

## Diagnosis of malaria infection

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### **Introduction**

The technical capability to perform a correct diagnosis of malaria infection is of the utmost importance in several settings, not only in endemic countries, for clinical, epidemiological and research purposes. Due to the practical nature of this Handbook, which is addressed to health workers in the field, this chapter will mainly concentrate on bedside simple technology to perform an adequate diagnosis of malaria infection, even though brief notes on the most updated and sophisticated diagnostic tools will also be offered to the reader. The correct and timely diagnosis of malaria infection in a ill patient is in fact of critical importance since symptoms of complicated malaria may suddenly develop, possibly leading to death despite intensive care efforts.

This Chapter will deal with (a) clinical presumptive diagnosis and laboratory confirmatory diagnosis of both (b) the direct evidence of the parasite presence and (c) the indirect presence of malaria parasite antigens or antibodies (immunodiagnosis).

### **Clinical presumptive diagnosis**

The appearance of clinical symptoms is usually the reason that urges the ill patient in industrialized non endemic countries to turn to the physician. Clinicians in western countries need to be well aware of the possibility of imported malaria cases. Diagnosis in these cases may be made difficult by the possibility that the exposure to the infective bite may date back to a long time before clinical symptoms appear, especially in the case of *P. vivax* or *P. malariae* infections. The accurate geographical history (*unde venis?*) should be a routine in every interrogation of every patient even in the malaria-free world. Finally, it is to be stressed that *P. falciparum* malaria in non immune subjects coming from non endemic western countries may be extremely severe, with high case-fatality rates if correct diagnosis and treatment is not carried out promptly.

In malaria endemic developing countries, on the contrary, economic and logistic reasons force entire populations to rely exclusively on self diagnosis and treatment, even in the case of moderate fever. It is to be noted, in fact, that semi-immunity usually leads to mild to moderate clinical disease in adults living in endemic countries. Unfortunately, the clinical symptoms of mild to moderate malaria in these cases are extremely aspecific (fever, headache, myalgias, etc.), mimicking a large series of other clinical conditions. On the contrary, clinical symptoms may be severe and disease may be lethal in children and in selected adult patients (large inoculum, malnourished patients, etc.) even in endemic areas.

The extremely wide spectrum of clinical signs and symptoms occurring during malaria infection is covered in chapter 6 (*Clinical features of malaria infection in man*) and exceeds the purpose of this chapter. A brief and non exhaustive list of possible differential diagnosis of *falciparum* malaria presenting symptoms and signs is nevertheless indicated in table 1.

Whenever possible, it is recommended to perform at least a simple panel of clinical chemistry blood examinations (white blood cell count, red blood cell count, haematocrit, haemoglobin level, platelet count, etc.), the predictive positive value of which may be very high in some endemic malaria areas. Polyfactorial haemolytic *anaemia* is very common in *falciparum* malaria and it is caused by direct lysis of red blood cells, inappropriate bone marrow response and autoimmune disorders (World Health Organization, 1990). Platelet levels are very seldom normal in malaria infections caused by all species and often fall below 50.000/ $\mu$ l in *falciparum* malaria. The predictive positive value for *falciparum* malaria given the *triade* fever, history of recent stay in tropical areas and *thrombocytopenia* below 100.000/ $\mu$ l has been found to be as high as 97% in imported malaria (Castelli et al., 1995). No specific diagnostic indications are given by the white blood cell count during malaria attack since leukocytosis is usually absent in the absence of concomitant bacterial infections (Wery, 1991).

Table 1. Differential diagnosis of the presenting clinical symptoms/signs in malaria infection (adapted from Gilles, 1988)

Presenting symptom/sign	Notes	Differential diagnosis
fever	often irregular or continuous at onset	influenza, dengue, brucellosis, sandfly fever, acute respiratory infections, viral hemorrhagic fevers, tuberculosis, enteric fever, trypanosomiasis, visceral leishmaniasis, rickettsial fever, relapsing fever
coma	usually (not always!) parasitaemia is present (not high is)	meningitis, encephalitis, typhoid fever, cerebrovascular accidents, trauma, alcoholism, hypoglycemia
jaundice	more common in severe malaria	infectious hepatitis, leptospirosis, disease of the biliary tract
hepatomegaly	usually tender in the acute phase	amoebic liver abscess, beri-beri, brucellosis, leptospirosis, relapsing fever, viral hepatitis, thalassaemia
splenomegaly	present in all types of malaria and usually tender in the acute phase.	brucellosis, relapsing fever, trypanosomiasis, typhoid fever, leptospirosis, thalassaemia
renal failure		G6PD deficiency, sickle cell disease, snake venoms, leptospirosis, traditional herbal remedies, heat stroke
gastrointestinal malaria	all kinds of	all types of dysentery, cholera,

	gastrointestinal symptoms (ranging from vomiting to choleraic diarrhea)	gall bladder disease, acute pancreatitis, acute appendicitis
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Wherever the laboratory confirmation of malaria is not available in highly endemic areas, the experienced clinician may diagnose malaria with sufficient predictive positive value for positive blood slide (Genton et al., 1994), even though contrasting reports exist indicating that the predictive positive value of clinical signs to diagnose malaria may be poor during low transmission seasons (Sowunmi and Akindele, 1993).

### **Laboratory diagnosis**

Once malaria is suspected on clinical grounds, it is mandatory to obtain the laboratory confirmation of the presence of malaria parasites in the patient's organism, whenever possible. The diagnosis of malaria may in fact be pursued by the direct demonstration of the parasite whole cell or of parasite's nucleic acid or products in the blood (*direct diagnosis*) or by the demonstration of the patient's immune response to the infection (*indirect diagnosis or immunodiagnosis*).

Direct diagnosis of malaria infection has been, and still is, the cornerstone of malaria diagnosis since 1880, when the French Army surgeon Alphonse Laveran first observed and described malaria parasites in human blood in Algeria.

The direct demonstration of the whole parasitic cell may be accomplished by several methods, from the old and simple (but still golden standard!) direct microscopic observation of stained blood specimen to the more recent and sophisticated concentration and staining techniques (Quantitative Buffy Coat ®, acridine orange method). Finally, the detection of the presence of the genome of the parasite is now possible by the Polymerase Chain Reaction (PCR) technique. New and sensitive immunological methods to detect *P. falciparum* antigens have yielded promising results for the individual diagnosis of malaria infection.

The evidence of seric specific antibodies is of little, if any, diagnostic value in endemic malaria areas but may provide important information in epidemiological studies and, in some selected setting, in blood donor screening.

