

## Introduction

About 300–500 million people worldwide are infected with malaria each year, and more than 1 million, mainly children, die from the disease. Most cases occur in developing countries, particularly in Africa. Malaria is caused by protozoan parasites of the genus *Plasmodium*, which contains four species important to humans: *P. falciparum* (which causes the most serious form of malaria and is responsible for the majority of deaths), *P. vivax*, *P. ovale* and *P. malariae*. The parasite attacks and destroys red blood cells, and it may affect vital body organs, including the brain: most deaths due to falciparum malaria are the result of cerebral malaria.

These *Bench aids for the diagnosis of malaria infections* present photomicrographs — with explanatory text — that show the various species and morphological forms of human malaria in thick and thin blood films. Descriptions of *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* are provided, as are instructions on the preparation and use of buffer and staining solutions. The photomicrographs, all at x1000 magnification and stained with Romanowsky stains, show many of the possible variations of malaria parasites. Differences in fixing and staining procedures result in highly variable staining of blood films; as a result, the background of the film and the organisms will differ considerably from slide to slide. Parasites in the photomicrographs of thick blood films tend to be less clearly defined than those in thin films: a thick film consists of several layers of cells, making it difficult to focus on different layers.

These bench aids are aimed at laboratory workers responsible for diagnosing malaria by microscopic examination of blood films, but will also be useful as teaching aids. Experienced health workers can often diagnose malaria from the way a patient looks and feels. To confirm the diagnosis, however, the patient's blood must be examined: for microscopy thick and thin blood films should therefore be prepared. A thick blood film usually allows an experienced microscopist to identify the species of *Plasmodium*, but where there is any doubt a thin blood film should be examined to confirm the identity. Accurate diagnosis is essential, since treatment varies according to species; diagnostic errors can be life-threatening for patients, particularly in *P. falciparum* infection.

The previous edition of the bench aids (*Bench aids for the diagnosis of malaria*), published by WHO in 1988, included colour illustrations of the different life-cycle stages of the four *Plasmodium* species that cause human malaria. In this edition, colour micrographs, which provide a more accurate representation of these stages, have replaced the illustrations. Biosafety guidelines for handling blood specimens have been added, in view of the increasing incidence of hepatitis and human immunodeficiency virus (HIV) infection/acquired immunodeficiency syndrome (AIDS).

## Life cycle and diagnosis

Malaria is transmitted by female *Anopheles* mosquitoes. Some 70 of the approximately 420 *Anopheles* species around the world are vectors of human malaria, of which about 40 are of major importance. The mosquito becomes infected with malaria parasites when it bites a person whose blood contains the sexual forms of the parasite (gametocytes). Female macrogametocytes and male microgametocytes ingested in this way become mature gametes in the midgut of the mosquito. Fertilization of a female gamete (macrogamete) by a male gamete (microgamete) results in a motile zygote (ookinete) which migrates to the gut wall and develops into an oocyst. Asexual division inside the oocyst yields as many as 10 000 elongated, spindle-shaped sporozoites, which are released when the oocyst eventually ruptures and migrate via the body cavity to accumulate in the salivary glands of the mosquito. When the infected female *Anopheles* takes its next blood-meal, the sporozoites are inoculated into the bloodstream of the human host. They are carried in the blood to the liver, invading the parenchymal cells where they develop into exo-erythrocytic schizonts.

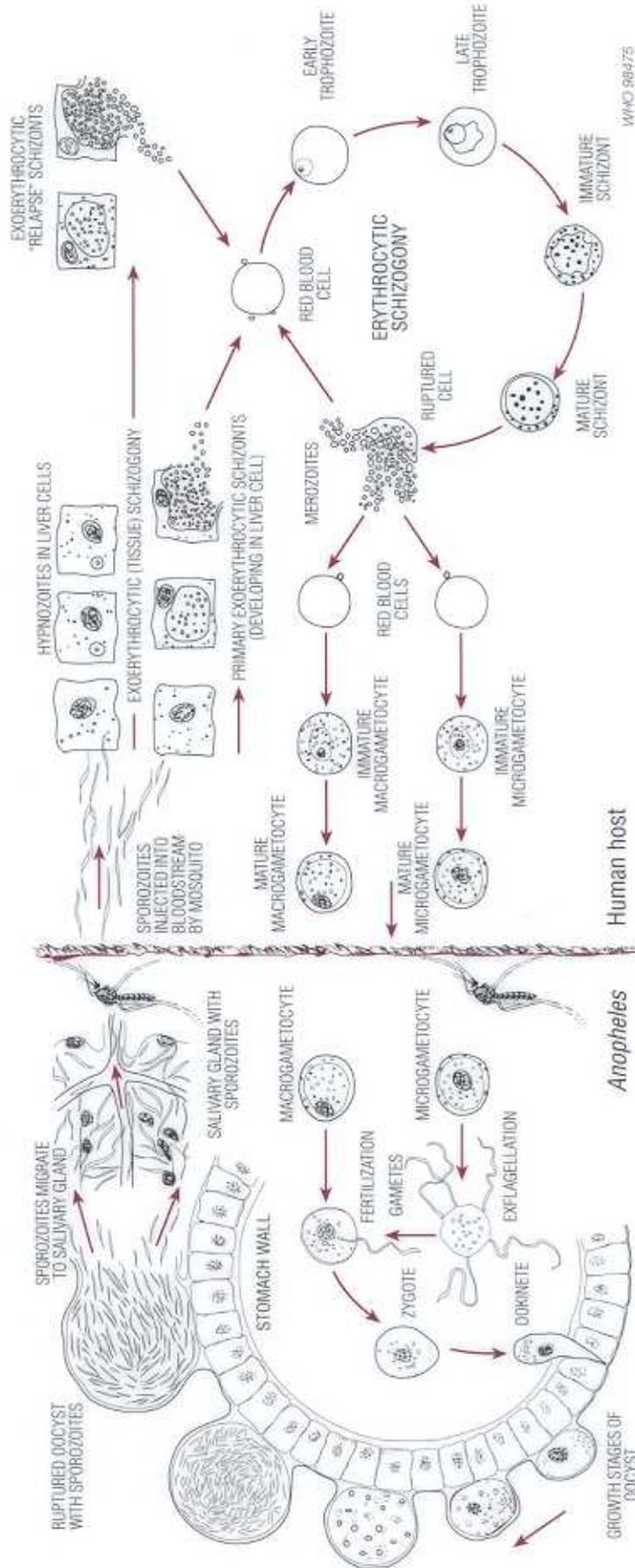
A multiplication phase follows, usually lasting between 5½ and 15 days depending on the species of *Plasmodium*. At the end of this time, the mature schizont bursts, releasing thousands of merozoites (up to 30 000 in *P. falciparum*) into the bloodstream. In *P. vivax* and *P. ovale* malaria, however, some sporozoites do not immediately develop into schizonts; they remain dormant in liver cells for months. Unless these dormant forms — hypnozoites — are destroyed in the liver by specific antimalarial drugs, their later development is responsible for the relapses seen in *P. vivax* and *P. ovale* infections. Since *P. falciparum* and *P. malariae* do not produce hypnozoites, they do not cause relapses, although recrudescence from erythrocytic forms is possible.

In the bloodstream, merozoites invade the red blood cells, where haemoglobin provides nutrition for their development into trophozoites; young trophozoites are known as ring forms because of their shape. Trophozoites develop into schizonts during this erythrocytic (red blood cell) phase and produce malaria pigment as a by-product of their metabolism. Reproduction at this stage is by asexual division (erythrocytic schizogony); after several divisions each mature schizont commonly contains 6–24 merozoites (range 6–40), depending on the *Plasmodium* species. Rupture of the infected red blood cells liberates the merozoites into the bloodstream, where they infect further fresh red cells and begin a new erythrocytic cycle. The repetition of this cycle results in an increasing parasitaemia. After several rounds of erythrocytic schizogony, some of the merozoites differentiate into microgametocytes and macrogametocytes which, when ingested by the female mosquito during a blood-meal, give rise to another cycle of malaria transmission.

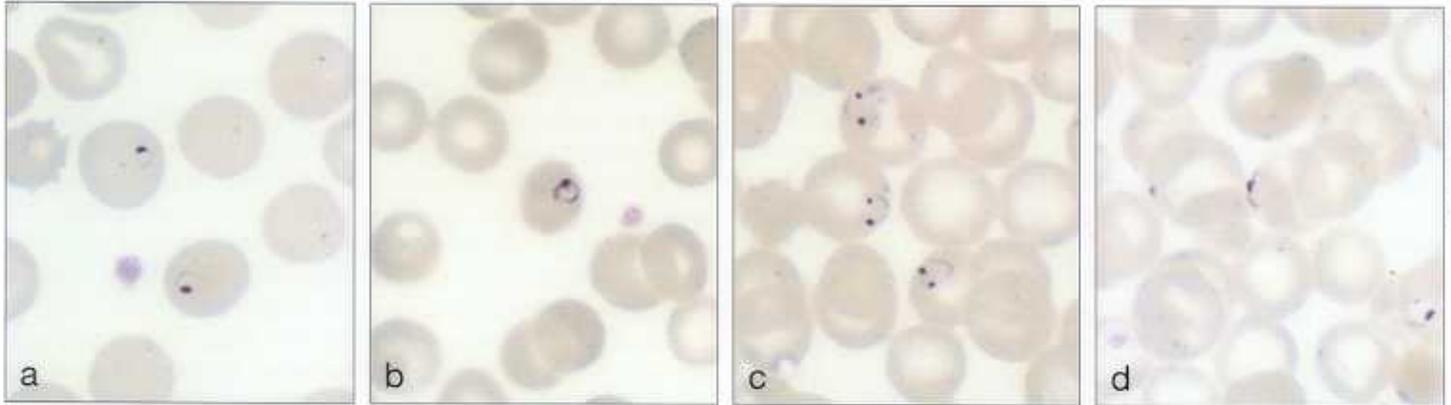


### Life cycle of malaria

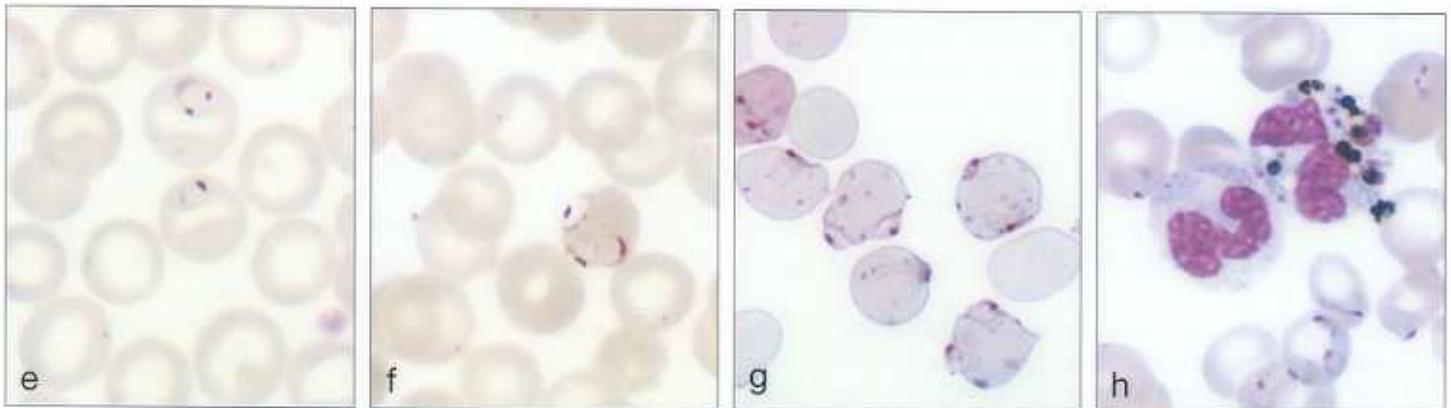
Figure reproduced, with minor amendments, from *Bruce-Chwatt's essential malariology*, London, Arnold, 1993, with the permission of H.M. Gilles and D.A. Warrell, eds.



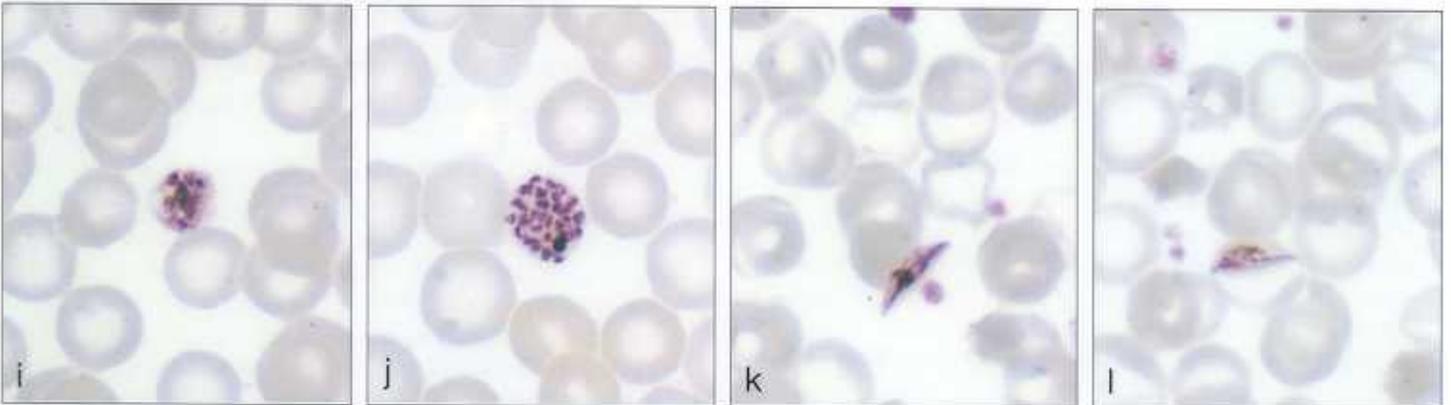
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*Plasmodium falciparum* thin film

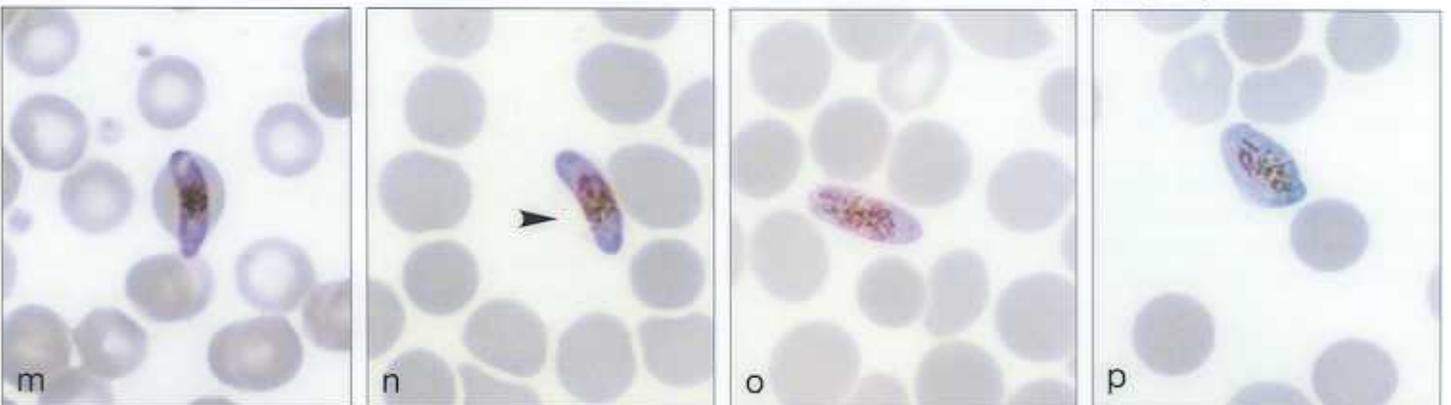
**Trophozoites:** Merozoites invade red blood cells of all ages. Trophozoites of *P. falciparum* are smaller than those of the other human malarials; tending to have a delicate, thin ring of blue cytoplasm, with a vacuole and a prominent red chromatin dot (a–e). Infected red cells with double chromatin dots (c, e) and multiple invasion of erythrocytes (f, h) are frequent features of this infection. The infected red blood cells are not enlarged. Parasites at the margin of red cells are referred to as accolé or appliqué forms (d); recognition



of these is useful in diagnosis. In some instances, these marginal forms are markedly displaced so that much of the parasite extends beyond the cell margin (f, g). Maurer's clefts (f, g) appear later in *P. falciparum* infection than do Schüffner's dots in *P. vivax* infection. Maurer's clefts are seen in red cells containing older trophozoites and stain best when the pH is alkaline (pH 7.2–7.6). Growing and mature trophozoites are not usually seen in peripheral blood films unless infection is severe and parasitaemia is high. Occasionally lumps of pigment can be seen within the cytoplasm of granulocytes.



**Schizonts:** These are rarely seen in peripheral blood films except in heavy infections. Mature schizonts are compact, rounded bodies usually containing between 16 and 24 (range 8–40) merozoites (i, j). Pigment in the schizont is usually fused into a single or double mass and may be anywhere in the infected erythrocyte (i, j).



**Gametocytes:** Initially these are rounded bodies lacking pigment and with no vacuole. As they mature, they become spindle-shaped (k, l) and then develop into characteristic banana- or sausage-shaped bodies with rounded ends (m–o). It is frequently possible to see the red-cell membrane during maturation of the gametocyte (n, arrow). In macrogametocytes, the cytoplasm stains blue and the chromatin is concentrated as a purplish mass (m, n); pigment tends to be more concentrated than in microgametocytes and appears as irregular granules or rodlets in the centre of the parasite. The cytoplasm of microgametocytes is often a pinkish purple and the chromatin is more diffuse (o); pigment granules tend to be more scattered than in macrogametocytes. Occasionally, gametocytes may assume bizarre shapes (p).



