Techniques for the detection of malaria parasites

P L Chiodini PhD MRCP **A H Moody** FIMLS *Department of Parasitology, Hospital for Tropical Diseases, London NW1 0PE*

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Globally, malaria shows no sign of coming under control. Reflecting this, and the increase in overseas travel plus increasing difficulties in providing effective but safe chemoprophylaxis, the Hospital for Tropical Diseases (HTD) is called upon to diagnose and to treat more and more patients with imported malaria. At the present time, approximately 30% of the United Kingdom cases of falciparum malaria, are managed at the HTD. However, the geographical spread of cases and the fact that the clinical presentation of malaria is non specific, means that patients may also present for diagnosis and treatment on general medical intake or to an accident and emergency department or general practitioner in any part of the UK. It is, therefore, essential that a high index of clinical suspicion plus the skills to achieve rapid accurate laboratory confirmation of malaria, are maintained and appropriately distributed throughout the National Health Service.

This review summarizes current methodology for the diagnosis of malaria in a clinical laboratory, and examines new methods under development with special reference to their potential for the diagnosis of an individual patient.

Current situation

Methodology

Samples In most cases, microscopic examination of peripheral blood is all that is required for the diagnosis. On occasions, centrifugation and examination of the buffy coat provides a means of increasing sensitivity, and thus can be helpful in light infections. Bone marrow aspiration is advocated by some authorities as sometimes being positive when peripheral blood smears are negative. However, it is not a comfortable procedure, and it is doubtful whether any additional benefit is obtained[1]. Intradermal puncture has been suggested as a way to detect parasites of *Plasmodium falciparum* sequestered in skin capillaries. In patients dying with a possible diagnosis of cerebral malaria, a postmortem smear of grey matter provides a means of confirming the presence of parasites sequestered in capillaries or post capillary venules. In obstetric practice, microscopy of cord blood, or a placental impression smear, can be used to demonstrate malaria parasites.

The thin blood film A small drop of blood (approx 3 mm diameter) is placed on a grease free microscope slide and a second slide, held at 45°, used to spread the blood smoothly and rapidly. The film is air dried, fixed in methanol for 2 min, then stained for 20 min in 10% Giemsa stain in phosphate buffered saline at pH 7.2. The film is rinsed in tap water, blotted dry and examined systematically under oil immersion microscopy. The area of the film where red blood cells are just overlapping provides optimal morphology of the parasites. Modified Field's stain provides a rapid (< 3 min) method for staining malaria parasites, but does not reliably demonstrate red cell stippling.

Thick blood film The thick film is prepared by spreading approximately 5 μl of blood over a 1 cm area on a clean grease-free microscope slide. The film is air dried, then stained with Field's stain or with dilute Giemsa stain. After staining, the film is carefully washed in tap water, and allowed to drain dry.

More detailed methodology is beyond the scope of this brief review, but can be found in the reviews of Bruce-Chwatt[1] or Shute[2].

Problems

Before a laboratory can receive and examine blood for malaria parasites, someone has to think of malaria as a possible diagnosis in the febrile (sometimes afebrile) patient. Neglecting to do so is a dangerous mistake which can result from failure to take a travel history or to consider transfusion or residence near an airport as possible risk factors[3]. In addition, the practice of needle sharing among some intravenous drug abusers provides another way to contract malaria without necessarily travelling overseas. Unfortunately, some individuals, medical and non-medical, still hold the erroneous belief that a history of antimalarial chemoprophylaxis excludes the diagnosis of malaria.

Problems with thin blood films Too acute or too obtuse an angle between the spreader and the slide, will produce too thick or too thin a film, respectively. Slow drying produces crenation of the erythrocytes and distorted parasite morphology. Failure to stain at the correct pH (7.2) will lead to poor demonstration of the altered staining properties of infected red blood cells; for example, Schuffner's dots due to *P. vivax* will not be seen if pH 6.8 (often employed in routine haematology laboratories for automated staining of blood films) is used.