### **Direct diagnosis**

This chapter will focus particularly on the simple direct microscopic observation of blood specimen, but informations on the newer diagnostic tools (QBC ®, PCR) will also be given to facilitate the reader's comprehension of the new developments in malaria operational research which may hopefully bear fruit at the field level in the new future.

#### **(A) Light microscopic observation**

At the end of the last century, it was recommended that the direct microscopic observation of malaria parasites be done in fresh unstained blood specimens flattened between a slide and a cover slip to appreciate the mobility of the parasite. It was then soon recognized by Romanowsky (1890) that the fixation and staining of blood specimens permitted a more accurate definition of the different stages and species, even though mobility was lost. The current standard methodology of Giemsa stained blood thick smears for the diagnosis of malaria dates back to 1903 (Ross, 1903).

Though simple in principle, the direct microscopic observation of stained blood specimens requires specific instrumentation and reagents (microscope, microscopic slides, pricking needle, staining reagents, water, electric or solar light, etc. - see Annex 1) and a trained professional to obtain to a correct diagnosis. Information on basic microscopy, the basic staining procedures and on the most common mistakes are given in this subchapter together with the morphological clues to make an adequate qualitative (stage and species diagnosis) and quantitative (parasitaemia) diagnosis of malaria infection at the district level.

Excellent reviews on microscopic malaria diagnosis, to which continuous reference will be made in the text, are those from Shute (1988), Lopez Antunano (1990), the World Health Organization (1991) and Gilles (1993).

### **Blood drawing technique**

Since parasite concentration is fairly constant in internal and peripheral blood, it is routine to draw the blood by pricking a finger (or the heel in young infants) with a sharp sterile needle. The best site to prick is the lateral side of the third phalanx of the second or third finger of the left hand (unless the patient is left-handed). After accurate cleaning with spirit-moisted cotton, pricking is carried out by the health worker and the first drop of blood must be removed with cotton. The following drops of blood, obtained by gentle squeezing of the finger by the health worker, are then collected on a glass slide that is lowered to touch the top of the drop (see fig. 1). It is to be stressed that the glass slides are to be carefully cleaned before use with clean water and detergent followed by 95% ethyl alcohol polishing to remove every trace of grease that may spoil the results. Slides, particularly in warm tropical climates, should be stored packaged in a dry atmosphere to prevent contamination with dust and mould. Blood obtained by syringe for routine examination purposes may also be used to prepare specimens for malaria diagnosis, if no anticoagulant is used (Shute, 1988).

### **Sample preparation (thin film and thick film)**

The microscopical observation of malaria parasites is optimal when parasites are fixed and observed in their natural location within red blood cells after appropriate staining. This is best accomplished with the *thin film* preparation technique. Unfortunately, thin film has a low sensitivity (100-200 parasites/ $\mu$ l of blood) and is thus inadequate for low parasitaemic infection. An adequate parasite concentration method is obtained by osmotic lysis of the red blood cells releasing the parasites, as is the case with *the thick film* preparation technique, the sensitivity of which is then increased (about 10 parasites/ $\mu$ l of blood). Experienced technicians may also prepare thick and thin films on the same slide (fig. 1), even though particular attention is to be paid in this case to avoid fixation of the thick film (see below). Both methodologies are described below..

*Thin film.* One end of the slide is allowed to touch the top of the blood drop on the patient's finger. Only the top of the drop should come into contact with the slide. The quantity of blood to be transferred to the slide should not exceed 1.5  $\mu$ l, usually corresponding to a diameter of 3-4 mm. The edge of a second slide (or a cover slip) is then laid on the drop of blood, that will spread on the entire line of contact between the two slides. The second slide, steadily held by the technician to form a 45° angle with the original slide, is then moved to the opposite end of the slide to which the drop was originally located. In the well prepared thin film, the blood film should end with multiple tails not touching the edges of the slide. Red blood cells should be visible one by one without overlapping. Abnormally thick slide may be the result of an exaggerated volume of blood or of an angle larger than 45°.

*Thick film.* A volume of blood twice to thrice the one used for a good thin film is needed for the preparation of a thick film (3.0 to 4.0  $\mu$ l). This is usually accomplished by touching three times the top of the blood drop at the top of the finger to obtain a triangle on the slide (fig. 1). The blood is then gently mixed for 20-30" using the corner of a second slide to defibrinate the blood and to obtain a round smear of about 1 cm in diameter. The thickness of the obtained film should allow the reading of the printed text of a usual newspaper through it (Gilles, 1993).

### **Fixation and staining procedures**

Thin and thick films may be allowed to dry in the air protected by dust, or actively dried by waving it. It is important to avoid abrupt exposure to heat (fire, sunlight) that may lead to fixation and fissure the preparations.

Thin films must undergo both processes of *fixation* and *staining*, while thick films must not be fixed to allow hemolysis of red blood cells and consequent dehemoglobinization. It is to be noted that fixation may also occur spontaneously (*autofixation*) with time (7 to 15 days, varying with humidity and temperature of the atmosphere). It is then important to process thick film as soon as possible to permit complete dehemoglobinization.

This chapter will review the more used fixing and staining procedures (Giemsa, Field, Leishman). Technical information on the composition of staining and fixing solutions are given in annex 2. For a more complete and detailed review, see Shute (1988) and Gilles (1993).

*Fixation.* Fixation may be achieved by heat and alcoholic solutions. Methanol (methyl alcohol) is the most widely used fixative for malaria thin films. Contact with methanol should be maintained for 10-20 seconds. If both thin and thick films are on the same slide, it is mandatory to avoid the contact of methanol (or even of its fumes!) with the thick film to avoid fixation.

*Giemsa staining procedure.* To achieve optimal results, it is important that the Giemsa staining solution is prepared with distilled or deionized water buffered to pH 7.2 (see annex 2). The Giemsa staining solution is prepared by diluting Giemsa stain with buffered water A ratio 1:10 and 1:20 is usually used for staining thin and thick films respectively, that is 2 to 1 drops of Giemsa per ml of buffered water. Grossly, 5 ml of the solution is necessary to stain each single slide. The Giemsa stain is then poured onto the slide (or the slide is immersed in the staining trough) and contact is maintained for 30-40 minutes depending on the local climatic conditions. The staining solution is then removed by gentle but accurate rinsing with buffered distilled water (don't pour the stain, but wash it off!) and the slide is then drained on filter paper and allowed to dry in air. When the clinical situation requires immediate action, thick films may be stained with a Giemsa accelerate procedure. The qualitative morphological results are however far worse than the usual ones. Briefly, the thick smear is allowed to dry under a heat source of up to 50°C (even sunlight is suitable if slides are protected from dust) and then stained for 8 minutes with a 10% Giemsa solution (2 drops/ml) before rinsing and drying.