## *Plasmodium falciparum*

This species of *Plasmodium* is the most important of the four human species of malaria. Its distribution is concentrated in the tropical and subtropical regions of the world, particularly Africa and Asia. In sub-Saharan Africa it is responsible for almost all recorded malaria and, along with measles, malnutrition, diarrhoea and pneumonia, accounts for most of the deaths in children. In highly malarious areas pregnant women may develop severe anaemia; malaria is an important cause of fetal death. In areas of low transmission all age groups are at risk and, from time to time, epidemics of *P. falciparum* kill thousands of people. Once a patient is infected with *P. falciparum*, it takes 7–27 (average 12) days for the first clinical signs to appear (incubation period). If the patient lacks immunity, infection may quickly develop into an acute form, producing serious damage to the brain (cerebral malaria) and other organs. Cerebral malaria is characterized by coma, which can lead to death. Some patients who recover from cerebral malaria may suffer lifelong neurological sequelae. As there are no hypnozoites in *P. falciparum* infection, only one generation of parasites is produced, but recrudescence may occur up to 1 year later.

In blood films the microscopist usually sees only young trophozoites (ring forms). Gametocytes may also be seen; the more mature trophozoites and schizonts are hidden

away (sequestered) in the organs of the body. In fact, when schizonts are found in blood films, it is usually a sign that infection is heavy and has reached a critical stage. Occasionally, the microscopist will see grains or clumps of pigment (digested haemoglobin), produced by the malaria parasite, within the cytoplasm of white blood cells that have destroyed mature malaria parasites (**h**). In some films only gametocytes are seen. Infected individuals who have only circulating gametocytes may lack signs and symptoms of the disease, but they can still infect the *Anopheles* mosquitos that bite them. The erythrocytic cycle of *P. falciparum* takes about 48 hours (i.e. it is repeated every third day), although shorter intervals are also possible.

**Diagnostic problems:** If only a few ring forms are found in blood films, they may be difficult to differentiate from other species of malaria. The presence of large numbers of ring forms in the absence of other stages is strongly suggestive of *P. falciparum* infection; accolé or appliqué forms and multiple infection of red blood cells would further strengthen the diagnosis. Gametocytes are not seen early in the infection, so their absence from blood films should not preclude a diagnosis of *P. falciparum*. Occasionally, only gametocytes may be found in peripheral blood films.

## Preparation of buffer solutions for malaria staining

A phosphate buffer solution, correctly balanced to pH 7.2, is essential for Giemsa staining of malaria parasites.

### Preparation of a solution for daily use

1. Dissolve 1.0 g of anhydrous disodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4$ ) and 0.7 g of potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) in 1000 ml of distilled or deionized water. Filtered rainwater or even tap water may be used if no other is available.
2. Check the pH with a pH meter or colour-based indicator.
3. If the pH is below 7.2, add small quantities of a 2%  $\text{Na}_2\text{HPO}_4$  solution; if it is above 7.2, add small quantities of a 2% solution of  $\text{KH}_2\text{PO}_4$ .
4. When balanced to pH 7.2, store the solution in a tightly stoppered bottle, preferably of dark glass, in a cool place away from direct sunlight.

This solution is good for some weeks, but needs to be regularly checked to ensure that growths or moulds do not become established. This may be done by shaking the solution; if cloudy, discard.

### Preparation of a concentrated stock solution (useful for field trips or dispatch to distant locations)

1. Dissolve 3.0 g of anhydrous  $\text{Na}_2\text{HPO}_4$  and 2.1 g of  $\text{KH}_2\text{PO}_4$  in 25 ml of distilled or deionized water.
2. If the pH is below 7.2, add small quantities of a 2%  $\text{Na}_2\text{HPO}_4$  solution; if it is above 7.2, add small quantities of a 2% solution of  $\text{KH}_2\text{PO}_4$ .
3. Store in a dark bottle away from direct sunlight; this solution will remain good for several weeks.
4. To make up a working solution, dilute 1 ml of the concentrate to 20 ml with distilled or deionized water.

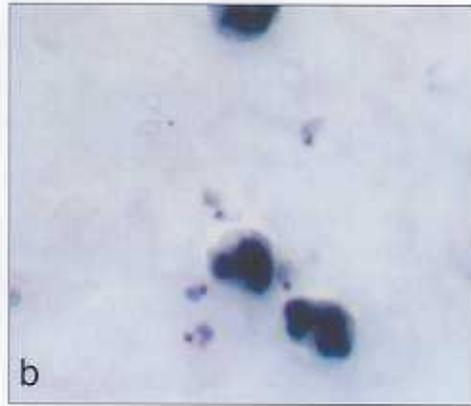
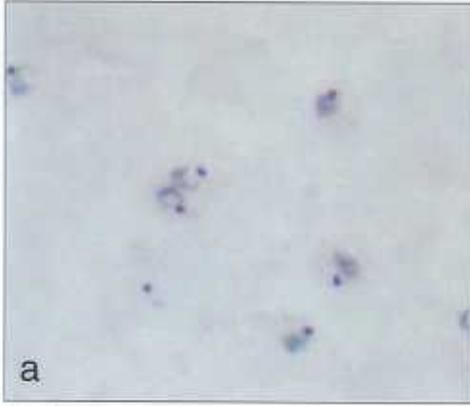
### Preparation of preweighed packs

The two phosphate salts can be preweighed and placed together in a clearly labelled, tightly stoppered tube or bottle or in a well sealed plastic bag stored in a screw-capped jar. To make the solution, add the contents to 1 litre of distilled or deionized water and adjust to pH 7.2.

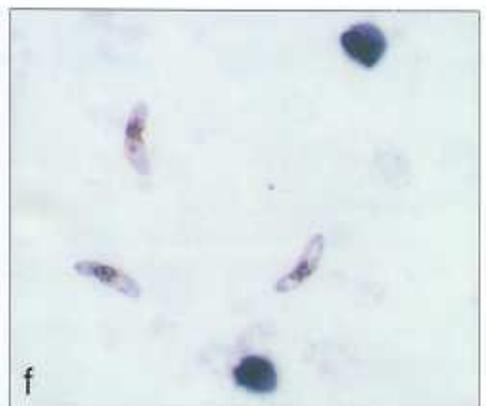
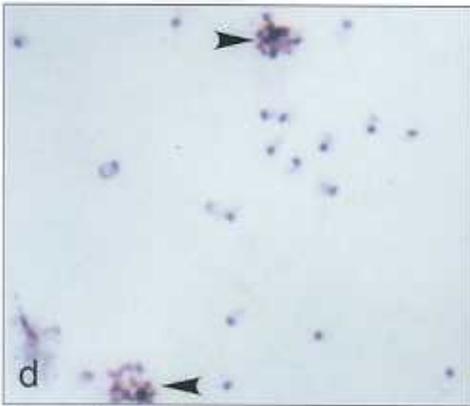
### Buffer tablets

Buffer tablets that produce a solution of pH 7.2 when dissolved are readily available from laboratory suppliers but are rather expensive.

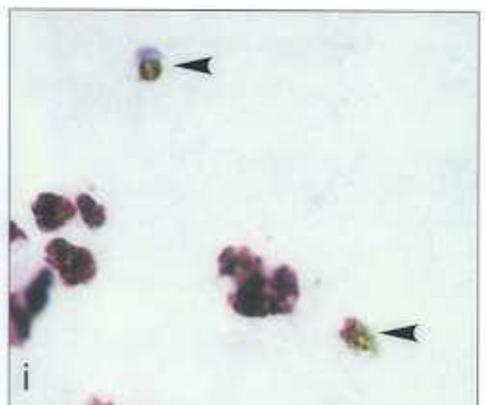
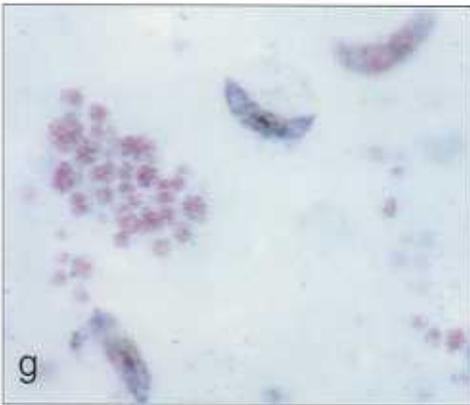
*Plasmodium falciparum* thick film



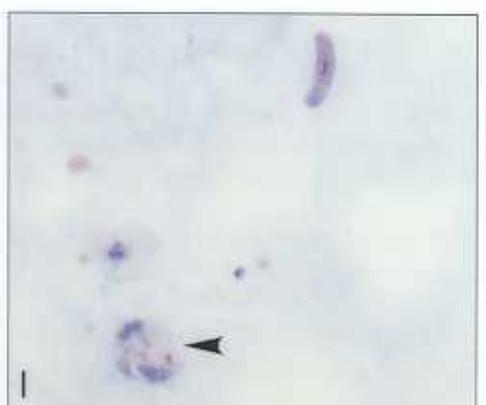
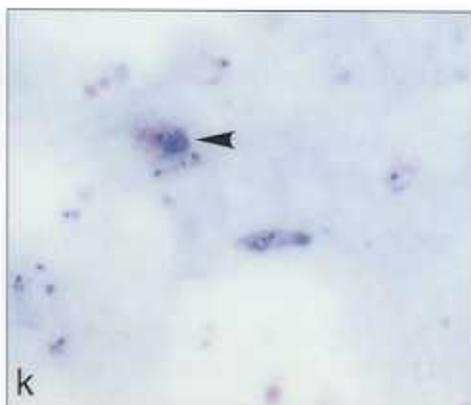
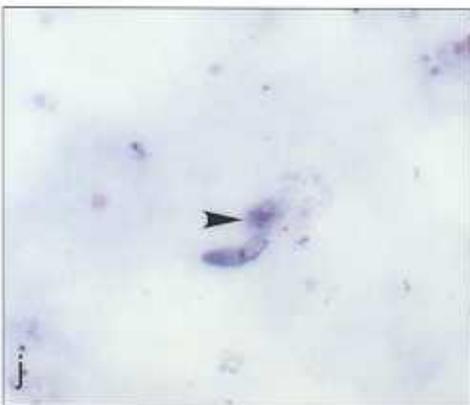
Ring forms (a–c) are usually small, often numerous, with delicate, scanty cytoplasm. Ring and comma forms (distorted ring forms) are common but older ring forms may have more cytoplasm (a). Ring forms with double chromatin dots are common. The presence of large numbers of ring forms in the absence of other morphological stages is generally diagnostic for this species (c).



In heavy infections, small, compact schizonts (d, arrows), usually containing between 16 and 24 (range 8–40) merozoites, may be found clustered around a small, dark mass of pigment (d); large numbers of ring forms are also present. Ring forms may be found with gametocytes (e) and, in some infections, gametocytes may be found in a field in the absence of ring forms (f).



Typical banana- or sausage-shaped gametocytes (g, h), sometimes referred to as crescents, are easy to identify. However, when damaged or altered in the process of making thick films, gametocytes (i, arrows), in the absence of ring forms, may be more difficult to recognize.



In mixed infections of *P. falciparum* and *P. vivax* (j–l), a typical gametocyte and numerous small ring forms of *P. falciparum* are visible, along with a *P. vivax* trophozoite (j, arrow). In (k) ring forms and a gametocyte are present with a large trophozoite of *P. vivax* (arrow); in (l) a gametocyte is visible along with a prominent trophozoite of *P. vivax* (arrow).



## Preparation of thick and thin blood films on the same slide

For routine malaria microscopy, thin and thick blood films are made on the same slide. The thin film is used as a label but, if well prepared, is also available for species confirmation. Examination should be done on a thick film.

The following items are needed for preparation of blood films: clean and wrapped slides; sterile lancets; 70% ethanol and water; absorbent cotton wool; surgical gloves; clean, lint-free cotton cloth; slide box (or cover to exclude flies and dust); record form or register; soft lead pencil; ball-point pen.

1. Holding the patient's left hand, palm upwards, select the third finger from the thumb. (The big toe can be used with infants. The thumb should never be used for adults or children.)

Clean the finger with a piece of cotton wool lightly soaked in 70% ethanol, using firm strokes to remove grease and dirt from the ball of the finger.

Dry the finger with a clean cotton cloth, using firm strokes to stimulate blood circulation.

2. Puncture the ball of the finger with a sterile lancet, using a quick rolling action.

Apply gentle pressure to the finger to express the first drop of blood and wipe it away with a dry piece of cotton wool. Make sure that no strands of cotton remain on the finger to be later mixed with the blood.

3. Working quickly and handling clean slides only by the edges, collect the blood as follows.

Apply gentle pressure to the finger and collect a single small drop of blood, about this size ●, on the middle of the slide. This is for the thin film.

Apply further pressure to express more blood and collect two or three larger drops, about this size ●, on the slide, about 1 cm from the drop intended for the thin film (see illustration). This is for the thick film.

Wipe the remaining blood away from the finger with a piece of cotton wool.

4. **Thin film.** Using a second clean slide as a "spreader" and, with the slide with the blood drops resting on a flat, firm surface, touch the small drop with the spreader and allow the blood to run along its edge. Firmly push the spreader along the slide, keeping the spreader at an angle of 45°. Make sure that the spreader is in even contact with the surface of the slide all the time the blood is being spread.

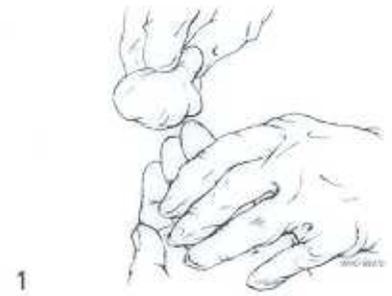
5. **Thick film.** Always handle slides by the edges or by a corner to make the thick film as follows.

Using the corner of the spreader, quickly join the drops of blood and spread them to make an even, thick film. The blood should not be excessively stirred but can be spread in circular or rectangular form with 3 to 6 movements. The circular thick films should be about 1 cm ( $\frac{1}{3}$  inch) in diameter.

6. Label the dry thin film with a soft lead pencil by writing across the thicker portion of the thin film the patient's name or number and the date. Do not use a ball-point pen for labelling the slide. Allow the thick film to dry with the slide in a flat, level position, protected from flies, dust and extreme heat.

7. Wrap the dry slide in the patient's record form and dispatch it to the laboratory as soon as possible.

8. The second slide used for spreading the blood films may now be used for the next patient and another clean slide from the pack will be used as a spreader.



1



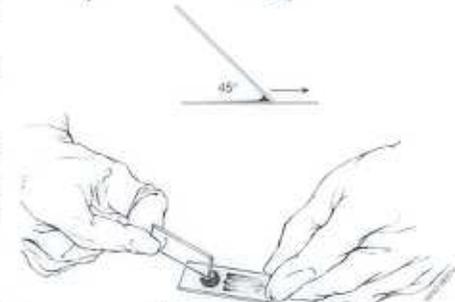
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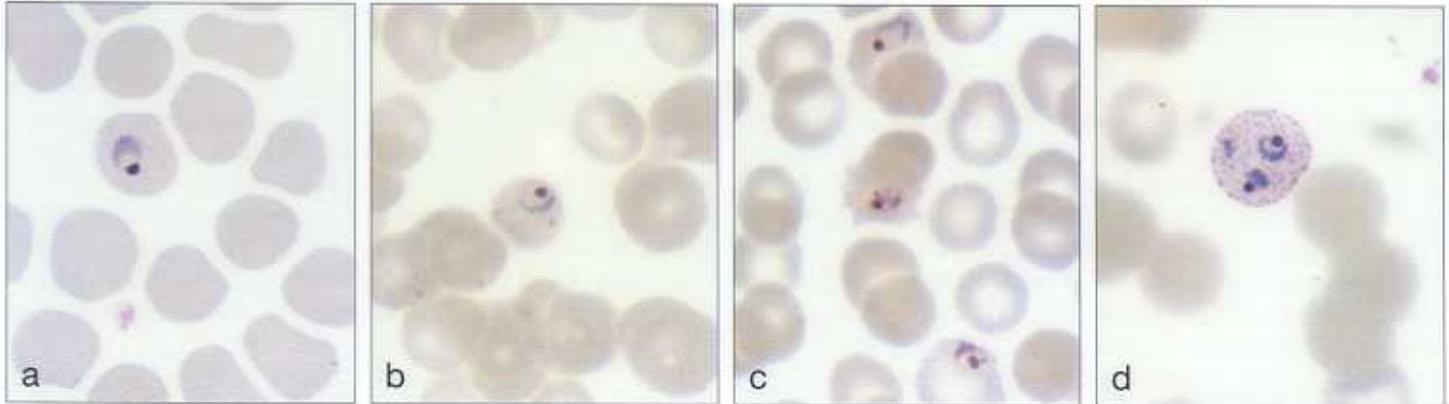


