Thin half-white slides, clean and free from grease, are best for this work; scratched or frosted slides should be avoided. In an emergency, slides as supplied by the makers, if held by the edges, breathed on, and polished with a clean cloth, will usually give satisfactory results. Whenever possible, however, the slides should be cleaned chemically by boiling for five minutes in a solution containing 6% each of potassium bichromate and sulphuric acid, and then washing in running tap-water for 10 minutes; the slides are then stored in spirit and polished before use. Chemical cleaning is essential in certain tropical regions to prevent the growth of moulds.

Leishman's Stain.—Sold as a powder or in tablets of known weight, the staining solution is prepared as follows:

Place 0.15 g. of the stain in a small mortar and measure out 100 c.cm. of pure methyl alcohol (acetone-free). Grind the dry powder and then add about 20 c.cm. of the methyl alcohol. Grind again for a few minutes and decant into a clean dry bottle with a well-fitting ground-glass stopper. Repeat with successive small quantities of the alcohol; the last two portions should be only faintly tinged with the stain. If pestle and mortar are not available satisfactory results may be obtained by placing the stain in the bottle, adding the methyl alcohol, and shaking vigorously for five minutes on three occasions.

Freshly prepared solution does not give the best results, so the stain should be kept in a dark cupboard for 10 days, shaking vigorously every day, and then placed on a shelf in ordinary daylight (avoiding direct sunlight) for seven days before being used. The stain should not be filtered, and no attempt be made to hasten its solution by heat. The bottle should be kept carefully stoppered, as contact with the air speeds deterioration. When required for use the stain should be removed from the bottle with a pipette; the last half-inch of solution is best discarded, as it always contains some granular deposit which may cause troublesome artefacts in a stained blood-film. If correctly made and stored, Leishman's stain keeps indefinitely in a temperate zone, but deterioration may be rapid when temperature and humidity are high.

Giemsa's Stain.—This is supplied in solution, and has only to be diluted for use. It is thus more suitable than Leishman's stain for occasional use away from a laboratory. The undiluted stain keeps well and is not affected by heat and humidity.

Distilled Water.—This is a most important reagent, and consistently good results cannot be obtained unless it has the correct pH concentration. The best staining of blood cells is obtained when the water used for dilution and washing has a pH value of 6.8 to 7.0. For malaria parasites, however, the best results are obtained at a pH of 7.2. At this pH value the red blood corpuscles give a slightly bluish staining, against which the bright red and deep blue of the parasites stand out boldly. In addition the weakly basic reaction accentuates the earliest changes in the parasite-containing corpuscles, which are an important feature in species diagnosis. As taken from the still, distilled water is almost always slightly acid owing to the absorption of carbon dioxide from the air in cooling. One of the following methods ensures that a diluent of the desired reaction (pH 7.2) is employed.

(1) 9 g. of anhydrous disodium hydrogen phosphate and 1.8 g. of anhydrous potassium dihydrogen phosphate are dissolved in 3 litres of distilled water. This buffer salt solution is recommended for use wherever frequent examinations are carried out.

(2) About 4 c.cm. of bromothymol blue indicator solution is added to a litre of distilled water. This gives a yellow colour owing to the acid reaction. A 0.5% solution of sodium carbonate is now added drop by drop, with vigorous shaking, until the water attains and retains a bluish-green colour. This indicates a

pH of about 7.2, but is less accurate than the use of buffer salts described above.

(3) In emergency, if distilled water or filtered rain-water is boiled vigorously for 5 to 10 minutes it usually attains a pH of 7 to 7.2 and allows fairly satisfactory staining. The acid reaction returns on standing.

Spreading the Blood Films

(a) **Thin Films.**—The slides are placed flat on a bench or bedside table and one with a perfectly smooth edge is chosen as a "spreader." Shorten the spreading edge by about 1/4 in. by chipping off one corner: both edges of the resulting smears can be readily examined. The patient's finger and the needle are cleaned and dried and a slight incision is made above the base of the nail. A small drop of blood is allowed to collect, and this is lifted with the under surface of the edge of the spreader, held by the edges in the right hand. The blood is now touched down on the slide to be spread, about an inch from the right end, and allowed to run across the width of the spreader. The free end of the spreader is lowered to make an angle of 20 to 25° with the horizontal, and is then pushed smoothly and firmly to the left end of the slide. A really thin film, with the red cells lying separated, is essential for the accurate diagnosis of malaria parasites. The two factors which control the thickness are the amount of blood and the angle at which the spreader is held—the less the amount of blood and the lower the angle, the thinner the resulting smear. The films are allowed to dry at room temperature, and should not be heated.

(b) **Thick Films.**—The advantages of the thick film technique may be entirely lost if attention is not paid to technical details. In particular, the beginner should eschew certain ultra-rapid methods that have recently become fashionable. The term "thick" is purely relative, and a common mistake is to make the smears too thick. A drop of blood similar to that used for a thin smear should be lifted on the centre of a slide and spread out with the blade of the needle until a page of print can be clearly seen through it. The film is allowed to dry horizontally, protected from dust and insects. Drying may be hastened by placing the slide for a few minutes in an incubator at 37° C.; but more vigorous heating interferes with subsequent dehaemoglobinization.

Staining

(a) **Leishman's Stain for Thin Films.**—The slide is supported in a horizontal position and 10 to 12 drops of the stain are placed on the film, using a clean dry pipette. The stain is spread out with the barrel of the pipette to cover the entire area of the film, and is allowed to act undiluted for about 30 seconds, thus fixing the blood. Double the quantity (20 to 24 drops) of pH-adjusted distilled water is then added, and is mixed with the stain by drawing up and expelling two or three times with the water pipette. The precise time the diluted stain should be allowed to act varies with different batches of stain. The period is usually between 10 and 20 minutes, and the best results are obtained by somewhat longer staining than for blood cells. At the end of the staining period the stain should not be poured from the slide but gently flushed off in a stream of distilled or tap water for a few seconds only. This prevents the scum which forms on the surface of the stain from settling on the film and showing as a granular deposit, but more prolonged washing will rapidly decolorize the parasites. The film should be allowed to dry in a semi-vertical position without heat or blotting.

(b) **Leishman's Stain for Thick Films.**—Haemoglobin is removed from the preparation by placing the dry unfixed film in an upright position in a jar of distilled water. When the film is uniformly white—in about 10 minutes with a fresh film—it is gently withdrawn and allowed to dry. The dehaemoglobinized film is then stained as the thin films, except for a rather shorter staining time (5 to 15 minutes).

(c) **Giemsa's Stain for Thin Films.**—As this is a watery solution the films must be treated with a fixing reagent before staining. The surface of the slide is flooded with absolute methyl alcohol, which is allowed to act for one or two minutes. The slide is then washed gently in a stream of tap-water and placed vertically or face downward in a staining jar or dish containing Giemsa's stain diluted in the proportion of two drops of the stain to each c.cm. of pH-adjusted distilled water. The staining is complete in 20 to 30 minutes; the slide is then quickly rinsed and allowed to dry in a semi-vertical position.

(d) **Giemsa's Stain for Thick Films.**—Without previous fixation the films are placed vertically or face downward in a staining jar or dish containing the diluted stain—one drop of stain to each c.cm. of distilled water. The vessel is gently agitated from time to time, and dehaemoglobinization and staining are complete in 20 to 30 minutes. The slides are then gently transferred to a dish of water for a few seconds only and finally allowed to dry in a semi-vertical position. Vigorous handling or washing in running water may cause the film to leave the slide. Dilutions of Giemsa's stain remain active for about 12 hours, during which time they may be used repeatedly.