Giemsa staining is the most commonly used method for both thin and thick films all over the world for the quality of the stain and, of greater importance, its stability in tropical climates.

*Field staining procedure.* Field staining is a good method to stain thick films but is not suitable for thin films. However, it has the remarkable advantage to be extremely quick (the smear may be stained in less than 1 minute). Two different Field solutions (Field solutions A and B) are to be prepared (see annex 2). Briefly, the slides are dipped first in Field solution A (3 seconds), then briefly drained by touching the edge of some filter paper and rinsed in distilled buffered water (3

seconds), dipped in Field solution B (1 second) and finally rinsed in distilled water to remove stain excess, drained and dried before observation.

*Leishman staining procedure.* Since Leishman staining solution uses methanol as a solvent, this method is only useful to stain thin films. Despite this drawback, the staining quality of the Leishman stain is excellent. Briefly, 7-10 drops of the staining solution (see annex 2) are dropped on the slide. After 20 seconds, a few drops (10-15) of buffered water are also dropped on the slide and mixed with the stain by gently rocking. The staining mixture is allowed to rest on the slide for 20-30 minutes and then washed off by rinsing with buffered water before drying and observation.

### **Basic microscopy**

The microscope is an essential component of the malaria diagnostic laboratory, even in field condition. The care and maintenance of the microscope is thus of the outmost importance. A basic knowledge of the main features of a microscope is also required (World Health Organization, 1991). Schematically, a light microscope is represented in fig. 2. A monocular microscope is best suited for a natural solar light source, but the quality and the ease of observation is greatly improved when a binocular microscope (requiring electric light supply) is used. A routine microscope usually has three objectives (10x, 40x, 100x magnifications). The 100x objective is also referred to as an “immersion objective” and has to be used to observe blood smears. The oculars also have their own magnification (6x or 7x are preferred for malaria diagnosis), the total magnification resulting from the multiplication of both lenses.

The following practical suggestions may be of help for a better use of this invaluable diagnostic tool:

1. Place the slide on the stage;
2. Keep the diaphragm completely open and raise the condenser to reach the brightest field;
3. Using the coarse adjustment, lower the stage (or lift the objective, depending on the microscope used) to the maximum distance from the objective revolver
4. Place 1 drop of immersion oil on the slide;
5. Using the coarse adjustment, lift the stage to allow the oil drop to touch the lens of the objective;
6. Using the fine adjustment, sharply focus by slightly lowering the stage again;
7. Modulate iris diaphragm to obtain optimal illumination;
8. Clean the objective lenses with a soft gauze or lens tissue. Use xylene (always avoid strong alcohol solutions or acetone as these substances may dissolve the glue used to fix the objective lenses) only from time to time to remove traces of dried oil.

### **The observation: the three key questions**

Three key questions need to be answered during the diagnostic itinerary to ascertain malaria infection:

1. Are malaria parasites present in the blood smear?
2. If yes, which species and stages do they belong to?
3. If yes, how many of them are present per  $\mu\text{l}$  of blood?

The answer to question n. 1 is better achieved by observing the thick film (especially in case of low level parasitaemia), while the answer to question 2 better requires the observation of thin films. Question 3 may be answered by observing thick or thin films, depending on the technique used (see below). The following paragraphs will provide useful information to answer the key questions and allow an adequate qualitative and quantitative diagnosis of malaria infection.

## **Morphological species and stage diagnosis**

Complete knowledge of the morphological features of the different blood stages of the different *Plasmodia* species represents the essential basis of a correct laboratory diagnosis confirmation of malaria infection. It is to be stressed that a correct diagnosis may be done only after attentive and careful observation of a number of microscopic fields (at least 100 microscopic fields should be observed before a thick film may be classed as negative) and of a number of different morphologic characteristics that draw a well defined picture of the species. Do not rely on single images, even if apparently pathognomonic. A crescent-shaped gametocyte in peripheral blood does not obligatorily mean that a *P. falciparum* infection is the cause of the actual clinical complaint and, however, does not rule out the possibility of mixed infection!

This chapter will review the most peculiar features of each species of *Plasmodia* in both thin and thick film examination (Giemsa stain), providing information also on the most common artifacts that may deceive the unexperienced observer. A comprehensive synopsis of the morphological characteristics of the host cell and of the different stages of *Plasmodia* by species is provided in tables 2 and 3. A graphic presentation of all *Plasmodia* stages is presented in plates 1 to 4 and microphotographs showing thin and thick films are shown in annex 3..

***Plasmodium falciparum* (plate 1).** In *P. falciparum* infection, only trophozoites and, in a later stage of the disease, gametocytes are found in the peripheral blood. The persistence of gametocytes even in treated patients is not surprising since the majority of antimalarial drugs are powerful schizonticides but weak gametocytocides and thus gametocytes may persist for weeks after the clinical attack. The observation of a schizont in peripheral blood is exceedingly rare, since schizogony usually occurs in red blood cell sequestered in visceral internal organs, and its occurrence is a sign of severe infection. At the onset of malaria attack, it is not uncommon to find trophozoites in different phases of maturation, reflecting the presence of different non synchronized broods of parasites entering the blood stream at different times. Usually, one brood prevails over the others and in few days synchronization takes place. *Plasmodium falciparum* penetrates both young and mature erythrocytes without preference.

In thin blood films the infected red blood cell is not usually enlarged and may show coarse red pigmentation only in mature trophozoite or schizont stages. *P. falciparum* trophozoites within red blood cells are ring-shaped and their dimension varies with age, the more mature the bigger. Young trophozoites may show the typical (and almost pathognomonic) presence of marginal forms (“*appliqué* forms”). Polyparasitized erythrocytes may be present and parasitaemia may be high. The presence of two purple red chromatin dots is not uncommon. When present, schizonts are compact, contain 15 to 30 nuclei and may have a single dark pigment mass in their context. Intraerythrocytic crescent-shaped *P. falciparum* gametocytes are blue with compact central coarse granules (female gametocyte) or pale violet, with scattered granules (male gametocyte). They may be seen in the second stage of the infection or in the convalescence phase.

In thick blood films, trophozoites assume various shapes (comma, ring, vibrio-like, dot, etc.) and are pale blue in colour, their size depending on the maturation stage. Gametocytes may also be seen in thick films and, when present, schizonts are usually distorted but multiple nuclei are easily visible.