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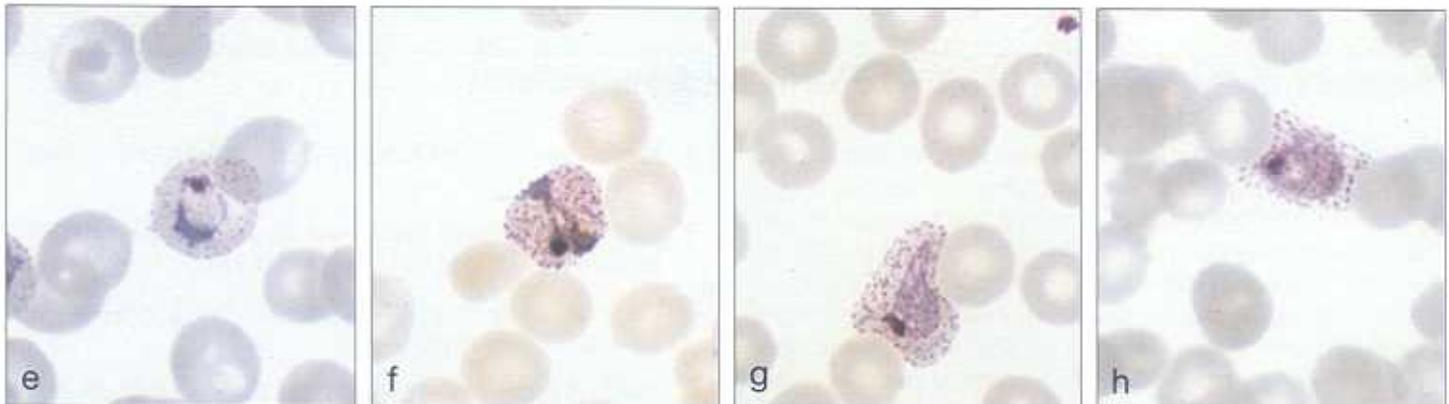


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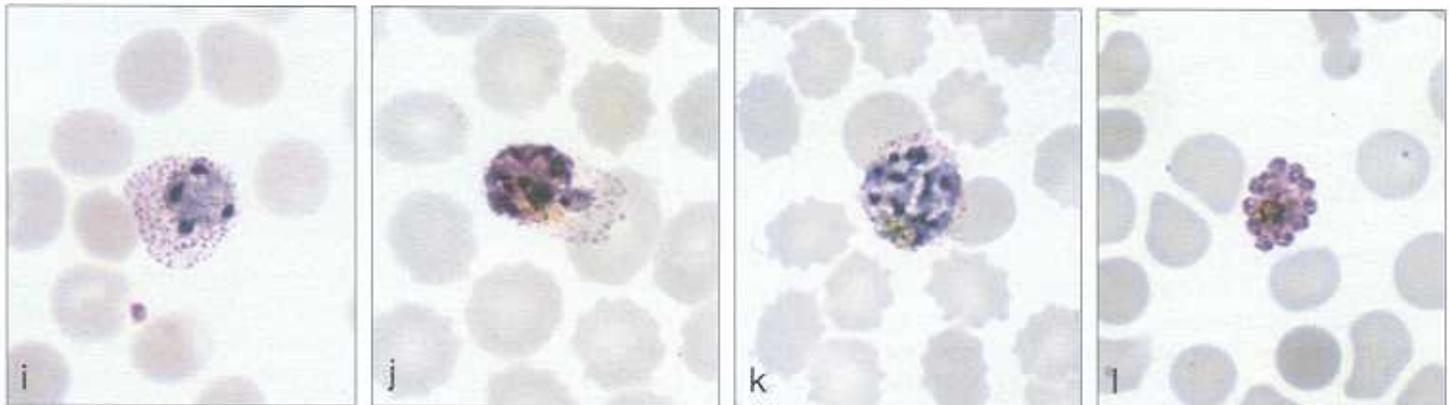
*Plasmodium vivax* thin film



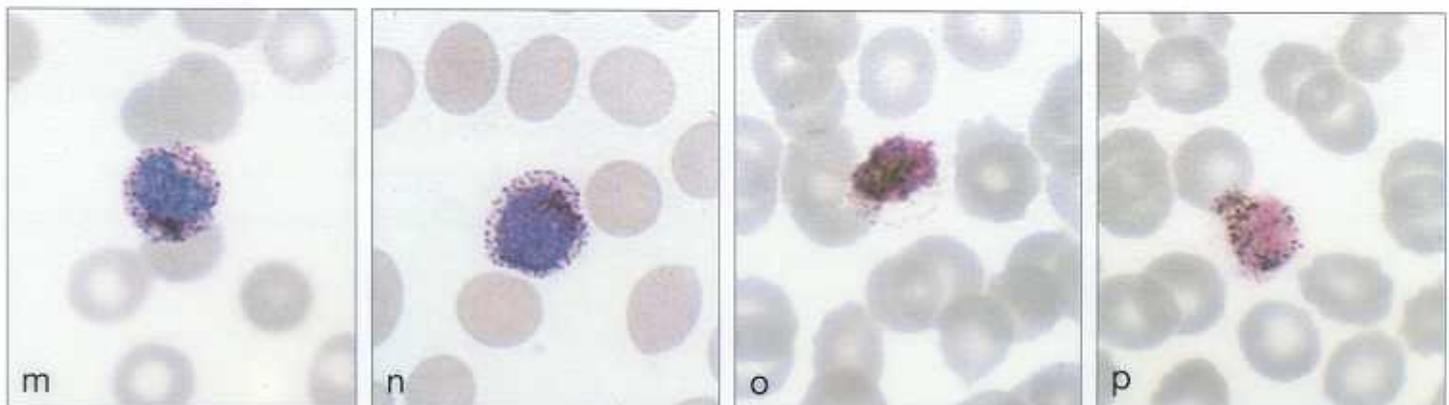
**Trophozoites:** Merozoites typically invade young erythrocytes. The infected red blood cells become enlarged, often by more than about 50% (about the size of a white blood cell), and may vary from round to oval. Very young trophozoites (ring forms) typically measure about one-third the diameter of the red blood cell; they are composed of a prominent red chromatin dot and a fine circle of blue cytoplasm (a–d). Occasionally there may be two chromatin dots. Young growing trophozoites have an increased mass of cytoplasm



and usually an irregular, amoeboid appearance (e). Older trophozoites become very large and markedly amoeboid and can fill the red blood cell (f–h). The chromatin mass is large and compact; grains of pigment are scattered throughout the cytoplasm and a conspicuous vacuole is almost always present (h). Mature trophozoites may be difficult to differentiate from gametocytes (h, m). In properly stained blood films, Schüffner's dots may be seen in red blood cells containing young trophozoites (e–g) and, rarely, earlier (d).



**Schizonts:** Early in its development, the schizont is large and amoeboid. The chromatin divides into small irregular masses, ultimately forming between 14 and 20 (range 12–24) merozoites (i–l). In the mature schizont, one or two clumps of pigment may be centred in the cluster of merozoites (l). Each merozoite is composed of a dot of chromatin surrounded by a small mass of cytoplasm (l). The mature schizont usually fills the enlarged red blood cell (k, l). Schüffner's stippling is usually evident in the infected cell (k).



**Gametocytes:** The parasites are usually round to oval and regular in outline (m–p). Macrogametocytes (m, n) are typically large and blue and have a small, eccentric, compact chromatin mass. Brown pigment is scattered throughout the cytoplasm and vacuoles are absent. The mature parasite nearly fills the enlarged red blood cell (m, n); this stage is often difficult to distinguish from a mature trophozoite (h). Microgametocytes (o, p) have a large, diffuse mass of pink-staining chromatin and light blue to pink or lavender cytoplasm containing scattered granules of dark pigment.



## *Plasmodium vivax*

*Plasmodium vivax* occurs throughout the tropics and subtropics and is also the predominant species in temperate climates. It is very rare in West Africa. The incubation period is typically about 13–17 days, although some strains may have prolonged incubation periods of up to 6–12 months. An important feature of this species is the presence and persistence of exoerythrocytic stages (hypnozoites) in the liver, which may produce relapses of infection repeatedly over a period of many years. The erythrocytic cycle usually requires about 48 hours and all the characteristic morphological stages of trophozoites, schizonts and gametocytes may be found in the peripheral blood.

**Diagnostic problems:** If only a few ring forms are found in blood films, diagnosis is very difficult. It may be necessary to make an extended examination of the slide for other stages and for Schüffner's stippling. Examination of thick blood films may be necessary to demonstrate additional parasites. It should be remembered that, in thick films, the periphery of the film may be poorly lysed and "ghosts" of red blood cells may demonstrate organisms as well as signs of stippling (Plate 5, f).

## Biosafety in the handling of blood specimens from patients

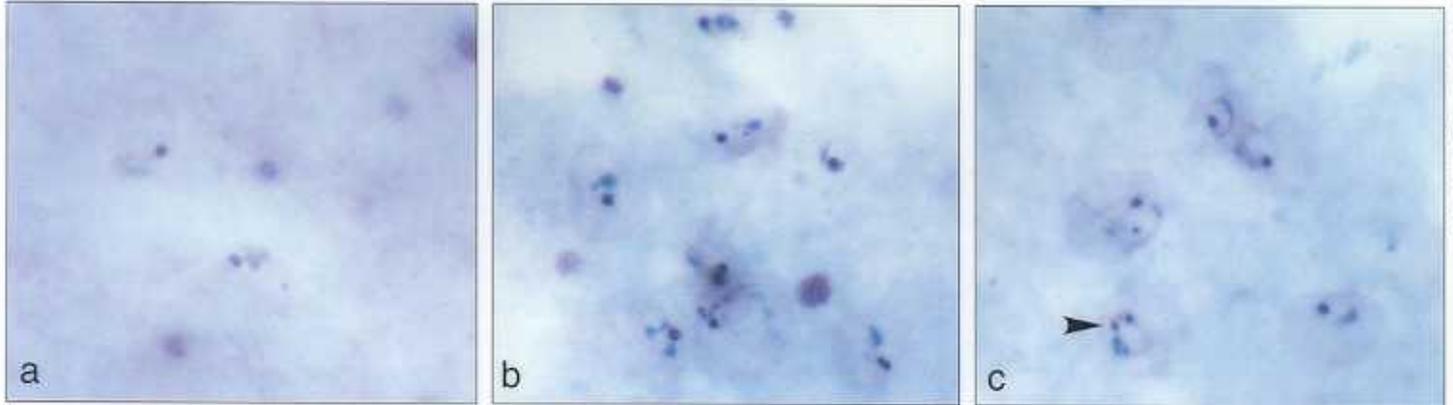
The hazards that technical staff encounter in laboratories are widely recognized. All laboratories should follow national guidelines on safety or they may have guidelines that have been developed locally. A number of guidelines are also available from WHO; these include *Safety in health-care laboratories*, *Guidelines on sterilization and disinfection methods effective against human immunodeficiency virus (HIV)*, 2nd ed., *Biosafety guidelines for diagnostic and research laboratories working with HIV* and *Preventing HIV transmission in health facilities* (see "Further reading" section).

All blood samples must be considered as potentially infectious. Two of the more dangerous bloodborne diseases are hepatitis (caused mainly by hepatitis viruses B and C) and HIV/AIDS. When blood samples are collected for diagnosis of malaria, biosafety guidelines must be followed.

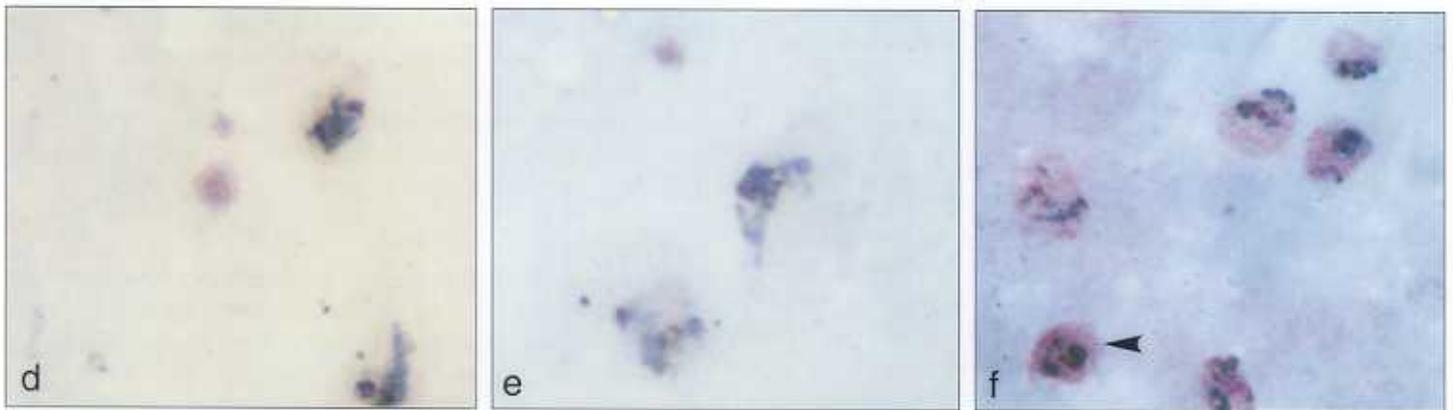
The major hazard to laboratory workers taking blood specimens is contamination of the hands and the mucous membranes of the eyes, nose and mouth by infectious blood. Such contamination occurs as a result of penetrating injuries caused by sharp objects, and the spilling or splashing of specimens. The guidelines given here outline practices and procedures designed to keep such accidents to a minimum.

1. All laboratory workers must be adequately trained, both in the duties they perform and in all aspects of laboratory work.
2. Standard operating procedures should be clearly written, which cover all steps in the procedures to be carried out.
3. Wear a laboratory gown, smock or uniform when in the laboratory. Remove this protective clothing before leaving the laboratory.

4. Wear gloves when taking and handling blood specimens.
5. Do not touch your eyes, nose or other exposed membranes or the skin with gloved hands.
6. Do not leave the workplace or walk around the laboratory wearing gloves.
7. Discard gloves whenever they are thought to have become contaminated, wash your hands, and put on new gloves.
8. Wash your hands with soap and water immediately after any contamination and after work is completed. If gloves are worn, wash your hands with soap and water after removing the gloves.
9. Puncture wounds, cuts and skin contaminated by spills or splashes of blood should be thoroughly washed with soap and water. Bleeding from the wound should be encouraged.
10. All spills, accidents and overt or potential exposure to infectious specimens should be reported immediately to the laboratory supervisor and appropriate action should be taken to prevent further occurrences.
11. Place used lancets in a puncture-resistant container.
12. Disinfect work surfaces when procedures are completed and at the end of each working day. An effective all-purpose disinfectant is a hypochlorite solution with a concentration that provides 0.1% available chlorine (1 g/litre).
13. Do not eat, drink or smoke in the laboratory.
14. Access to the laboratory must be restricted to authorized personnel only.

*Plasmodium vivax* thick film

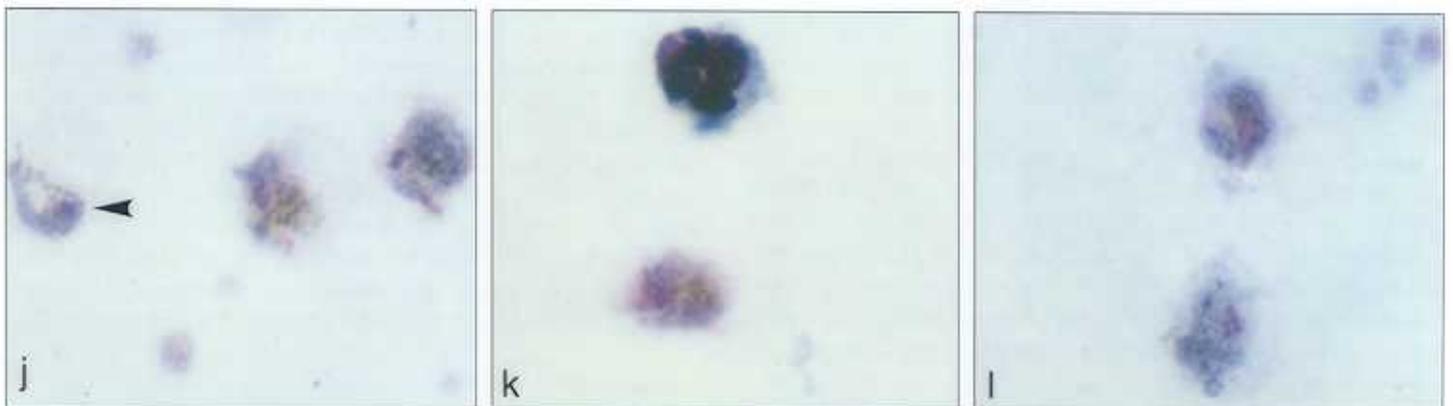
Typical ring forms (a–c), and one with a double chromatin dot (c, arrow), vary somewhat in size and have prominent red chromatin dots with varying amounts of light blue chromatin. The ring forms are typically larger than those of *P. falciparum*, often without a complete circle of blue cytoplasm. Young trophozoites (b, c) are visible in "ghosts" of erythrocytes.



Trophozoites (d–f) of this species may vary in both size and the numbers in which they are present. The cytoplasm is darker and thicker (d, e) than that seen in the "ghosts" of red blood cells containing amoeboid organisms with irregular and fragmented cytoplasm (f). Schüffner's stippling is evident in several of the infected erythrocytes (f). A gametocyte is also visible (f, arrow). Large trophozoites are often dense, compact and darkly staining and contain scattered pigment; they can be confused with macrogametocytes.



Immature (g, h) and mature (i) schizonts are usually large and present in small to moderate numbers. Mature schizonts usually contain between 16 and 24 merozoites and have a loose mass of pigment. Individual chromatin masses are irregular in shape and often quite large in immature schizonts. Immature schizonts may be confused with those of *P. malariae*.



Gametocytes (j–l) of *P. vivax* are usually larger than those of other species. Mature forms are usually large and round; pigment granules are fine and dispersed throughout the non-vacuolated cytoplasm. Chromatin masses are dense and may or may not be well defined. Differentiation of gametocytes, especially immature forms, from mature trophozoites (j, arrow) is often difficult.