Problems with thick blood films Films which are too thinly spread will have diminished sensitivity (small sample size), and those which are too thick may lyse poorly and be difficult to see through. Poor lysis may also be due to the film being too old, and thus partly fixed by drying. Films may detach from the slide if stained too quickly after preparation, or if they are too thick. Nevertheless, a properly prepared thick film, examined by a competent microscopist has an approximate sensitivity of 20 parasites per microlitre, or 0.0004% parasitaemia[4].

Importance of parasitaemia It is essential when reporting infection with *P. falciparum* to provide information on the level of parasitaemia present. This is of practical importance in taking management decisions, as the complication rate, morbidity and mortality of falciparum malaria are higher with higher parasitaemias. Estimates of parasitaemia can be obtained in a variety of ways. Firstly, using the thin film, one thousand erythrocytes can be counted and the number of cells containing asexual stages enumerated and converted to a percentage of the total cells (% parasitaemia).

Secondly, using the thick film, the number of asexual parasites per 200 white blood cells can be counted. This is converted to a parasite count per microlitre by knowing the white cell count for that patient; alternatively but less accurately, an assumption of 8.0×10^9 white blood cells per litre can be made for the calculation.

Thirdly, the Earle and Perez method[5] counts the number of asexual parasites per known volume of blood (usually 5 μl) spread as a thick film. This method is probably the most accurate, though there are always some losses of parasites from thick films during the staining procedure.

Errors in interpretation of blood films Artefacts in the form of stain granules, bacteria or algae growing in contaminated buffer solutions, are examples of false positive 'malaria'. Platelets, if superimposed on erythrocytes, can also cause confusion. On occasions, the reverse occurs, and genuine malaria parasites are dismissed as artefacts by the inexperienced observer. Poor staining technique, especially incorrect attention to the pH of buffer solutions, contributes to misidentification of the species of malaria parasite present, especially *P. vivax* or *P. ovale*, where erythrocytic stippling is an important diagnostic criterion. Failure systematically to scan the film may produce a bias towards higher parasitaemia as the observer may be diverted in counting towards these areas of the film where parasites are seen. Use of an eyepiece graticule can reduce this tendency.

'Blood film negative' malaria Occasionally there is strong clinical suspicion of malaria, but the blood film is reported as negative. Unfortunately, observer error accounts for a large proportion of these cases. However, there are instances where the film is genuinely negative. Suppressive prophylaxis or partial treatment may render the thick film temporarily negative without achieving cure or alleviation of the clinical illness. Sequestration of *P. falciparum* in the deep tissue capillaries may, if multiplication is synchronous, cause the blood film to become temporarily negative. This is a very pernicious parasite, and the possibility of sequestration must be considered and covered by repeating the blood film several hours later, whether or not empirical treatment has been started.

Advantages of blood film examination
Blood film examination can establish the diagnosis of malaria very rapidly (in less than one hour), and is remarkably sensitive. The species can be identified and mixed infections diagnosed. Asexual parasites can be distinguished from gametocytes and a rapid estimation of parasitaemia obtained.

Serology Antibody detection is unhelpful in the diagnosis of an acute attack of malaria. However, serodiagnosis is valuable in providing retrospective confirmation of an attack of malaria, for diagnosing the tropical splenomegaly syndrome (hyperreactive malarial splenomegaly) and in field surveys. Blood bank screening is another area where serology is potentially useful. The indirect fluorescent antibody test (IFAT) is widely used, and is available to asexual blood stage antigens of *P. falciparum*, *P. vivax* and *P. malariae*. A fuller discussion of the role of serology in malaria diagnosis can be found in Gillespie and Chiodini[6].

New techniques

The exciting entry of molecular biological techniques into parasitology has already produced several new techniques which are undergoing evaluation for the diagnosis of malaria infection.