***Plasmodium vivax* (plate 2).** In *Plasmodium vivax* infection, all stages of the schizogonic parasite developmental cycle may be seen in peripheral blood because schizogony takes place in circulating infected red blood cells non adherent to the endothelium of internal organs. In the early stage of the erythrocytic cycle, the possible presence of two or more non synchronized broods accounts for the concomitant presence of different maturative stages. *P. vivax* better penetrates younger

erythrocytes, the natural deformability of which is essential to enable the blooming growth of the parasite.

In thin films, the host red cell is usually enlarged (up to twice the normal diameter) and is often filled with red fine membrane-derived granules (*Schuffner's dots*) that are extremely useful, when present, for identification purposes being present only in *P. vivax* and *P. ovale* infections. *P. vivax* trophozoites are extremely mobile due to the continuous emission of pseudopodi which confer the typical ameboid shape. Their dimensions increase as the parasite grows, sometimes to fill the entire red blood cell. In Giemsa stained thin blood films, the cytoplasm of *P. vivax* trophozoites is pale blue, while the chromatin dots (that may be double) are stained purple red. Schizonts are common in *P. vivax* infection and appear as large bodies containing 12 to 24 nuclei and a loose pigmented body. Gametocytes are also commonly seen and appear as round mononuclear bodies with a large nucleus (purple red and dense in female gametocytes, pink and loose in male forms).

In thick films, the Shuffner's dots (when present) may appear as an halo surrounding the parasite. *P. vivax* trophozoites appear as falsely fragmented cytoplasmic dots (joined by thin non visible cytoplasmic bridges) only one of which is provided with a voluminous purple red chromatin dot.

**Plasmodium ovale (plate 3).** As in *P. vivax* infection, to which its morphological and clinical features much resemble, all schizogonic developmental stages may be seen in peripheral blood in *P. ovale* infection. Trophozoites are ring to ameboid in shape and are contained in enlarged erythrocytes stippled with fine red dots (Schuffner's dots, also referred to as *James' dots* in *P. ovale* infection). The morphological appearance of schizonts and gametocytes is often also superimposable to the corresponding stage of *P. vivax*. The differential diagnosis between *P. ovale* and *P. vivax* infection (which is not always possible) is mainly based on the geographical setting (see table 4 and chapter 3 - *Epidemiology of human malaria Plasmodia* - for further details) and on the peculiar oval shape with *fimbriae* at one pole that is occasionally, but not always, assumed by infected erythrocytes.

**Plasmodium malariae (plate 4).** All schizogonic developmental stages may be observed in peripheral blood of subjects infected with *P. malariae*, whose merozoites preferably penetrate older red blood cells. No pigmentation is usually observed in the host erythrocytic cell, that are normal in size. Parasitaemic levels are usually much lower than in the other species of Plasmodia. Low level parasitaemia persisting for many years has been observed. Younger trophozoites are ring form, regular and small in size, with no pseudopodi. Occasionally, in blood smears, the trophozoites assume the typical equatorial band form. Older trophozoites may be irregularly ringed, occupying most of the host cell. Schizonts are compact, with 8-10 merozoites arranged in a rosetta-like fashion around the central pigmented area. Gametocytes are difficult to distinguish from late trophozoites and have coarse pigment granules scattered around the parasite body.

Table 2. Morphological features of the different stages of Plasmodia by species in stained thin blood films

	<i>P. falciparum</i>	<i>P. vivax</i>	<i>P. ovale</i>	<i>P. malariae</i>
<b>Trophozoites</b>	Always present in peripheral blood. Ring-shaped, small to medium size in dimension ( $\varnothing = 2-4 \mu\text{m}$ ) depending on maturation. Young form may lay in marginal position. Polyparasitism and double chromatin dots possible.	Polymorphous in shape from large ring forms (younger forms) to ameboid mass occupying the entire red blood cell (mature forms).	Polymorphous in shape from ring forms often showing a central clear vacuole surrounded by regular cytoplasm (younger forms) to large ameboid masses (mature forms). Their dimensions are slightly inferior to <i>P. vivax</i> .	Ring form, small and regular in shape, with no pseudopodes. Older forms may be large, with vacuole. Occasionally, equatorial band form present
<b>Schizonts</b>	Solely present in more severe infections. Small and compact, containing 15 to 30 merozoites and a dense dark brown pigmented residual body.	Normally present in peripheral blood. Large ( $\varnothing = 12-16 \mu\text{m}$ ), round bodies containing 12 to 24 merozoites and loose golden brown residual pigmentation	Normally present in peripheral blood. Large ( $\varnothing = 10-12 \mu\text{m}$ ), round bodies containing 4 to 12 merozoites and dark pigmentation	Compact, rosetta-like forms with 8-10 merozoites surrounding a central pigmented area
<b>Gametocytes</b>	Present in the second phase of the erythrocytic cycle. Crescent-shaped with coarse rice-like granules and pigment. The female is blue in colour and granules are in central position, while the male form is violet and granules are scattered over the parasite	Round regular bodies with a single voluminous nucleus (dense and red purple in female gametocytes, loose and pink in male forms).	Round regular bodies with a single voluminous nucleus (dense and red purple in female gametocytes, loose and pink in male forms). Their dimensions are usually inferior than in <i>P. vivax</i>	Compact large single dense purple nucleus (female form) or loose violet nucleus (male form). Scattered coarse pigment granules are present
<b>Parasitic density</b>	may be very high (average 20-500.000, max 2.000.000)	intermediate level (average 20.0000, max 50.000)	usually moderate (average 9.000, max 30.000)	usually very low (average 6.000, max 20.000)



Table 3. Morphological features of the host red blood cell by species of Plasmodia in stained thin blood films

	<i>P. falciparum</i>	<i>P. vivax</i>	<i>P. ovale</i>	<i>P. malariae</i>
<b>Age</b>	Young and old erythrocytes infected	Young erythrocytes infected	Young erythrocytes infected	Older erythrocytes infected
<b>Dimensions</b>	Normal	Enlarged	Enlarged, sometimes assuming oval shape with <i>fimbriae</i> at one pole	Normal
<b>Color</b>	Normal to dark	Normal to pale	Normal	Normal
<b>Granules</b>	Unusual coarse scattered red stippling in mature trophozoites or schizonts ( <i>Maurer's clefts</i> )	Frequent fine red diffuse stippling in all stages of erythrocytic developmental cycle ( <i>Schuffner's dots</i> )	Frequent fine red diffuse stippling in all stages of erythrocytic developmental cycle ( <i>Schuffner's dots</i> , also called <i>James' dots</i> )	None
<b>Pigment</b>	Dark brown and usually compact.	Golden brown and usually loose	Brown coarse pigment granules	Brown coarse scattered pigment granules
<b>Leucocytes</b>	The presence of malaria pigment in neutrophils and monocytes is a prognostic marker of severe disease (Hoan Phu et al., 1995)			