## Staining blood films for malaria parasites

The use of Giemsa stain is the recommended and most reliable procedure for staining thick and thin blood films to demonstrate malaria parasites. It is available as a commercially prepared solution or in powder form; however, since the quality of the stain may vary it should be acquired from a reputable manufacturer and each batch of prepared stain should be evaluated before being used to stain large numbers of slides. Further information on stain and buffer solution preparation and staining procedures is provided in the WHO publication *Basic laboratory techniques in medical parasitology* (see "Further reading" section).

### Preparation of stock solution of Giemsa stain

*Giemsa stain formula:* Giemsa powder, 3.8 g; methanol, 250 ml; glycerol, 250 ml.

### Giemsa stain preparation

1. Put 50 solid glass beads in a dark bottle. If a dark bottle is not available, use a chemically clean, dry, clear glass or polyethylene bottle of suitable size. Pour in the measured amount of methanol and add the stain powder.
2. Tightly stopper the bottle. Allow the stain powder to sink slowly through the methanol until it settles to the bottom. Shake the bottle with a circular motion for 2–3 minutes.
3. Add the measured amount of glycerol and repeat the shaking process. Continue to shake the bottle for 2–3 minutes at half-hourly intervals for at least six times.
4. Leave the bottle for 2–3 days, shaking it 3–4 times each day until the stain is thoroughly mixed. This is the stock solution. Keep some of this stock solution in a small bottle for routine use to avoid contamination of the stock solution.

Each newly prepared batch of stock solution should be properly labelled, including date of preparation, and should be tested for optimum stain dilution and staining time. Always keep the bottle, tightly stoppered, in a cool place, away from direct sunlight. Clear glass bottles should be covered with thick dark paper to exclude the light.

### Giemsa staining technique — regular method

Ideally, for optimum staining, thick and thin films should be made on separate slides. This is often not possible and thick and thin films are generally made on the same slide. When this is done, good-quality staining of the thick film is of primary importance. Allow the thick film to dry in a flat, level position, protected from flies, dust and extreme heat. It is important to note that, in tropical countries, autofixation may occur. This is a process by which blood films gradually become fixed through exposure to the atmosphere. In tropical conditions, this process may become well advanced with films stored for only a few days. When storing thick films for later staining it is particularly important to keep them in a dry atmosphere; a desiccator may be used for this purpose.

### Fixation procedure

Once the thin film has dried, fix it by adding a few drops of methanol, or by dipping it into a container of methanol for a few seconds. With prolonged fixation it may be difficult to demonstrate Schüffner's dots and Maurer's clefts. Allow the thin film to dry thoroughly by evaporation. Exposure of the thick film to methanol or methanol vapour should be avoided. If the methanol does fix the thick film, dehaemoglobinization will not be possible.

### Staining procedure (for 20 or more slides)

Place the slides in a staining trough. Prepare a 3% Giemsa solution in buffered distilled or deionized water, pH 7.2, in sufficient quantity to fill the trough and cover the slides. Stain for 30–45 minutes, out of sunlight. Pour clean water gently into the trough to float off the iridescent scum on the surface of the stain. Rinse rapidly in clean water. Remove the slides one by one and place them, film side downwards, in a slide rack to drain and dry, making sure that the film does not touch the rack.

### Staining results

On the thick film, the background should be clean and free from debris; leukocyte nuclei should be a deep, rich purple, and the malaria parasites should have deep red chromatin and pale purplish blue cytoplasm. At the periphery of the thick film, erythrocytes are not lysed and Schüffner's stippling may be apparent in *P. vivax* and *P. ovale* infections.

### Giemsa staining technique — rapid method

This is a satisfactory procedure but uses much more stain than the regular method.

### Fixation procedure

Once the thin film has dried, fix it by adding a few drops of methanol or by dipping the film in a container of methanol for a few seconds. To permit dehaemoglobinization, the thick film should not be fixed; exposure of the film to methanol or methanol vapour should therefore be avoided. Allow to dry thoroughly by evaporation.

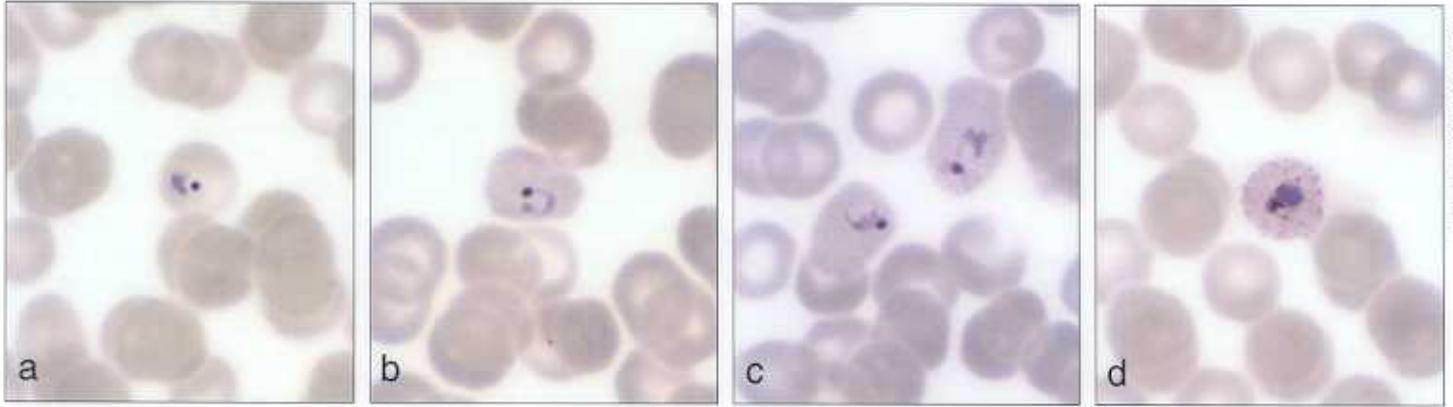
### Staining procedure

Prepare a 10% Giemsa solution in buffered distilled or deionized water, pH 7.2; if a small quantity is being used, 3 drops of stain per ml of buffered water will give the correct concentration of Giemsa solution. One slide requires about 3 ml of stain solution. Gently pour the stain onto the slide or use a pipette. Alternatively, the slide can be placed face-down on a concave staining plate and stain introduced underneath the slide. Stain for 5–10 minutes. Gently flush the stain off the slide by adding drops of clean water; do not tip off the stain and then wash, as this will leave a deposit of scum over the film. Place slides, film side downwards, in a slide rack to drain and dry, making sure the film does not touch the rack.

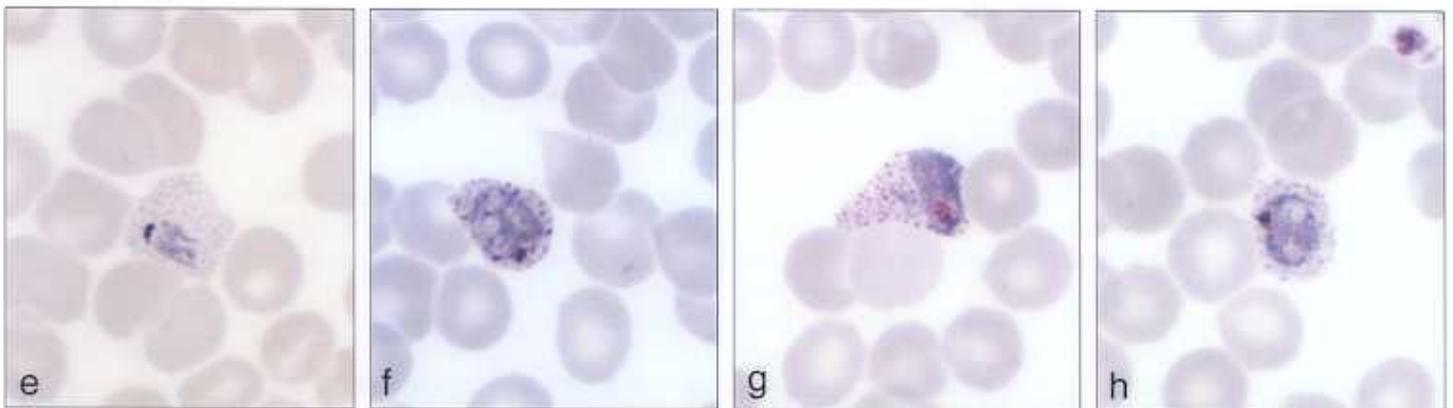
### Staining results

These are the same as are obtained in the regular method of Giemsa staining.

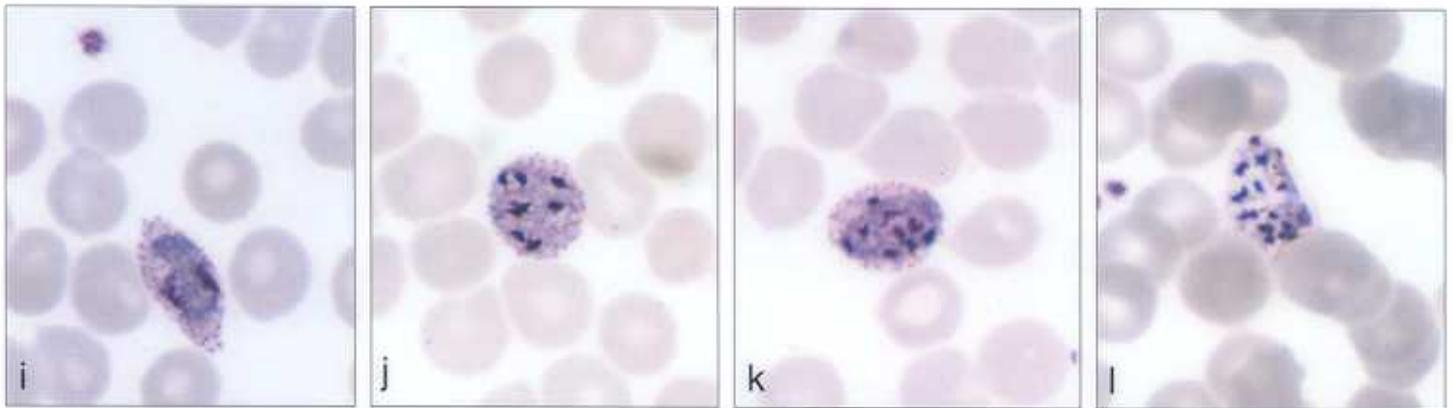
*Plasmodium ovale* thin film



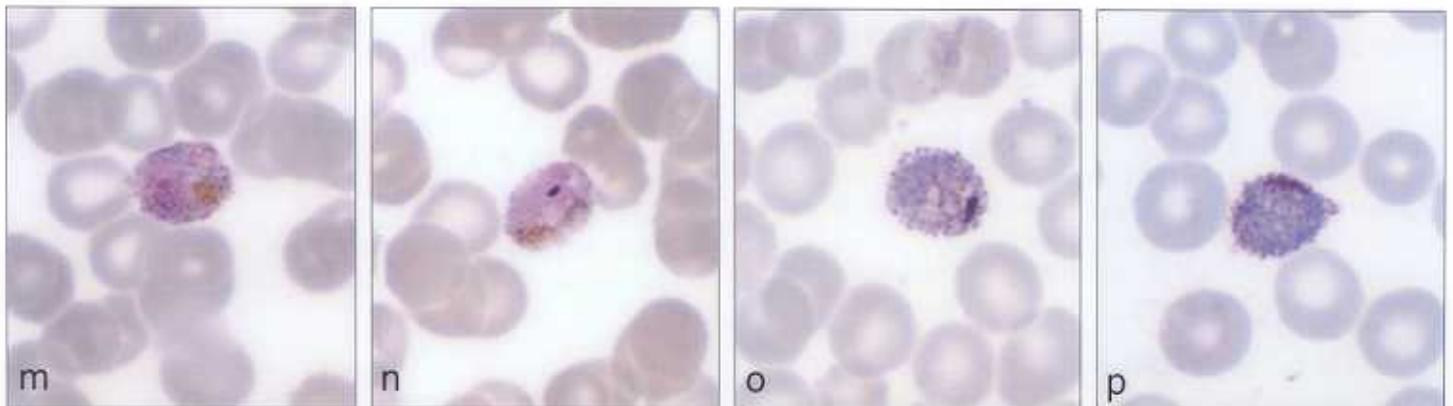
**Trophozoites:** Merozoites, like those of *P. vivax*, invade young erythrocytes; the ring stage resembles that of the other human malaria species (a, b). Infected red blood cells may be round but are often oval in shape and may or may not have an irregular margin (c, d). In young trophozoites, the mass of chromatin may be large and irregular in shape



and may show Schüffner's stippling (c–e). More mature trophozoites are often in slightly enlarged red cells (f), which may be oddly shaped with fringed (fimbriated) margins (g, h). The trophozoites tend to be less amoeboid in appearance than those of *P. vivax*.



**Schizonts:** Developing (i–k) and mature (l) schizonts are frequently found in oval-shaped cells, many with irregular margins (i). Erythrocytes may be only slightly enlarged. Mature schizonts usually have 6–12 merozoites, but occasionally up to 18 (k, l). Clumps of pigment are often found at the centre of the cluster of merozoites. Schüffner's stippling is usually prominent.



**Gametocytes:** Mature gametocytes typically fill the host red blood cells, which may be round or oval with sometimes irregular margins (m–p). Pigment granules are scattered throughout the cytoplasm or, in microgametocytes (m, n), concentrated towards the periphery of the organisms. The gametocytes of *P. ovale* are often difficult to distinguish from those of *P. vivax*. Microgametocytes (m, n) are usually smaller than macrogametocytes and have diffuse pink chromatin. In macrogametocytes (o, p), chromatin is compact and usually dark red. Schüffner's stippling is prominent in the infected red cells.



## *Plasmodium ovale*

The global distribution of *P. ovale* is more limited than that of other *Plasmodium* species; it is found mainly in tropical Africa where *P. vivax* is rare. It is also present in New Guinea and the Philippines, with occasional reported occurrences in other parts of south-east Asia. Like *P. vivax*, *P. ovale* has an incubation period of approximately 16–18 days, an asexual cycle of about 50 hours and persistent exoerythrocytic stages (hypnozoites) in the liver which can produce relapses of infection. As in *P. vivax* infection, red blood cells typically show Schüffner's stippling; generally it develops much earlier in young trophozoites and the granules tend to be more prominent. All erythrocytic stages are usually present in the bloodstream.

**Diagnostic problems:** This species is the most difficult to diagnose because of morphological similarities to both *P. vivax* and *P. malariae*. Schüffner's stippling is similar to that seen in *P. vivax*. Oval or elongated infected red blood cells, which are very common in *P. ovale* infections, are occasionally seen in *P. vivax* infections. A history of the patient's residence and travel is important in establishing the diagnosis.

## Rapid staining of blood films for malaria parasites

A rapid staining technique for thick blood films which can produce excellent results involves the use of Field's stain; this procedure is especially useful when single thick films are submitted for evaluation. Further information on stain and buffer solution preparation and staining procedures is provided in the WHO publication *Basic laboratory methods in medical parasitology* (see "Further reading" section).

### Preparation of Field's stain

This stain is useful for rapid detection of malaria parasites; it is usually used for thick blood films. Schüffner's dots are not always stained with this procedure. Both Field's stain A and Field's stain B must be made up for staining slides, as both are used in the procedure.

#### Field's stain A — stock solution

Field's stain A stock solution may be prepared in two different ways, depending on whether prepared powders or original stains and chemicals are being used.

##### *Preparation from prepared powders:*

1. Add 5.9 g of Field's stain A powder to 600 ml of hot (approx. 60 °C) distilled water.
2. Mix until dissolved.
3. Filter when cool.

##### *Preparation from original stains and chemicals:*

1. Dissolve 10.0 g of anhydrous disodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4$ ) and 12.5 g of potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) in 1000 ml of distilled water.
2. Pour half of this solution into a 1-litre bottle containing a few glass beads. Add 1.6 g of methylene blue (medicinal) and 1.0 g of azur I and mix well.
3. Add the remainder of the phosphate solution.
4. Mix well and filter.

#### Field's stain B — stock solution

Field's stain B stock solution may be prepared in two different ways, depending on whether prepared powders or original stains and chemicals are being used.

##### *Preparation from prepared powders:*

1. Add 4.8 g of Field's stain B powder to 600 ml of hot (approx. 60 °C) distilled water.
2. Mix until dissolved.
3. Filter when cool.

##### *Preparation from original stains and chemicals:*

1. Dissolve 10.0 g of anhydrous disodium hydrogen phosphate ( $\text{Na}_2\text{HPO}_4$ ) and 12.5 g of potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) in 1000 ml of distilled water.
2. Add 2.0 g of eosin (yellow, water-soluble).
3. Mix until dissolved.
4. Filter.