DNA probes
At the time of writing, DNA probes exist only for the diagnosis of *P. falciparum*. Those currently developed use a highly repeated 21 base pair sequence from *P. falciparum* DNA, either cloned (for example in *Escherichia coli*) or synthetic. The majority of probes use radioisotope labelling to detect binding of probe to test DNA, though biotin/avidin or alkaline phosphatase labelling has also been used. Franzen *et al.*[7] using a phosphorus-32 labelled 21 base pair recombinant probe, were able to detect *P. falciparum* down to a parasitaemia of 0.001%. They reported no cross reaction with *P. vivax* or with human DNA. McLaughlin *et al.*[8] developed a ^{32}P labelled synthetic 21 nucleotide *P. falciparum* DNA probe. They were able to detect 0.1 ng of *P. falciparum* DNA after overnight exposure, with a 10-fold increase in sensitivity if exposure was extended to one week.

There are still unresolved problems in the use of DNA probes for malaria diagnosis. Some have high background signals due to a non-specific binding to blood components other than malaria. Most still use radioisotopes, and the methods are uniformly much slower than blood films for the detection of malaria infection. At present, sensitivities are, depending on the probe, at best equal, but not uncommonly inferior to those achievable by microscopy. The future will produce probes with much greater sensitivity, and it is likely that probes will be developed for each species infecting man. A non-specific 'malaria' probe would be useful for initial screening prior to speciation. Blood bank screening (where parasitaemias in asymptomatic donors are usually below the sensitivity of microscopy), would be a most useful area for deployment of highly sensitive probes as transfusion induced malaria can still occur in the UK[3]. Epidemiological surveys could also provide a market for DNA probes, with tests being performed at sophisticated base laboratories after transport of partially processed specimens from the field.

For a more detailed discussion of DNA probes for malaria see Chiodini[9].

RNA probes
Preliminary work with RNA probes suggests that they have greater sensitivity than DNA probes, and give lower background signals. Waters and McCutchan[10] developed RNA probes for all four species of human malaria parasite, reporting the

detection of fewer than 10 parasites by autoradiography in an overnight exposure.

Antigen detection

Avidor *et al.*[11] reported a radioimmunoassay (RIA) system for the diagnosis of *P. falciparum* infection. They obtained good specificity, with only one false positive in 530 blood samples examined microscopically. However, its sensitivity was below that of conventional microscopy: the RIA had a detection limit of approximately 200 parasites per microlitre of blood. Immunoradiometric assay[12] has also been deployed. Iodine 125 labelled monoclonal antibody used in an antibody 'sandwich' permitted detection of 0.02 *P. falciparum* parasites per 10^6 red blood cells. The authors reported a lack of cross reaction with *P. vivax* and stated that detection of *P. falciparum* antigen post treatment, was possible for a longer period than achieved by microscopy.

Londner *et al.*[13] using a dot Elisa method (inhibition of antibody binding) reported detection of a 0.001% *P. falciparum* parasitaemia. The system was economical in use of reagents compared with standard Elisa methods and the test could be read visually as well as spectrophotometrically.

Acridine orange stain

Spielman *et al.*[14] reported detection of malaria parasites by centrifugation of acridine orange stained blood samples in a sealed tube, visualizing the parasites by fluorescence microscopy. They reported sensitivity greater than they achieved by microscopy. The system does require fluorescence microscopy, a disadvantage in the tropics and it is doubtful whether speciation will be as good as that achievable using Giemsa stained blood films. However, it may fulfil a useful role in screening blood samples in parallel to Romanowsky staining.

Conclusions

The newer techniques for detection of malaria parasites are of great interest, and are likely to fulfil a major role in studies of malaria epidemiology. When sensitivity is improved and more rapid methods devised DNA or RNA probes may become valuable tools for diagnosis of individual patients. However, at the present time, the best way to confirm malaria is to think of the diagnosis and have thick and thin blood films examined by a competent microscopist. Failure to do so could have fatal consequences.

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