Table 4. Geographical distribution of the different species of Plasmodia in the tropics

	<i>P. falciparum</i>	<i>P. vivax</i>	<i>P. ovale</i>	<i>P. malariae</i>
North Africa	frequent	predominant	absent	frequent
West Africa	predominant	very rare	frequent	rare
Central Africa	predominant	very rare	rare	frequent
East Africa	predominant	rare	rare	frequent
Indian Ocean	predominant	rare	rare	frequent
Central America	frequent	frequent	absent	rare
South America	frequent	predominant	absent	rare
Indian subcontinent	frequent	predominant	very rare	rare
South-East Asia	predominant	frequent	rare	rare
Pacific Islands	frequent	frequent	rare	rare

from Basic Laboratory Techniques, WHO, 1982, modified

**Artifacts.** A number of possible artifacts may act as misleading factors for the unexperienced observer and particular attention and exercise is needed to rule out the possibility of confounding factors (fig. 3).

The most frequent confounding elements are *platelets* which are superimposed on red blood cells. The absence of chromatin dots, the reddish stains and the different refrangment patterns while focusing are useful in differentiating platelets from malaria parasites. Among other possible misleading artifacts, *bacteria, dust particle, fungi spores, stain deposits* and *blood cell ghosts* are the most frequent

### **Quantitative parasitaemia count**

The determination of the number of circulating parasites is exceedingly important for clinical purposes to monitor the evolution of the disease and the efficacy of therapy. Different methods have been proposed:

1) Number of parasites/μl of blood (thick film). This method requires the observation of as many microscopic fields (100x) necessary to count 200 leukocytes. The number of asexual parasitic forms (trophozoites, schizonts) present in these microscopic fields has also to be recorded separately from the number of gametocytes. The calculation of the total number of parasites/μl of blood requires the knowledge of the total white blood cell (WBC) count value. If the haemogram is not available, the value of 8000 WBC is generally assumed (WHO, 1991):

$$\frac{\text{number of observed asexual parasites} \times \text{total WBC count}/\mu\text{l}}{200} = \text{parasites}/\mu\text{l of blood}$$

2) Number of parasites/μl of blood (thin film). This method requires the preliminary determination of the number of erythrocytes present in the average microscopic field. This value is usually around two hundred, but it may vary considerably depending upon the quality of the smear and the magnification used. The actual red blood cell count (RBC) value is also needed. If the haemogram is not available, the value of 5.000.000 RBC/μl (males) and 4.500.000 RBC/μl (female) is generally assumed (Shute, 1988) even though the frequent occurrence of anaemia consequent to malaria infection may render these estimations grossly inaccurate. The number of asexual parasites is then counted in at least 25 microscopic fields and the total parasite count//μl of blood is calculated as follows:

$$\frac{\text{number of observed asexual parasites} \times \text{total RBC count}/\mu\text{l}}{\text{total n. of RBC scanned in 25 microscopic fields}} = \text{parasites}/\mu\text{l of blood}$$

3) Proportion of parasitized erythrocytes/total RBC count (thin film). This method requires the preliminary determination of the number of erythrocytes present in the average microscopic field. This value is usually around two hundred, but it may vary considerably depending upon the quality of the smear and the magnification used. The number of parasitized erythrocytes (asexual forms) present in 25 microscopic fields is counted divided by the total number of erythrocytes present in these fields, and multiplied by one hundred. The result will indicate the percentage of erythrocytes that are infected by malaria parasites.

4) Semi quantitative count (thick film). This method is fairly inaccurate and should be used only when it is not possible to perform one of the more accurate methods described above. It uses the following semi-quantitative scale:

- + 1-10 asexual parasites per 100 thick film fields
- ++ 11-100 asexual parasites per 100 thick film fields
- +++ 1-10 asexual parasites per single thick film field
- ++++ more than 10 asexual parasites per single thick film field

### The commonest mistakes

A number of technical mistakes may hamper the correct diagnosis of malaria infection and are briefly listed below in table 5.

Exercise and experience will teach the microscopist when one mistake has occurred during the preparation of the slide.

Table 5. Commonest technical mistakes in microscopic diagnosis of malaria

<b>Mistake</b>	<b>Result</b>
Pricking on non accurately dried finger	The parasites and host cells may be fixed by the alcoholic detergent solution
Use of unclean slides	The blood smear will not be spread evenly
Excessive use of blood	The blood smear will not be spread evenly due to the beginning of the coagulation process
Too much blood is used for thin films	Erythrocytes are laid on multiple layers. Observation is impossible
Inadequate quantity of blood is used for thin films	Parasites may be virtually absent if parasitaemia is low
The slide is not immediately marked with the identification name or code	Confusion may arise leading to unidentified positive or negative slides
Use of incorrectly buffered water	Staining is poor. In particular, Schuffner's dots may not be evident
Use of hypertonic staining solution	Acantocytosis of erythrocytes
Drainage (and not washing) of stain from the slide	Stain deposits may render the observation difficult
Excessive time elapses before preparation and staining of thick films	Autofixation occurs and haemolysis is impossible
Exposure of thick films to excessive heat	Autofixation occurs and haemolysis is impossible
Thick films are dried too slowly	<i>P. falciparum</i> gametocytes may exflagellate

### **(B) Quantitative Buffy Coat (QBC ®) and the direct acridine orange staining**

The Beckton Dickinson QBC ® test is a sensitive microscopic test based on the ability of acridine orange to stain nucleic acid containing cells (Rieckmann et al., 1989). Briefly, a microhaematocrit tube containing acridine orange stain and anticoagulant is filled with the patient's blood (55-65 µl), obtained by a conventional finger pricking method as described above. After insertion of a float, the microhaematocrit tube is centrifuged at 12000 g for 5 minutes and immediately placed under an ultraviolet light source microscope for observation. The blood cellular components sediment in the tube so that platelets, lymphomonocytes, granulocytes and erythrocytes are separated and forced against the tube wall by the internal float.

The parasites are seen as fluorescent bodies standing at different levels of the sedimentation column depending on the stage and species of the parasite.