## Staining with Field's stain (thick and thin films)

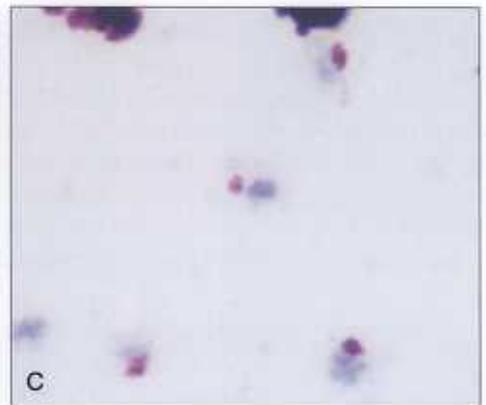
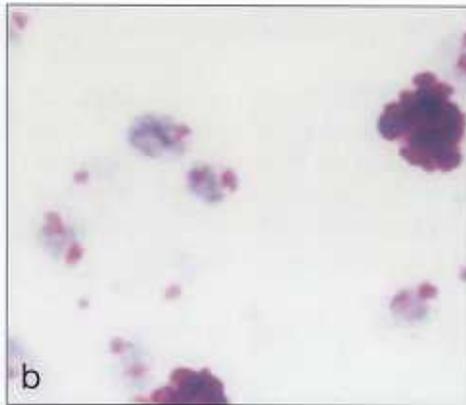
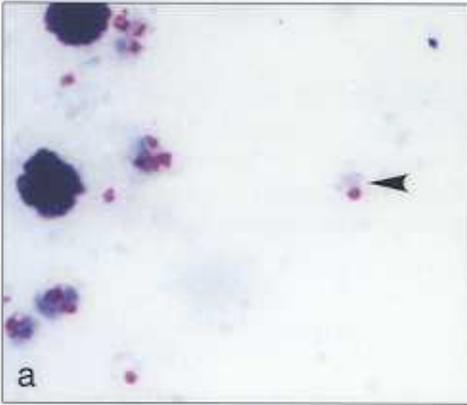
### Procedure for staining thick films

1. Dip unfixed film into a jar containing Field's stain A solution for 3 seconds.
2. Wash gently by dipping (once) into a jar of clean water.
3. Dip slide into a jar containing Field's stain B solution for 3 seconds.
4. Wash slide gently as in step 2.
5. Place slide upright in a draining rack to air-dry.

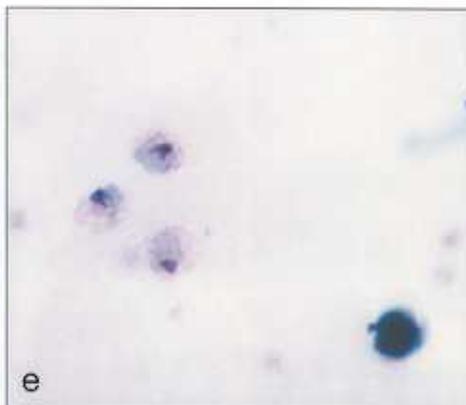
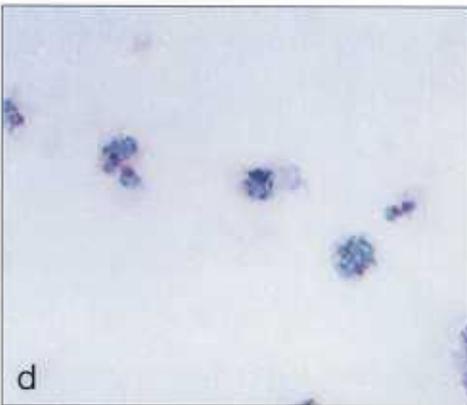
### Procedure for staining thin films

1. Fix film in methanol for 1 minute.
2. Wash off methanol with water.
3. Using a pipette, cover the film with diluted Field's stain B (1 part by volume of stock stain solution plus 4 volumes of distilled water buffered at pH 7.2).
4. Immediately add an equal volume of Field's stain A solution and mix well by tilting the slide.
5. Allow to stain for 1 minute.
6. Wash off stain with clean water.
7. Place slide upright in a draining rack to air-dry.

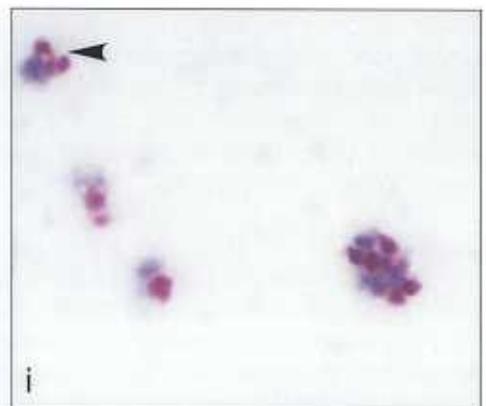
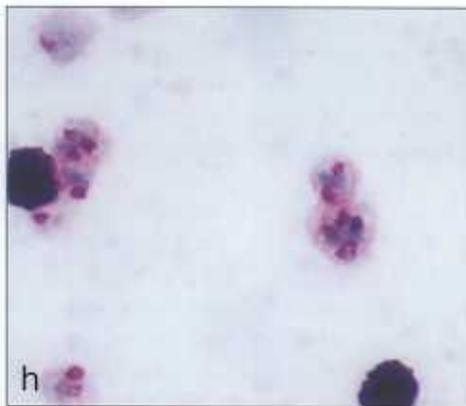
*Plasmodium ovale* thick film



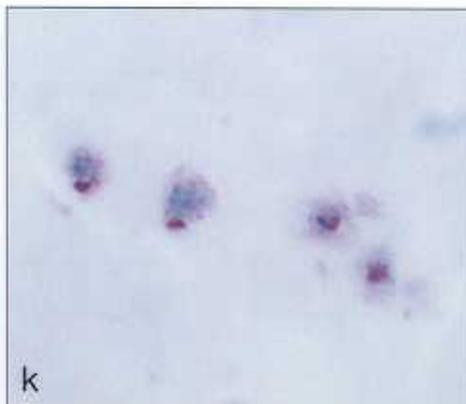
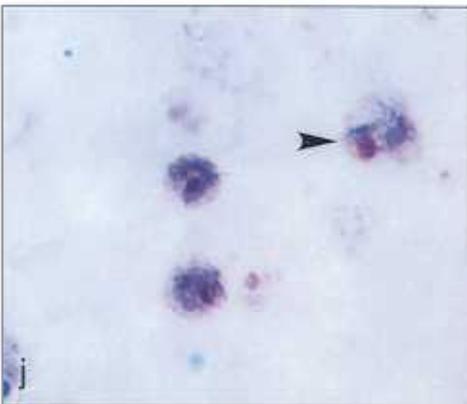
Ring forms and young trophozoites (a–c) of *P. ovale* are similar to those of *P. vivax*. A typical ring form with a prominent chromatin dot and wisp of blue cytoplasm is present (a, arrow), but most of the forms (a–c) are young trophozoites with a prominent mass of blue.



Compact, young trophozoites are present (d), but many of the trophozoites seen (e, f) are mature and have a somewhat amoeboid form. Stippling is frequently pronounced and appears as a pinkish "cloud" around the parasite (d–h).



Schizonts of *P. ovale* are usually few in number and similar in size to those of *P. malariae*. Immature schizonts with little pigment are evident (g). Mature schizonts contain 6–12 merozoites and pigment is usually seen as a concentrated mass (h). A mature schizont with a compact mass of pigment can be distinguished, along with two trophozoites and an immature schizont (i, arrow).



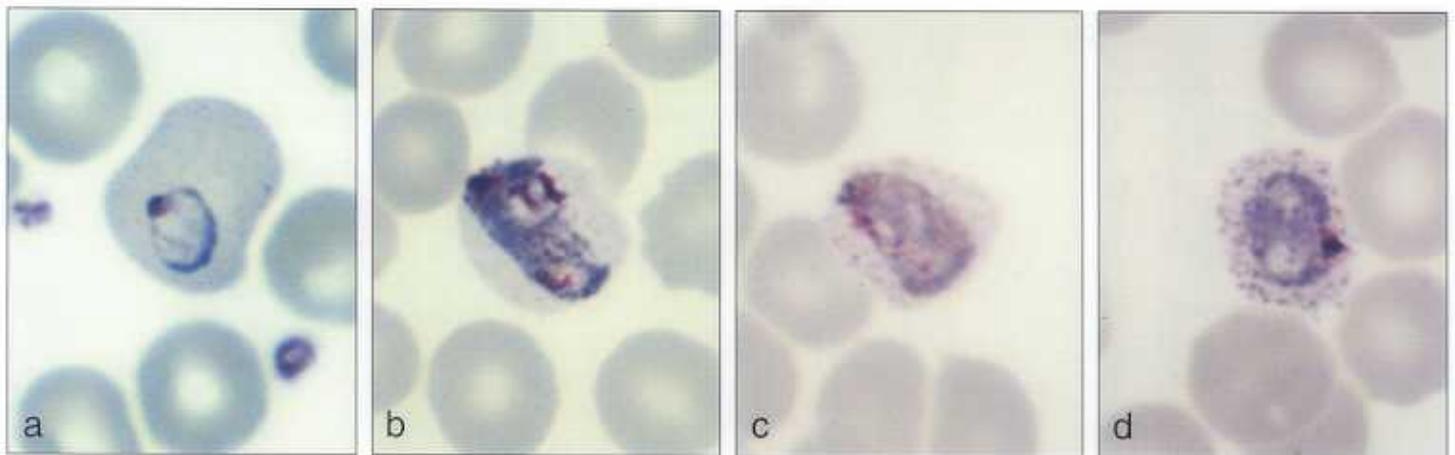
Immature and mature gametocytes of *P. ovale* may be difficult to distinguish from mature trophozoites. In addition, gametocytes of this species are similar in size and morphology to those of *P. vivax*. Two rounded gametocytes with considerable coarse pigment and a trophozoite (j, arrow) illustrate how difficult it is to distinguish between these stages. Two trophozoites (right) and two gametocytes (left) are visible (k), and also a single gametocyte with pigment (l).



## Effect of pH on Giemsa staining of malaria parasites

These four figures of *P. vivax* illustrate the effect of pH on Giemsa staining of malaria parasites and blood elements. In (a), note the markedly greenish blue staining of the erythrocytes at a pH of 7.6. A trophozoite is recognizable but the Schüffner's stippling in the erythrocyte cytoplasm is faint. At a pH of 7.4 (b), the erythrocyte containing a

trophozoite has a light greenish blue tinge and again Schüffner's stippling in the cytoplasm is pale and barely recognizable. At the more ideal pH values of 6.8 (c) and 7.0 (d), the red blood cells have a pinkish appearance, the trophozoites stain well and Schüffner's stippling is prominent.



## Routine examination of blood films for malaria parasites

### Examining thin films

Routine examination of thin films is not recommended as it takes at least three times as long to examine the equivalent amount of blood in a thin film as it does in a thick film. The exceptions to this rule are for poorly prepared thick films or when confirmation of species identification is necessary.

#### Procedure

It is usually in the distal third of the blood film that cells: (i) are most evenly distributed; (ii) are in a single layer; and (iii) have minimum distortion. This is where the microscopist should devote most of his or her attention. Place the slide on the mechanical stage, and position the x100 oil-immersion objective as shown by the letter X in Fig. 1. Add a drop of immersion oil to the slide and lower the objective until it touches the oil. Examine the blood film following the pattern of movement shown.

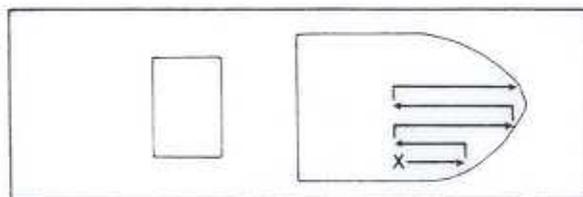


Fig. 1

### Examining thick films

#### Procedure

Routinely, it is thick films that are examined. Provided that they have been well made and stained before autofixation could occur, there should be no problems in identifying the species of malaria parasites. Place the slide on the mechanical stage, and position the x100 oil-immersion objective as shown by the letter X in Fig. 2. Add a drop of immersion oil to the slide and lower the objective until it touches the oil. Examine 100 fields, following the pattern of movement shown. If parasites are found, examine another 100 fields before confirming the identification of species present; this allows for possible detection of a mixed infection. To assist in examination, use a hand tally counter to count fields. Record findings on the appropriate record form. A parasite count may be included.

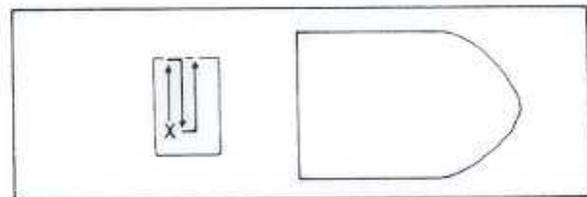
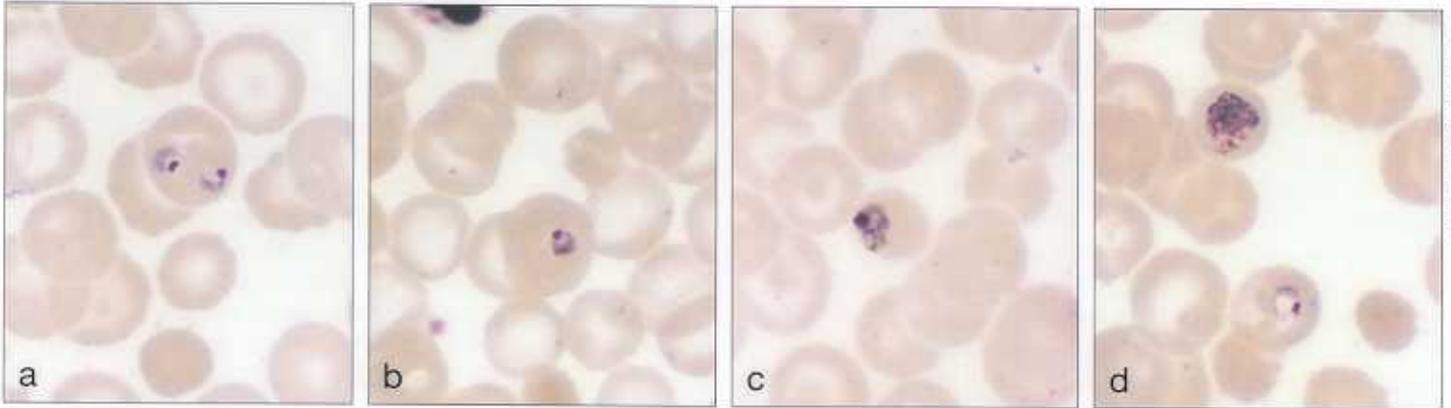
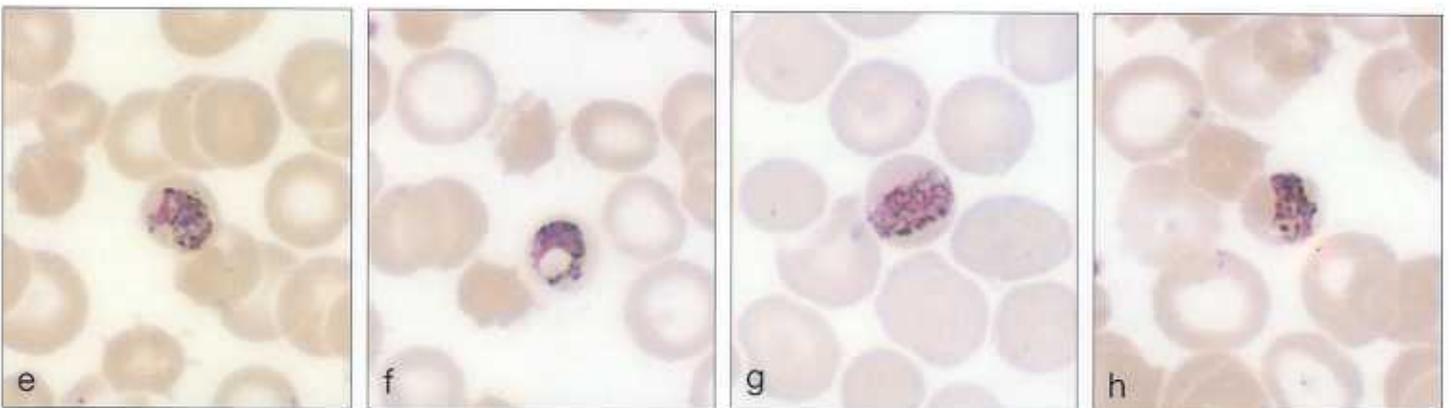


Fig. 2

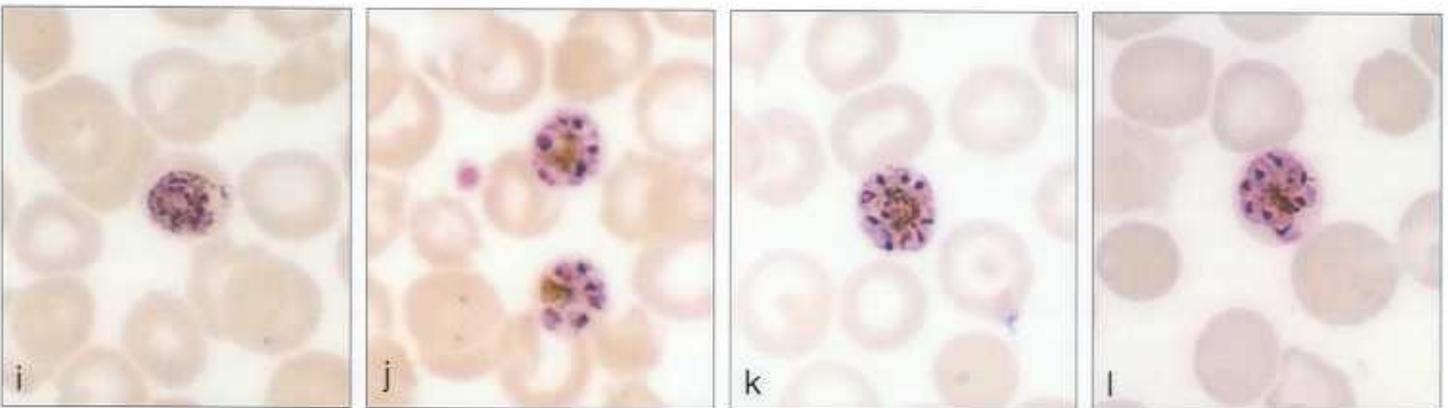
*Plasmodium malariae* thin film



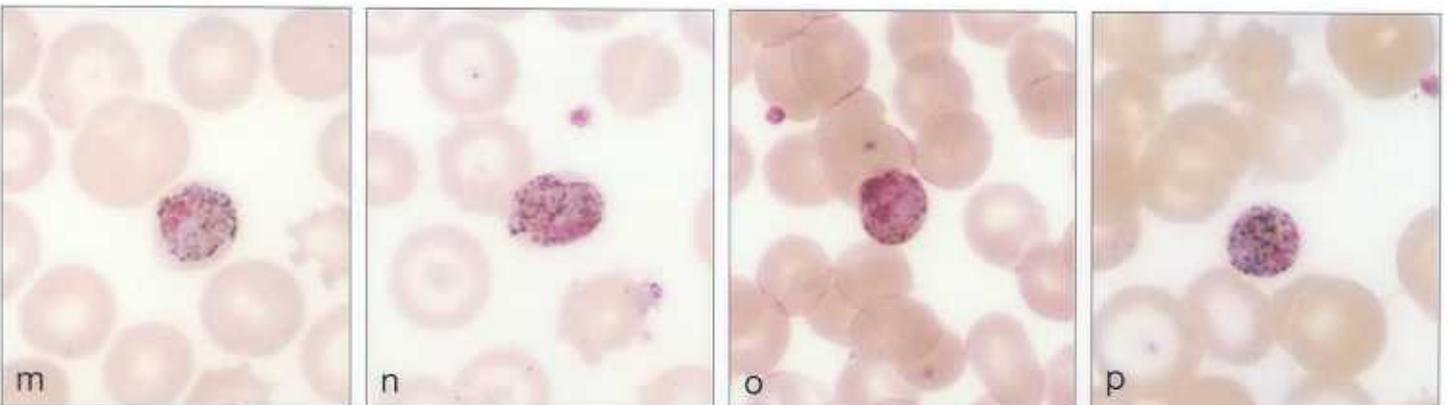
**Trophozoites:** Merozoites invade mature red blood cells. Ring forms are fairly large and the chromatin dot may be peripheral (a–d). Occasionally the chromatin dot may be located in the centre of the vacuole. Double chromatin dots and invasion of red cells by more than one organism (a) are uncommon. Trophozoites grow slowly and often they may stretch



across the equator of the red cell to produce the typical band forms associated with this species (g, h). Other trophozoites may exhibit a large vacuole surrounded by dense cytoplasm and an elongated mass of chromatin, producing a basket-like appearance (f). Mature trophozoites have considerable coarse, brown pigment and almost fill the erythrocyte (d, e).