There is no consensus on the potential of the QBC ® technique in replacing conventional microscopy in the field and many advantages and disadvantages have been pointed out at different times (Baird et al., 1992; Anonymous, 1992; Bawden et al., 1994; Petersen and Marbiah, 1994). Among the advantages, the *short time* and *ease of execution* (5-10 minutes) and the higher *sensitivity* than standard thick films at least in *P. falciparum* infections (as high as 3-4 parasites/µl of blood) are the most striking ones (Rieckman et al., 1989). Among the disadvantages, the *need for a fairly sophisticated instrumentation* (a 12.5x ocular and 50x objective and an ultraviolet light supplied microscope are recommended for optimal observation), *cost* (estimated at 1.5 ECU/test), the limited performance when *Plasmodia other than P. falciparum* are involved and the *difficulties in species determination and parasite quantification* are of concern (White N.J. and Silamut K., 1989).

In our opinion, the QBC ® technique is highly reliable and user-friendly (always coupled with conventional microscopy for species determination and quantification purposes) in a hospital setting and in the hands of well trained and experienced microscopists although still needs to be validated for field use. QBC ® should be used together, but should not replace, conventional standard thick and thin blood films for malaria diagnosis under field conditions until its major drawbacks have been overcome.

A direct acridine orange (fluorochrome) staining of thin and thick films (see annex 2) have been recently proposed to provide an economically convenient alternative to the QBC ® technique for use in the field by using specially designed interference filters that may be connected to conventional light (even sunlight) microscopes (Kawamoto and Billingsley, 1992). No definite advantages over conventional Giemsa staining have however been ascertained so far (Delacollette and Van der Stuyft, 1994). Fluorochrome staining with benzothiocarboxypurine has also been proposed with claimed satisfactory results (Makler et al., 1991).

### **(C) DNA probes and Polymerase Chain Reaction**

Conventional microscopy is usually satisfactory for the large majority of clinical situations, but may be inadequate for very low parasitaemias (below the detection threshold of 10 parasites/µl of blood) and to detect drug-resistant parasites. The possibility that modern molecular biologic techniques overcome these drawbacks has thus been explored. The initial studies in nucleic acid-based malaria diagnosis used the parasite's repetitive DNA found throughout the *Plasmodium* genome as the diagnostic target (Franzen et al., 1984). Therefore, after the sequencing of two small subunits (18S) rRNA genes from *P. falciparum* and *P. vivax* (Mc Cutchan et al., 1988; Waters et al., 1989), species-specific regions of the rRNA genes have been exploited in developing a sensitive and

specific diagnostic procedure. In fact, ribosomal RNA probes are considered to have greater potential than DNA probes since the RNA content in *Plasmodium* is one and a half times the amount of DNA and rRNA represents 85-95% of total cellular RNA. Once the Polymerase Chain Reaction technique became widely described, several investigators switched to designing PCR assays to detect *P. falciparum* and *P. vivax* because of the inherent increase in analytical sensitivity compared to DNA-RNA probes assays.

The PCR based method to diagnose parasitic infection has been recently reviewed (Weiss, 1995).

### PCR characteristics

Several PCR assays to detect Plasmodium DNA in human blood by using different approaches to increase sensitivity and specificity have been described. The PCR can detect a single *P. falciparum* in 20µl of human blood when primers based on moderately-repetitive DNA probe pBRK1 are used (Tirashopon et al., 1991), 100-fold more sensitive than the standard thick blood film. Therefore, an improvement on sensitivity was obtained using oligonucleotides complementary to the 18S rRNA genes (Snounou et al., 1993). The PCR-based techniques have also proven highly species-specific, a quality which is particularly useful in clinical practice where microscopically undetected mixed infections may be demonstrated by PCR (Brown et al., 1992). Another very interesting and recently described characteristic of PCR based techniques is the possibility of processing dried blood spots collected on filter paper, without the need for difficult laboratory steps in the field, even 6 months after collection. This method may reduce the risk of contamination in the laboratory and has yielded satisfactory sensitivity results for *P. falciparum* (Long et al., 1995).

### PCR Applications

The high sensitivity of PCR based techniques has been applied to the screening of donor blood with promising results and relatively acceptable costs in Viet Nam (Hang et al., 1995) and other Developing Countries. In this application, the possibility of false positive results is not a major concern because it implies the discharge of few donor blood units compared to a substantially lower incidence of transfusion malaria cases from asymptomatic low parasitaemia donors in highly endemic areas.

An other area of major interest where PCR based techniques may play an important role is the epidemiological study of the distribution of different Plasmodia (mainly *P. falciparum*) isolates within the insect and human hosts in a given geographical area, so providing intimate information on the biology of the parasite and pathogenesis of the disease in the population at risk (Viriyakasol S et al., 1995). Finally, it is known that *P. falciparum* drug resistance to different antimalarial drugs is due to point mutation in different amino acids (i.e. amino acid 108 in dehydrofolate reductase is responsible for resistance to antifolate drugs). The possibility to discriminate drug-resistant *P. falciparum* strains from drug-sensitive ones without the need for labour-intensive culture has already been demonstrated (Peterson et al., 1991).

### PCR drawbacks

Nevertheless, a number of drawbacks hamper the use of the PCR technique for the diagnosis of malaria infection as well as of other viral, bacterial or parasitic infections not only in the field but also in well equipped laboratories in industrialized countries, the most important of which being contamination, yielding false positive results, and high costs. Stringent, extensive and costly precautions (rarely obtainable in real field conditions) are required to diminish the rate of contamination of PCR with previously amplified products (Wilson, 1993). Finally, actual costs

confine the polymerase technique to selected laboratories, mainly in industrialized malaria-free Countries for research purposes.

It is hoped that the new biotechnological advances will soon offer a real inexpensive, sensitive and user-friendly PCR-based tool to be used in real field conditions for clinical, preventive and epidemiological purposes.

A detailed review of the technical features of Polymerase Chain Reaction (PCR) based malaria diagnostic techniques far exceeds the aims of this Handbook and will not be addressed here.