**Schizonts:** Immature schizonts show fewer divisions of chromatin into irregular sizes and shapes (i). Mature schizonts usually have 8 or 10 merozoites (range 8–12), which are characteristically arranged in a rosette formation surrounding a mass of brown pigment (k). However, many schizonts, which usually fill the red blood cell (j, k), may have the pigment clumped at the periphery of the organism (l).



**Gametocytes:** It is difficult to differentiate between gametocytes and mature trophozoites in this species. Macrogametocytes (m, n) have a bluer cytoplasm and the chromatin is smaller, redder and more compact than that seen in microgametocytes. The cytoplasm of the microgametocyte is a light bluish pink (p) and the chromatin is diffuse, pinkish blue (o). Brown pigment is conspicuous with granules scattered throughout the cytoplasm (m–p).



## *Plasmodium malariae*

This parasite has a very broad distribution in tropical and subtropical areas of the world, including tropical Africa, south-east Asia and, uncommonly, the Americas. Its prevalence is considerably less than that of *P. vivax* and *P. falciparum*. *P. malariae* has a long incubation period, typically 28–30 (range 23–69) days. As for *P. falciparum*, there is only one cycle of exoerythrocytic schizogony. Recrudescence of infection, arising from persistent infections with low or undetectable levels of parasitaemia, is possible. Mixed infections often occur, particularly with *P. falciparum*. All erythrocytic infections occur in the peripheral blood. Parasitaemia is usually low. Infected red blood cells are not enlarged, and pale pink stippling (Zieman's stippling) is seen only rarely. The erythrocytic cycle requires 72 hours.

**Diagnostic problems:** *P. malariae* infections tend to have lower parasitaemia than do the other species of human malaria and this contributes to difficulties in demonstrating organisms. In thin blood films, trophozoites in the characteristic band form and schizonts arranged in rosette formation around a clump of pigment are important diagnostic traits. In thick blood films, mature trophozoites may be difficult to differentiate from gametocytes of the same species. The most distinctive stage of *P. malariae* in thick films is the mature schizont.

## Methods of counting malaria parasites in thick blood films

It is often necessary to determine the density of malaria parasites in thick blood films so that the physician is aware of the severity of the malaria infection and how the parasites are responding to treatment.

Two methods can be employed to count malaria parasites in thick blood films: determination of parasites/ $\mu\text{l}$  of blood, and the plus system.

1. Determination of parasites/ $\mu\text{l}$  of blood is accomplished by enumerating the number of parasites in relation to a standard number of leukocytes/ $\mu\text{l}$  (8000). Initially, the blood film is examined for presence of parasites and species identification. Using two hand tally counters, one for counting leukocytes and the other for parasites, follow one of these two procedures:

(i) if, after counting 200 leukocytes, 10 or more parasites are found, record the results on the record form in terms of the number of parasites/200 leukocytes;

(ii) if, after counting 200 leukocytes, the number of parasites is 9 or fewer, continue counting until you reach 500 leukocytes and then record the number of parasites/500 leukocytes.

After procedure (i) or (ii), use a simple mathematical formula, multiplying the number of parasites by 8000 and then dividing this figure by the number of leukocytes (200 or 500). The result is the number of parasites/ $\mu\text{l}$  of blood.

It is normal practice to count all the asexual forms of *P. falciparum* present. This is particularly important when monitoring the response to schizonticidal drugs, which would not be expected to have any effects on gametocytes.

(parasites counted/number of leukocytes)  $\times$  8000 = parasites/ $\mu\text{l}$  of blood

### Example:

If 200 leukocytes are counted:

(50 parasites  $\times$  8000/200 leukocytes) = 2000 parasites/ $\mu\text{l}$  of blood

If 500 leukocytes are counted:

(5 parasites  $\times$  8000/500 leukocytes) = 80 parasites/ $\mu\text{l}$  of blood

2. A simpler method of counting parasites in thick blood films is to use the plus system. This system is less satisfactory, however, and should be used only when it is not possible to carry out the more acceptable count of parasites/ $\mu\text{l}$  of blood. The system entails using a code of between one and four plus signs, as follows:

+ = 1–10 parasites per 100 thick film fields

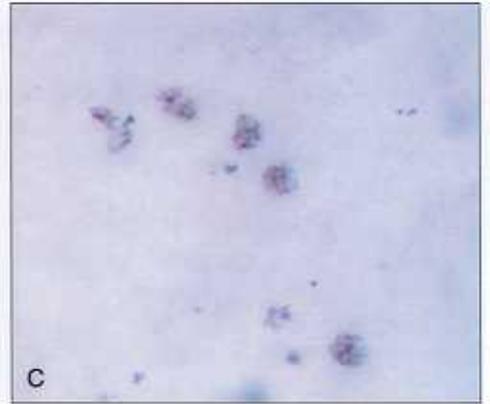
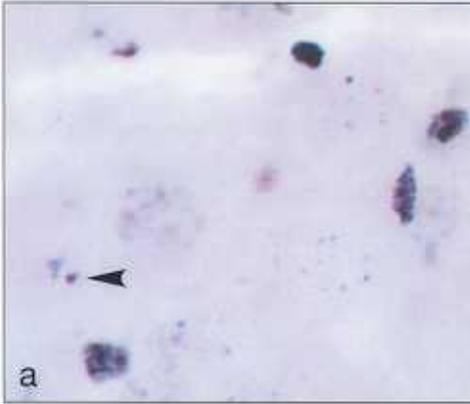
++ = 11–100 parasites per 100 thick film fields

+++ = 1–10 parasites per single thick film field

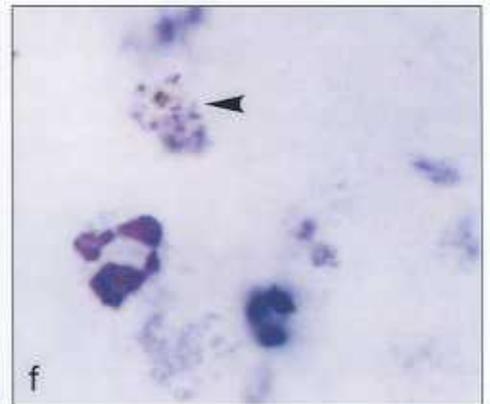
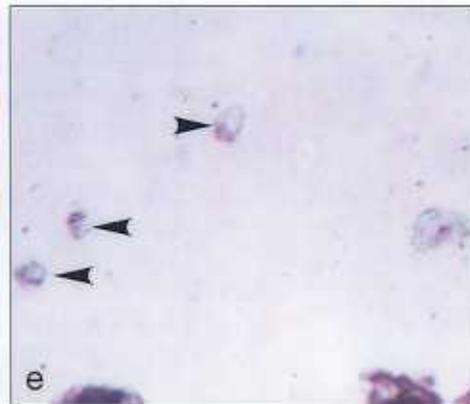
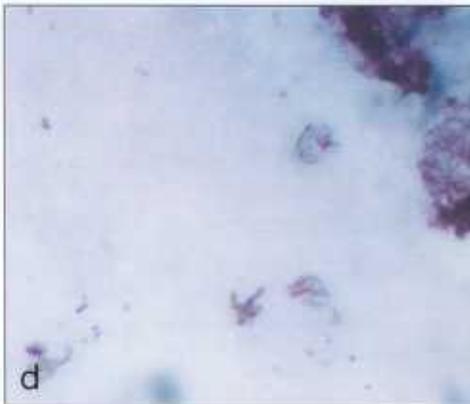
++++ = more than 10 parasites per single thick film field.

Remember: for proper identification and reliable parasite counting, use clean slides and well made and well stained thick films.

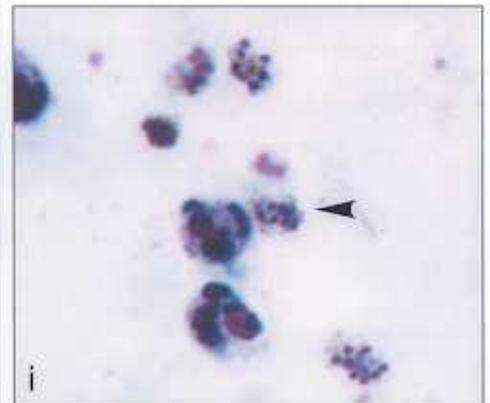
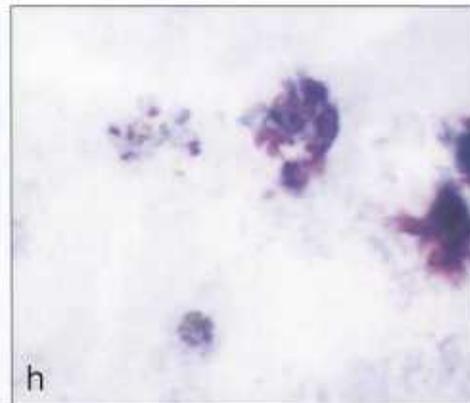
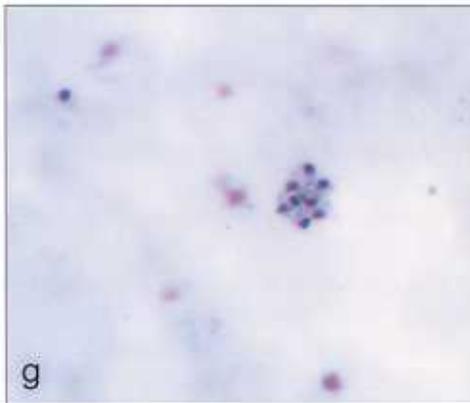
**Plasmodium malariae** thick film



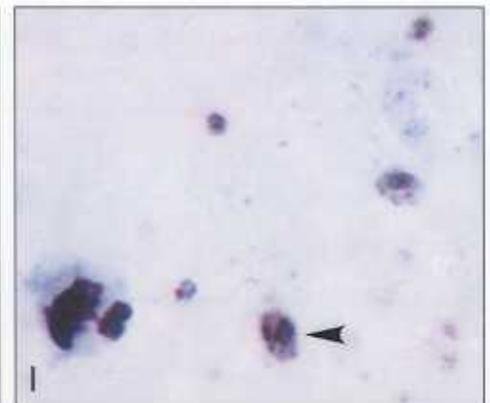
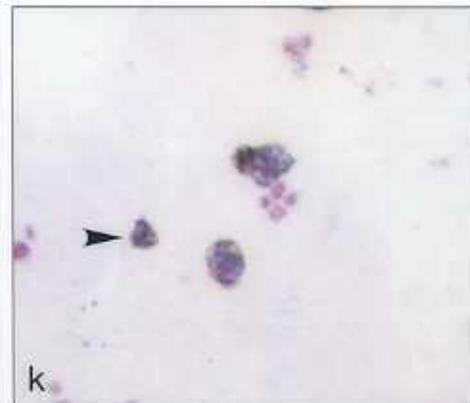
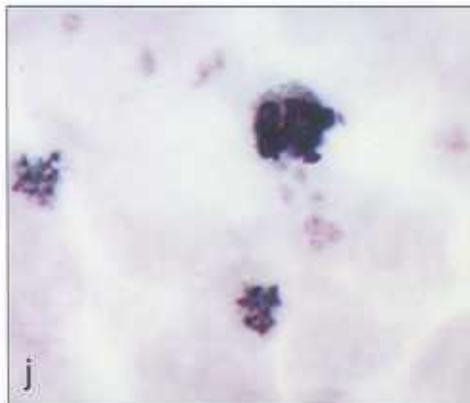
Ring forms tend to be small and few in number and have large chromatin dots and a small amount of cytoplasm, often without a vacuole (a, b). Ring forms may (a) lack a complete circle of cytoplasm, but in a field containing several trophozoites a complete ring form (b) can be seen. Early trophozoites (c) may lack a vacuole. Pigment forms early in this species and is present as dark, coarse grains (b, c).



Growing trophozoites (d, e) vary in shape. Three small, rounded trophozoites are present (e, arrows), along with a growing trophozoite (right). A schizont (f, arrow) containing 8 merozoites and a compact mass of pigment is visible, along with a few rounded trophozoites and two leukocytes.



Mature schizonts, containing 8 merozoites, are visible (g-i); small, dark, concentrated masses of pigment occur in each of the schizonts. In (h), the schizont and a rounded trophozoite are present. An immature schizont (i, arrow) is seen in the same field as three mature schizonts (i).



Two mature schizonts and a leukocyte are visible (j). Two gametocytes are larger (k) than the small, rounded trophozoites (k, arrow). A single gametocyte (l, arrow) is seen with small ring forms and growing trophozoites. Gametocytes may be difficult to distinguish from mature trophozoites; coarse pigment grains are often peripherally distributed in gametocytes but not in trophozoites.



## Common faults in making blood films

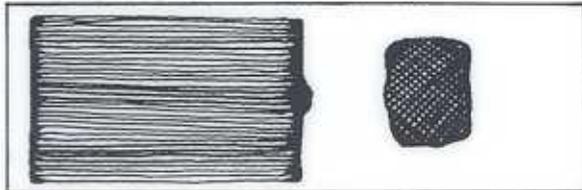
A number of faults are common in making blood films. These can affect the labelling, the staining or the examination, and sometimes more than one of these.

### Badly positioned blood films

Care should be taken that the blood films are correctly sited on the slide. If they are not, it may be difficult to examine the thick film. Also, portions of the films may even be rubbed off during the staining or drying process.

### Too much blood

After staining of films with too much blood, the background to the thick film will be too blue. There will be too many white blood cells per thick film field, and these could obscure or cover up any malaria parasites that are present. If the thin film is too thick, red blood cells will be on top of one another and it will be impossible to examine them properly after fixation.



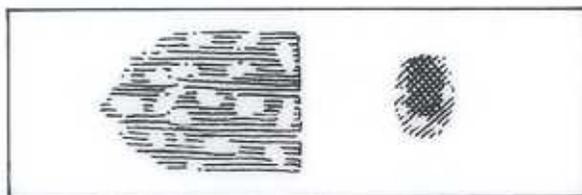
### Too little blood

If too little blood is used to make the film, there will not be enough white cells in the thick film field and you will not examine enough blood in the standard examination. The thin film may be too small to use as a label.



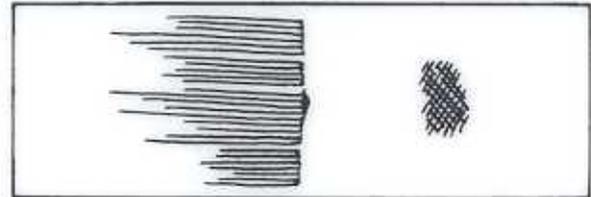
### Blood films spread on a greasy slide

The blood films will spread unevenly on a greasy slide, which makes examination very difficult. Some of the thick film will probably come off the slide during the staining process.



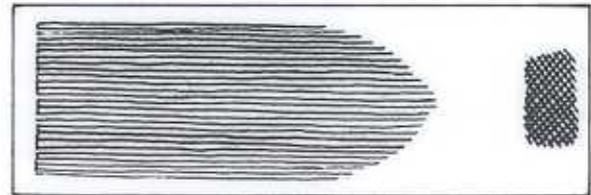
### Edge of spreader slide chipped

When the edge of the spreader slide is chipped, the thin film spreads unevenly, is streaky and has many "tails". The spreading of the thick film may also be affected.



### Thin film too big, thick film in the wrong place

If the thin film is too large, the thick film will be out of place and may be so near the edge of the slide that it cannot be seen through the microscope. During staining or drying, portions of the thick film will probably be scraped off by the edges of the staining trough or drying rack. It may be very difficult, or impossible, to position the thick film on the microscope stage so that it can be examined.



### Other common faults

Other faults that occur commonly in the preparation of blood films include the following:

- Flies, cockroaches or ants eat the dry blood and damage the films.
- Blood films are made on badly scratched slides or on slides with "frozen" or iridescent surfaces.
- The thick film is allowed to dry unevenly.
- Autofixation of the thick film occurs with the passage of time or through exposure to heat, and staining then becomes difficult or unsatisfactory.
- Slides are wrapped together before all the thick films are properly dried, and the slides stick to one another.

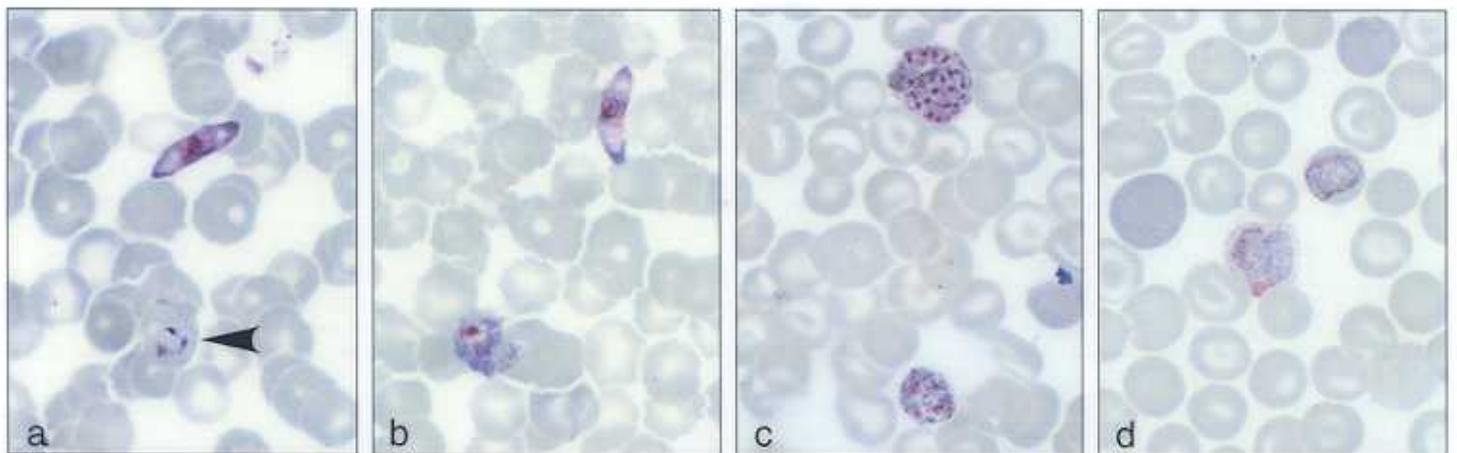
## Mixed infections

Infections with more than one species of malaria parasites may be encountered in many areas where two or more species are endemic. These infections may be overlooked by microscopists for a variety of reasons. If only thin blood films are made for diagnosis, as happens in many laboratories, low-density parasitaemias of one or the other species may be masked by the predominant species. Microscopists may also stop examining a blood film when the first parasite is found and identified. In general, successful detection of mixed infections usually requires longer and more careful study of thin blood films submitted for diagnosis.

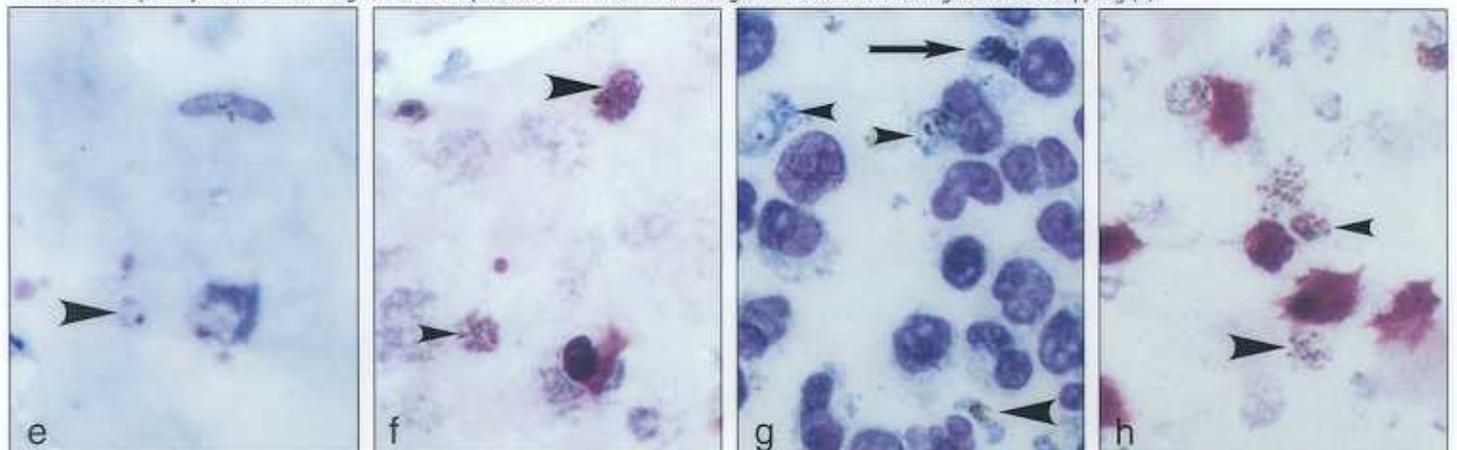
Thin and thick blood films are prepared from each patient on the same slide; the thick film should be examined first. Mixed infections will be found most easily in thick films because of the larger volume of blood examined. Microscopists who routinely examine thick blood films are more likely to detect low-density parasitaemias as well as have a better opportunity to find key diagnostic stages of each

malaria species present. When there is doubt about the presence of more than one species, multiple infections can be confirmed by careful evaluation of the thin film prepared on the same slide.

The most common mixed infection is probably *P. falciparum* with *P. vivax*. However, any combination is possible, depending on the geographical area. Although mixed infections with two plasmodial species are the most common, infections with three species are not uncommon. Mixed infections occur more commonly in children than in adults. As in the diagnosis of any malaria infection, species identification in mixed infections depends on recognition of the key diagnostic stages (e.g. schizonts or gametocytes in some cases), or parasite features (e.g. enlargement of erythrocytes, and presence or absence of stippling in some species). Ring forms (early trophozoites) of each species are typically distorted in thick blood films and are rarely useful in species determinations. The images below help to clarify these points.



**Mixed infection in thin blood films:** A sausage-shaped gametocyte of *P. falciparum* (a) and a young trophozoite (a, arrow) of *P. vivax* in an erythrocyte showing light Schüffner's stippling. A *P. falciparum* gametocyte with a mature *P. vivax* trophozoite in an enlarged red cell (b). A mature schizont of *P. vivax* with many merozoites and a large pigment mass at one side, and a smaller schizont of *P. malariae* containing fewer merozoites and only small pigment granules (c). A band-form trophozoite of *P. malariae* in a normal-sized erythrocyte and a much larger, mature trophozoite of *P. vivax* in an enlarged red blood cell showing Schüffner's stippling (d).

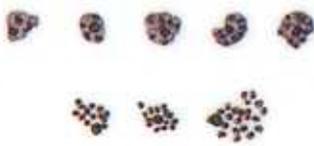
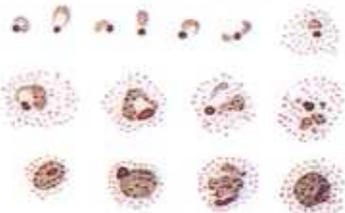
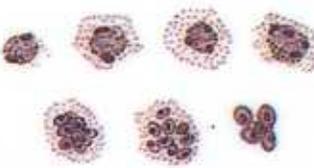


**Mixed infections in thick blood films:** A clearly visible, typical gametocyte of *P. falciparum* (e) with early (e, large arrow) and growing (e, small arrow) trophozoites of *P. vivax* adjacent to each other. A large, mature schizont of *P. vivax* (f, large arrow) demonstrating conspicuous pigment, and a typically smaller, mature schizont of *P. malariae* (f, small arrow). A triple infection (g), with a gametocyte of *P. falciparum* (g, large lower arrow), a mature schizont of *P. malariae* (g, large upper arrow) and two mature trophozoites of *P. vivax* (g, small arrows). A mixed infection of *P. vivax* and *P. malariae* with heavy parasitaemias of both (h); in the centre of the field, mature schizonts of *P. vivax* with many merozoites and conspicuous pigment, and a smaller *P. malariae* (h, small arrow) adjacent to it; a second schizont of *P. vivax* (h, large arrow) and trophozoites of both species scattered throughout the field.



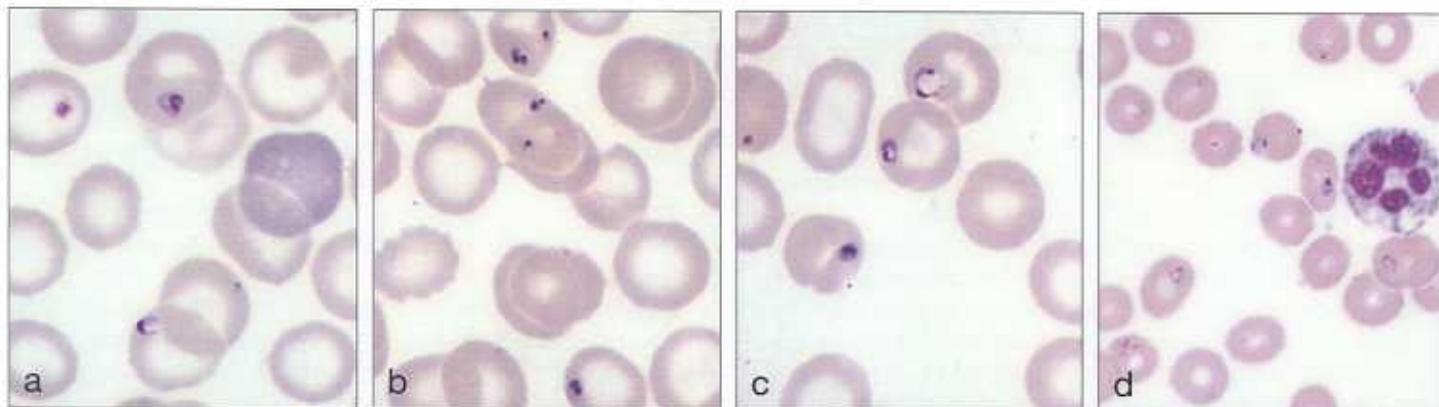
Summary table

Same figure of old bench aids plate #8

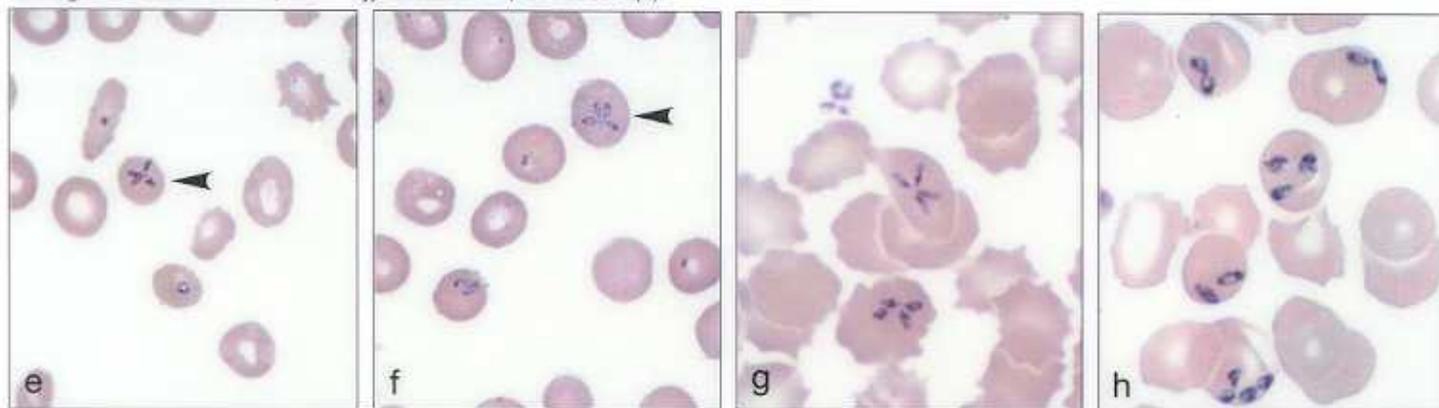
Species		Stage of parasite in peripheral blood		
		Trophozoite	Schizont	Gametocyte
<i>Plasmodium falciparum</i>	Young, growing trophozoites and/or mature gametocytes usually seen.	 <p><i>Size:</i> small to medium; <i>number:</i> often numerous; <i>shape:</i> ring and comma forms common; <i>chromatin:</i> often two dots; <i>cytoplasm:</i> regular, fine to fleshy; <i>mature forms:</i> sometimes present in severe malaria, compact with pigment as few coarse grains or a mass.</p>	 <p>Usually associated with many young ring forms. <i>Size:</i> small, compact; <i>number:</i> few, uncommon, usually in severe malaria; <i>mature forms:</i> 12–30 or more merozoites in compact cluster; <i>pigment:</i> single dark mass.</p>	 <p>Immature pointed-end forms uncommon. <i>Mature forms:</i> banana-shaped or rounded; <i>chromatin:</i> single, well defined; <i>pigment:</i> scattered, coarse, rice-grain like; pink extrusion body sometimes present. Eroded forms with only chromatin and pigment often seen.</p>
		 <p><i>Size:</i> small to large; <i>number:</i> few to moderate; <i>shape:</i> broken ring to irregular forms common; <i>chromatin:</i> single, occasionally two; <i>cytoplasm:</i> irregular or fragmented; <i>mature forms:</i> compact, dense; <i>pigment:</i> scattered, fine.</p>	 <p><i>Size:</i> large; <i>number:</i> few to moderate; <i>mature forms:</i> 12–24 merozoites, usually 16, in irregular cluster; <i>pigment:</i> loose mass.</p>	 <p>Immature forms difficult to distinguish from mature trophozoites. <i>Mature forms:</i> round, large; <i>chromatin:</i> single, well defined; <i>pigment:</i> scattered, fine. Eroded forms with scanty or no cytoplasm and only chromatin and pigment present.</p>
<i>P. ovale</i>	All stages seen; prominent Schüffner's stippling in 'ghost' of host red cells, especially at film edge.	 <p><i>Size:</i> may be smaller than <i>P. vivax</i>; <i>number:</i> usually few; <i>shape:</i> ring to rounded, compact forms; <i>chromatin:</i> single, prominent; <i>cytoplasm:</i> fairly regular, fleshy; <i>pigment:</i> scattered, coarse.</p>	 <p><i>Size:</i> rather like <i>P. malariae</i>; <i>number:</i> few; <i>mature forms:</i> 4–12 merozoites, usually 8, in loose cluster; <i>pigment:</i> concentrated mass.</p>	 <p>Immature forms difficult to distinguish from mature trophozoites. <i>Mature forms:</i> round, may be smaller than <i>P. vivax</i>; <i>chromatin:</i> single, well defined; <i>pigment:</i> scattered, coarse. Eroded forms with only chromatin and pigment present.</p>
		 <p><i>Size:</i> small; <i>number:</i> usually few; <i>shape:</i> ring to rounded, compact forms; <i>chromatin:</i> single, large; <i>cytoplasm:</i> regular, dense; <i>pigment:</i> scattered, abundant, with yellow tinge in older forms.</p>	 <p><i>Size:</i> small, compact; <i>number:</i> usually few; <i>mature forms:</i> 6–12 merozoites, usually 8, in loose cluster; some apparently without cytoplasm; <i>pigment:</i> concentrated.</p>	 <p>Immature and certain mature forms difficult to distinguish from mature trophozoites. <i>Mature forms:</i> round, compact; <i>chromatin:</i> single, well defined; <i>pigment:</i> scattered, coarse, may be peripherally distributed. Eroded forms with only chromatin and pigment present.</p>
<i>P. malariae</i>	All stages seen.			

## Babesiosis

Human babesiosis (piroplasmiasis) is an animal disease that can occasionally be transmitted to humans (i.e. a zoonosis) by the bites of infected ticks. *Babesia* spp. are intraerythrocytic protozoans belonging to the phylum Apicomplexa, which infect vertebrates throughout the world. The human infections include *B. microti*, a rodent parasite, *B. divergens*, a bovine parasite, and *B. equi* (reclassified as *Theileria equi*), an equine parasite. Like human malarias, *Babesia* spp. invade red blood cells; however, they do not produce pigments or cause enlargement of the host red blood cells. *Babesia* spp. differ from *Plasmodium* spp. in that they do not have an exoerythrocytic cycle, proper schizogony or gametocytes. Reproduction is by "budding" and produces only four merozoites, which are often arranged in a "Maltese Cross" pattern (e, arrow) or scattered at random in the red blood cells (h). Human cases have been recorded in Asia, Europe and North America, but not in Africa. Inside the host red blood cells the parasites are small (1–2.5 µm in diameter) and closely resemble the early ring forms of *P. falciparum*, with which they are most often confused. The diagnosis of *Babesia* spp. in blood films is therefore often difficult; certainly, many infections are incorrectly diagnosed as *Plasmodium* spp., especially as *P. falciparum*. *Babesia* spp. are rarely reported in humans; patients who have had their spleens removed are particularly at risk of symptomatic infection. Increased awareness of this disease may increase reporting, and microscopists should be alert to the possible presence of this infection.