#### **(D) Detection of *P. falciparum* antigen**

Contrary to the presence of antibodies, the presence of parasitic specific antigens may reflect active infection. The methods used to detect antibodies have also been adapted to the detection of antigens, with variable results. Recently, the advent of biotechnology has disclosed new horizons in the field of antigen detection. A brief outline of the most promising techniques is reported below. The production of histidine-rich protein II (HRP-II) antigen by blood stages of *Plasmodium falciparum* (Parra et al., 1991) forms the basis for the development of ELISA antigen test (Taylor and Voller, 1993) and, more recently, of the dipstick Becton Dickinson ParaSight®-F test, not needing microscopy for its execution, that has recently been proposed for the field diagnosis of malaria (Shiff et al., 1994). The latter test has shown promising results and is dealt with in some detail below. After the deposition in a plastic well, a drop of blood is haemolysed by a detergent and put into contact with a anti-HRP-II monoclonal antibody coated dipstick. In the case of a positive reaction, the latter addition of anti-HRP-II monoclonal antibody conjugated with sulphorhodamine B as a marker produces a red coloration at the site of immunological reaction (a red line appears one cm from the bottom of the dipstick) after few minutes. The test is simple, quick to execute (11 minutes according to the manufacturers), stable for months even in warm and humid climates and does not need trained specialized personnel or equipped laboratories, as recently demonstrated by village health workers in rural Tanzania (Premji et al., 1994) and by field malaria control activities in Brazil (Dietze et al., 1995). These characteristics makes the ParaSight®-F a good candidate for malaria diagnosis in the field or in blood bank in endemic areas where a large number of tests are to be carried out daily, even though costs are far higher than conventional microscopy. The test may also be of outstanding value in clinical settings in non endemic areas. The sensitivity of the test nevertheless decreases linearly with the decline of parasitaemia (from 96-100% in specimens with more than 60 *P. falciparum* parasites/ $\mu$ l to 11-67% in specimens with 10 parasites/ $\mu$ l or less) (Beadle et al., 1994) and is useless in the detection of *Plasmodia* infection other than *P. falciparum*. Furthermore, the test does not permit quantitative determination (useful in highly endemic areas where subclinical parasitaemia is common) and may give false positive results some day after therapy-induced clearance of parasites from the blood due to the persistence of the histidine-rich protein II in the blood..

#### **(E) Other direct diagnostic methods**

The determination of blood levels of parasite-specific lactate dehydrogenase (pLDH) has been evaluated as an indirect method of quantifying parasitaemia and also drug resistance (Makler and Hinrichs, 1993). The method may be automated allowing the processing of large numbers of samples, but its sensitivity has proven to be quite low (Knobloch and Henk, 1995).

## Indirect diagnosis (immunodiagnosis)

The limitation of conventional microscopy (low sensitivity, labour intensive, well trained personnel needed) and of the new diagnostic direct techniques (sophisticated instrumentations required) has stimulated the research for immunodiagnostic methods that might couple the advantage of easy processing a large amount of samples in a standardized way. The detection of Plasmodia specific antibodies is briefly delineated below.

### Detection of Plasmodia specific antibodies

The presence of the malaria parasite in the patient's organism elicits the production of a wide range of antibodies, both specific against Plasmodia antigens and non specific against leukocytes, red blood cell, rheumatoid factor, etc. (Ferreira, 1990). Stage-specific, species-specific and genus-specific antibodies are detectable in the blood some days after parasite invasion of the bloodstream and may persist for long periods of time after infection has occurred (from a few months in case of short-lived and therapy-terminated infection to many years in case of chronic or relapsing infection) (Voller, 1988) (fig 4)..

Antibodies against all blood stages of the parasite schizogonic cycle, and even against exoerythrocytic schizonts, have been demonstrated but commonly available serological tests are aimed at the detection of antibodies against asexual blood stages for practical purposes (supply of antigen) (Voller, 1988). The best antigens are *obviously homologous antigens* (*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*), but an adequate supply is practically obtainable only for *P. falciparum*, which is cultured *in vitro*. For the serological diagnosis of the other species of Plasmodia, heterologous antigens are to be used (*P. cynomolgi* for *P. vivax* and *P. brazilianum* for *P. malariae*).

The first serological test to be used for malaria antibodies was immunofluorescence (IFAT), which may give quantitative results for both G and M specific immunoglobulins. Its specificity and sensitivity largely rely on the laboratory technician's expertise. Results higher than 1:20 are considered to be positive and high titers (> 1:200) probably reflect recent infection. The indirect haemoagglutination test (IHA) is simple and suitable for field studies, but its sensitivity and specificity are poor. The enzyme-linked immunosorbent assay (ELISA) has similar sensitivity and specificity characteristics than the IFA test, but the interpretation of the results may be better standardized. Finally, for research purposes, radioimmunoassay (RIA) is sometimes used but needs well equipped research laboratories and personnel.

The characteristics of the different immunologic methodologies used to detect Plasmodia specific antibodies are summarized in table 6.

Table . Currently used immunological tests to detect Plasmodia specific antibodies (from Gilles, 1993, adapted)

Test	Current application	Notes
Indirect Immunofluorescence (IFAT)	diagnosis of infection epidemiological studies	erythrocytic schizonts as antigens fair sensitivity and specificity fluorescent microscope needed trained personnel needed difficult to standardize

Indirect haemoagglutination (IHA)	epidemiological surveys	low sensitivity and specificity simple material needed suitable for field conditions
Enzyme-linked immunosorbent assay (ELISA)	diagnosis of infection epidemiological studies	fair specificity, sufficient sensitivity possible in field conditions
Radioimmunoassay (RIA)	research studies	expensive sophisticated instruments needed

Since the presence of specific antibodies only reflects past infection, it is obvious that seroprevalence rate is very high in populations living in malaria endemic areas, where it linearly increases with age. The detection of specific antibodies has therefore little role to play in the individual diagnosis of actual clinically relevant infection in malaria endemic areas.

### **Application of immunological methods**

Though the protective importance of antibodies to prevent or decrease the clinical expression of malaria symptoms and signs has been established, their role in the individual diagnosis of clinical feverish episodes is now considered poor, at least in endemic areas.