Erythrocytes infected with *Babesia microti* (a–d). Tiny ring forms, which may be confused with the delicate ring forms of *Plasmodium falciparum*, are characteristic of this infection. Great variation in the morphology of *Babesia* is common, as is multiple infection of erythrocytes. Tetrad formation occurs but is not as frequent as with some other species of *Babesia* that infect humans. Note how the size of the organisms (a–c) varies, in particular the tiny ring forms (d) seen in several erythrocytes at a somewhat lower magnification. Four organisms within a red cell, not in a typical tetrad form, are also seen (d).



Typical tetrad forms of *Theileria equi* (formerly *Babesia equi*) are present in human blood films (e, f); other morphological forms of the parasite are visible in several other infected erythrocytes. An undescribed species of *Babesia* from California, USA, demonstrates tetrad formation in two erythrocytes (g). The pear-shaped organisms (h) in several red cells of a human blood film are similar in morphology to those of *Babesia divergens*.



## Cleaning and storing microscope slides

### Preparation

Slides for the preparation of blood films must be scrupulously clean and free from grease and moisture. Blood films made on dirty or greasy slides may wash off during the staining process. Scratched, "frozen" or iridescent slides should not be used for blood films (but can be used for other purposes in the laboratory).

### Cleaning

New slides should be soaked in water with a reliable detergent for 30–60 minutes. Rinse slides under running tap water or in several changes of clean water. Dry each slide using a clean, lint-free cloth. Previously used slides must be soaked for at least 1 hour in hypochlorite solution before being washed. Soak these slides in water

containing detergent for 1–2 days. Using gauze or cotton wool, remove all traces of the old blood film and immersion oil. After cleaning, rinse slides under running tap water or in several changes of clean water. Dry each slide using a clean, lint-free cloth.

### Storing

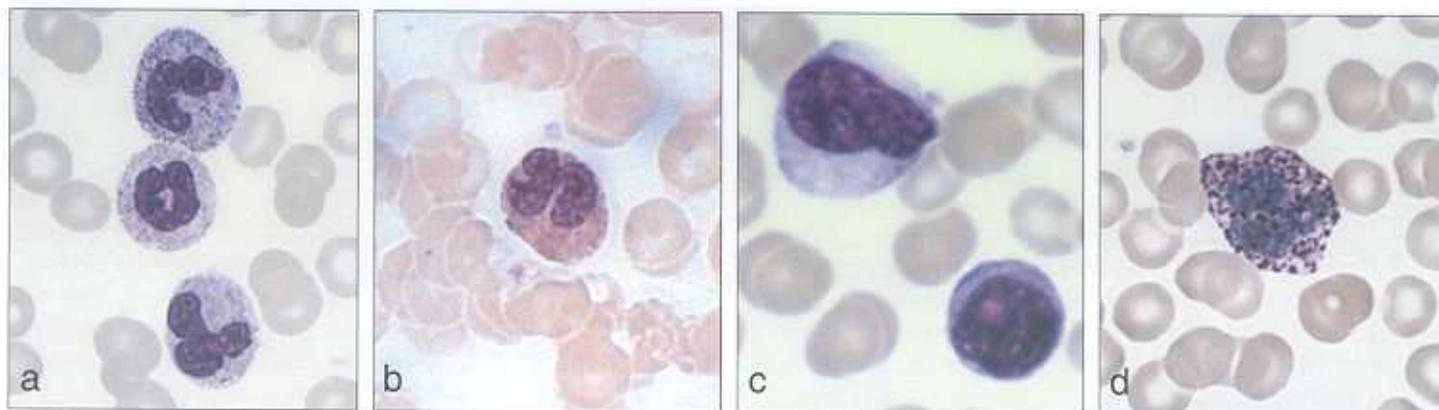
Clean slides (new or used) should be wrapped, in batches of 10, in thin paper secured with adhesive tape or rubber bands; slides can be stored and transported in cardboard slide boxes. In tropical and subtropical areas, clean slides should be stored in a dry place or in a warm-air cabinet. If stored at room temperature with high humidity, slides will stick together after a few weeks.

## Care of the microscope

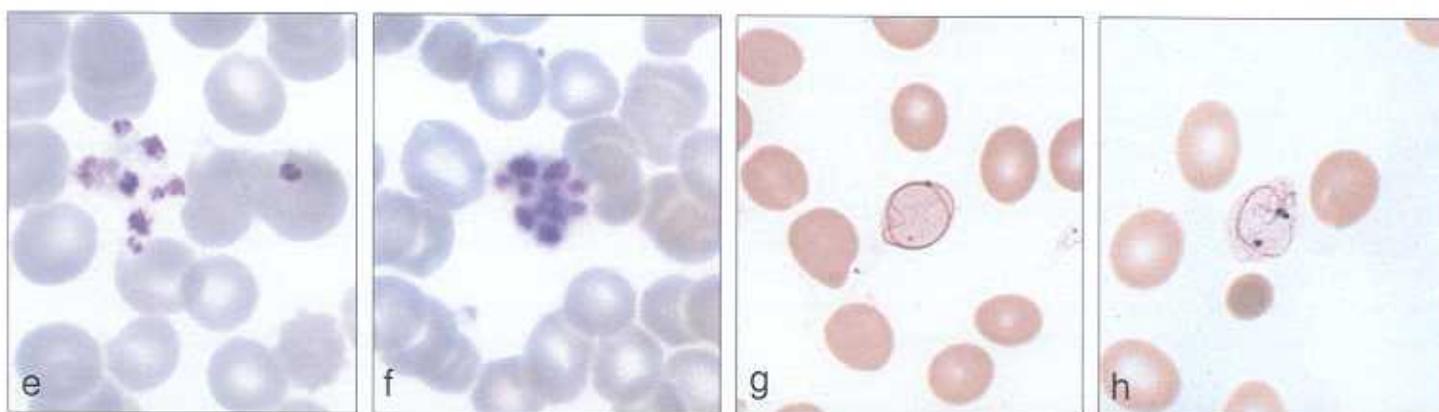
- **Do** always keep the microscope covered with a clean plastic or cloth cover when it is not in use to protect it from dust, especially in hot dry climates.
- **Do** protect the microscope from fungus growth in warm humid climates by one of the following: storing it in a continuously air-conditioned or dehumidified room; fixing a 15-watt bulb, which is left constantly lit, in the microscope box; or connecting a number of 15- or 25-watt bulbs, left constantly lit, inside a cupboard with tightly fitting doors.
- **Do** clean the immersion oil from the immersion objective every day.
- **Do** quote the model number and, if possible, the instrument and part number when ordering replacement parts.
- **Don't** dismantle the microscope to clean inaccessible parts.
- **Don't** use alcohol to clean the microscope.
- **Don't** clean the eyepieces with anything but dry lens tissue.
- **Don't** leave the lens ports empty: use the appropriate cover provided or close them with sealing tape.
- **Don't** exchange lenses or parts of the microscope.
- **Don't** store separate eyepieces and objectives without sealing each in an airtight plastic bag with a sachet of self-indicating silica gel. Self-indicating silica gel is blue when active and turns pink when it has absorbed all the water that it can. It can be reactivated by heating and will become blue again as this reactivation takes place.
- **Don't** store the microscope in its box for long periods or transport it without the retaining screw.

Artifacts and cellular elements in blood films

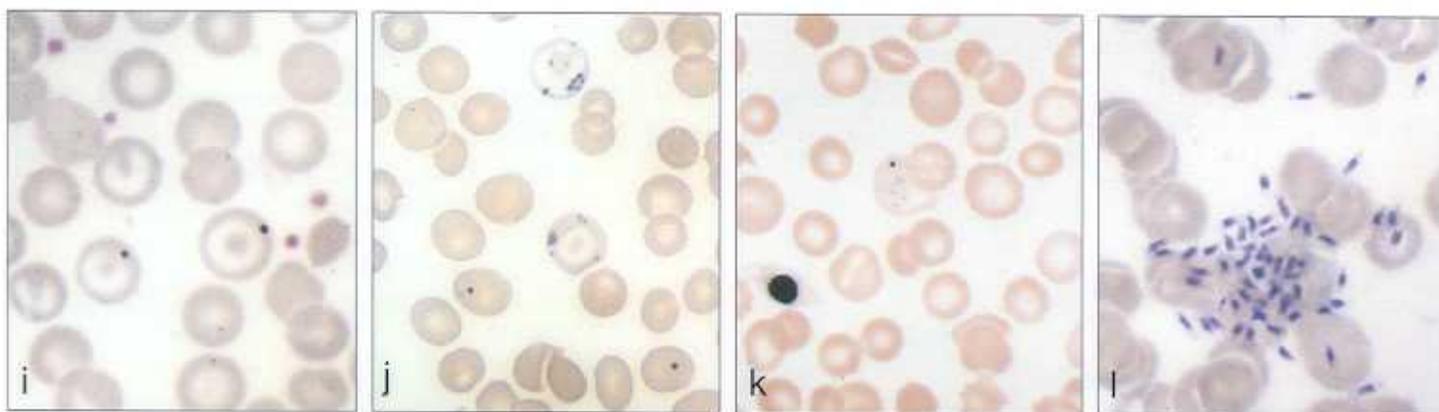
© World Health Organization 2010



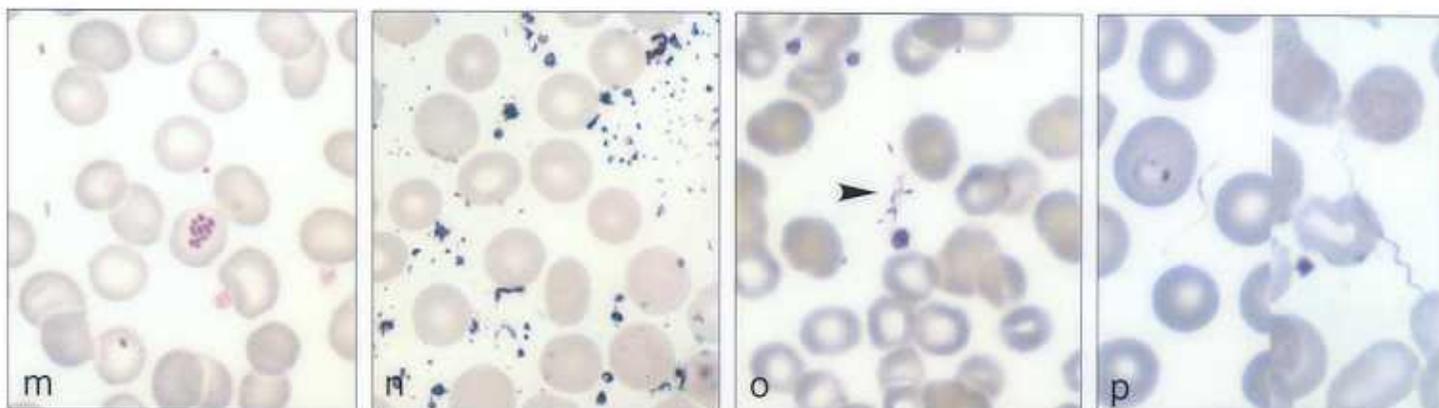
White blood cells (leukocytes) in blood films (a–d): neutrophils (a), an eosinophil (b), a lymphocyte (the smaller cell) and a monocyte (c), and a basophil (d) are commonly found when blood films are examined.



Platelets (e, f) lying free among red blood cells: when individual platelets are superimposed upon erythrocytes (e), or if they form a cluster (f), they superficially resemble schizonts and may be wrongly identified. Cabot's rings (g, h), a type of red cell inclusion that often takes the form of an oval ring, occur in severe anaemias and are thought to be remnants of spindle fibres forming during mitosis.



Howell–Jolly bodies (i, j) are purple-staining granules and represent nuclear (DNA) fragments. They are sometimes confused with the chromatin dots associated with malaria parasites. Pappenheimer bodies are small, irregular, basophilic deposits in erythrocytes; they may occur in association with Howell–Jolly bodies (j) or may be found alone in the erythrocyte (k). Other objects (l) that cannot be readily identified may also be confused with malaria parasites by inexperienced microscopists.



Bacterial organisms may contaminate blood films, and in this case a cluster of bacteria are superimposed on an erythrocyte (m). Blood films that are poorly rinsed following staining may retain the stain (n). In anticoagulated malarious blood kept at room temperature for several hours or more, microgametocytes may undergo exflagellation, releasing microgametes (o, arrow). In the split image (p), a single microgamete (note central nucleus) lies adjacent to a *P. vivax*-infected erythrocyte (left). On the right, a typical spirochaete of *Barrelia* is seen in a case of relapsing fever. Note the similarity to a malaria microgamete; the spirochaete is longer and thicker and has no nucleus.



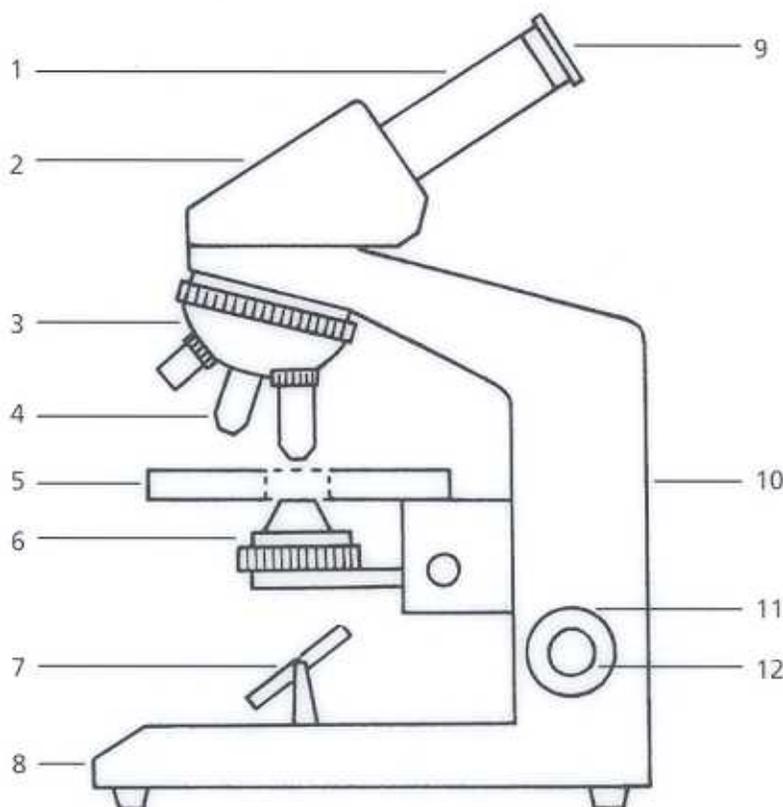
## Use of the microscope

A lens combination of a x10 eyepiece and a x100 objective, to give a total magnification of x1000, is the standard used in most microscopes currently available. The microscope may have one eyepiece (monocular) or two (binocular). Binocular microscopes are easier and less tiring to use, particularly for long periods. All microscopes need a good source of light, either natural (daylight) or artificial (a microscope lamp powered by mains electricity, batteries or a generator). Most modern microscopes have a built-in lamp. Some microscopes have a detachable lamp that can be replaced with a mirror. Microscope lamps must also have a blue "daylight" filter to convert the "yellow" electric lamp light to "white" natural light. Daylight filters are used to minimize the differences in colour of stained malaria parasites in the blood film, which in turn makes diagnosis easier. The ability to control the intensity of the light source is essential for good microscopy, and most microscope lamps have an adjustable light output. All microscopes have a built-in substage condenser and iris diaphragm combination with which to achieve the final optimal light intensity. When a mirror is used with artificial light, the flat side should be used; when daylight is the light source, the concave side should be used without the substage condenser.

To set up the microscope, switch on the lamp or angle the mirror to reflect the light. Raise the substage condenser to its fullest extent and open the iris diaphragm to two-

thirds of its maximum aperture. Remove an eyepiece and look down the tube. If necessary, align the condenser/lamp or condenser/mirror to put the brightest light in the centre of the condenser. Replace the eyepiece. Raise the objective clear of the microscope stage. Place a drop of immersion oil on the rounded end of the thin blood film. In some countries anisole is used for work with the oil-immersion objective; this product has the same refractive index as immersion oil. Place the slide on the stage. Looking from the side of the microscope, lower the objective with the coarse adjustment until it is just touching the immersion oil. The film is now ready to be examined. Bring the microscopic field into focus using the fine adjustment. Adjust the light to a comfortable intensity. Use the same procedure to examine the thick blood film.

At the end of the work session the oil-immersion lens must be properly cleaned. If anisole is used, it evaporates from the blood film after some time, so that the film does not need to be cleaned and there is less chance of its being damaged. Use of anisole also means that the objective lens does not need to be cleaned. On the other hand, immersion oil must be gently removed with a lens paper slightly moistened with xylene. If examined blood films are to be kept they should be dipped in xylene, carefully wiped dry with tissue and placed in storage boxes.



Parts of a typical compound microscope

1. Main tube
2. Body tube (prism) } inclined head
3. Revolving nosepiece
4. Objective
5. Stage (mechanical stage)
6. Substage condenser with iris diaphragm
7. Mirror
8. Base (foot)
9. Ocular (eyepiece)
10. Arm (limb)
11. Coarse adjustment
12. Fine adjustment