Immunodiagnosis may provide useful informations in the following fields of application:

- 1) Diagnosis of clinical attacks. The detection of specific antibodies has no role in the individual confirmatory diagnosis of suspected clinical attacks in endemic areas. Outside endemic areas, it may retain some clinical utility only in ruling out malaria in microscopically negative feverish patients returning from short trips to malaria endemic areas. This is particularly true for patients who took chemoprophylaxis or self-treatment during the feverish attack abroad without diagnostic confirmation.
- 2) Epidemiological studies. Conventional antibody serology has provided exceedingly useful species-specific epidemiological information in malaria control programmes to define malaria transmission areas and monitor effectiveness of preventive intervention strategies. The possibility of processing easily large numbers of samples and to evaluate the past exposure to the infecting agent (microscopy, PCR and antigen detection only point out present prevalence of infection) are important factors that support the use of conventional serology for epidemiological purposes. The detection of the highest transmission altitude in Africa and of the presence of residual transmission foci in Tunisia after control activities, the confirmation of malaria eradication from Mauritius and Greece, the validation of chemoprophylactic malaria control in Panama are only a few examples of its possible applications in epidemiological studies (reviewed in Voller, 1988).
- 3) Donor blood screening. Transfusion malaria is one of the most dreaded public health problems in highly endemic malaria Countries sometimes occurring also in malaria free western Countries. Microscopy is an unsuitable screening method in malaria-free areas since low parasitaemia is usually undetectable and long performing time is needed. In these areas, the “five year rule” (all persons reporting stays in malaria endemic areas will be excluded from donation for a five year period) is safe, but the increasing number of travels to the tropics for touristic and commercial reasons will probably render it too stringent in the coming years. Serology will then provide a good alternative to evaluate the persistence of low level malaria parasites in non endemic areas. On the contrary, serology has no role in blood screening activities in malaria endemic areas where continuous exposure occurs.

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**Annex 1 - Laboratory material needed for light microscopical observation of thin and thick films for malaria diagnosis**

Optic microscope with 100x oil immersion objective  
Electric supply of adequate mirrors for sun light  
Stain stock solution  
Buffered distilled/deionized water  
Graduated cylinders  
Staining recipients  
Clean glass slides  
Cover slips (optional)  
Disinfectant alcoholic solution  
Sterile pricking needles  
Gauze or cotton

## Annex 2 - Stock stain solutions for malaria microscopy

after: Shute G.T. **The microscopic diagnosis of malaria**. In *Malaria. Principles and practice of malariology* (Wernsdorfer W.H., McGregor I eds), 1988: 781-814

World Health Organization. **Basic Laboratory Techniques**. World Health Organization, Geneva (Switzerland), 1982

### a) Field stock solution

#### Solution A

<i>Medicinal methylene blue</i>	0.8 g	
<i>Azur I</i>	0.5 g	
<i>Disodium hydrogen phosphate anhydrous Na<sub>2</sub> HPO<sub>4</sub></i>	5.0 g	
<i>Potassium dihydrogen phosphate KH<sub>2</sub>PO<sub>4</sub></i>	6.25 g	
<i>Distilled water</i>		500 ml

#### Solution B

<i>Eosin</i>	1.0 g	
<i>Disodium hydrogen phosphate anhydrous Na<sub>2</sub> HPO<sub>4</sub></i>	5.0 g	
<i>Potassium dihydrogen phosphate KH<sub>2</sub>PO<sub>4</sub></i>	6.25 g	
<i>Distilled water</i>		500 ml

For both solution A and B, prepare first the phosphate solutions and pour them into a hard glass bottle containing glass beads. Add the stains and mix accurately. The stock solution needs to be filtered every two weeks and to be stored in tightly stoppered bottles to avoid the growth of moulds. Solution A and B may also be prepared from the commercially available (Field A powder 5.0 g must be accurately dissolved in 600 ml of hot water and Field B powder 4.8 g must be dissolved in 600 ml of hot water)

### b) Giemsa stock solution

<i>Giemsa stain (powder)</i>	3.8 g	
<i>(Giemsa Azur II 0.8 g + Azur II eosin 3.0 g)</i>		
<i>Methanol</i>	250 to 300 ml	
<i>Glycerol</i>	200 to 250 ml (to total 500 ml)	

Mix in a clean hard glass bottle containing about 50 glass beads and shake vigorously for a few minutes. Repeat shaking each day for 4 days before using the solution.

### c) Leishman stock solution

<i>Leishman powder</i>	1.5 g
<i>Methanol</i>	1000 ml

The powder and the methanol are mixed in a clean hard glass bottle containing glass beads. Screw the cap tightly to avoid humidity and mix accurately

d) **Acridine orange stain for thick and thin films**

Acridine orange      Sigma Chemicals n. A-4921

See Kawamoto F., Billingsley P.F. **Rapid diagnosis of malaria by fluorescence microscopy.** *Parasitology Today*, 1992; 8: 69-71 for further details on the technical procedure of staining thick and thin films

e) **Buffered water**

Stock solution I

di-sodium hydrogen phosphate	9.5 gr
distilled water	1000 ml

Stock solution II

potassium di-hydrogen phosphate	9.07 gr
distilled water	1000 ml

To obtain one liter of buffer solution, mix the 2 solutions as follows:

pH 6.8 =	Sol I (ml 68.4)	+ Sol II (ml 126.8)	+ distilled water (ml 804.8)
pH 7.0 =	Sol I (ml 89.5)	+ Sol II (ml 99.2)	+ distilled water (ml 811.3)
pH 7.2 =	Sol I (ml 105.0)	+ Sol II (ml 77.2)	+ distilled water (ml 817.8)

### **Annex 3 - Color plates and figures of malaria blood parasites in thin and thick films**

**Figure 1 Preparation of thick and thin blood films on the same slide**

(from World Health Organization. Basic malaria microscopy. Part I. Learner's guide Geneva (Switzerland), 1991

**Figure 2 Parts of a typical compound microscope**

World Health Organization. Basic malaria microscopy. Part I. Learner's guide. World Health Organization, Geneva (Switzerland), 1991

**Figure 3 Artifacts that may cause confusion in diagnosis**

World Health Organization. Basic malaria microscopy. Part I. Learner's guide. World Health Organization, Geneva (Switzerland), 1991

**Figure 4 Evolution of parasitaemia and antibodies in malaria (immunofluorescence)**

(Amended from Voller and Draper, 1982)

**Plate 1 Appearance of *Plasmodium falciparum* stages in Giemsa stained thin and thick films**

World Health Organization. Basic malaria microscopy. Part I. Learner's guide. World Health Organization, Geneva (Switzerland), 1991

**Plate 2 Appearance of *Plasmodium vivax* stages in Giemsa stained thin and thick films**

World Health Organization. Basic malaria microscopy. Part I. Learner's guide. World Health Organization, Geneva (Switzerland), 1991

**Plate 3 Appearance of *Plasmodium ovale* stages in Giemsa stained thin and thick films**

World Health Organization. Basic malaria microscopy. Part I. Learner's guide. World Health Organization, Geneva (Switzerland), 1991

**Plate 4 Appearance of *Plasmodium malariae* stages in Giemsa stained thin and thick films**

World Health Organization. Basic malaria microscopy. Part I. Learner's guide. World Health Organization, Geneva (Switzerland), 1991