

Laboratory Diagnosis of Malaria

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Rapid and accurate diagnosis is a key to the success of an effective treatment of malaria. The global impact of malaria has spurred interest in developing diagnostic strategies that will be effective not only in resource-limited areas but also in developed countries, where expertise in malaria diagnosis is often lacking. Accurate diagnosis of malaria is necessary to prevent morbidity and mortality while avoiding the unnecessary use of antimalarial drugs. Currently, there are three direct methods for laboratory diagnosis of malaria which include microscopic examination of stained slides (Giemsa, Field, Wright, or acridine orange stained films), detection of parasite antigens (histidine-rich protein 2, plasmodial lactate dehydrogenase), and molecular biology methods (polymerase chain reaction (PCR) and multiplex real-time PCR).

Microscopic examination

Conventional light microscopy is the established method for the laboratory confirmation of malaria. The careful examination of well prepared and well stained thick and thin blood films by an expert microscopist remains currently the "gold standard"¹ based on its ability to allow speciation, quantification of parasitemia, and assessment of the distribution of parasite forms. These latter two functions can help in the assessment of disease severity and sometimes influence the choice of therapy. Microscopy offers many advantages. The sensitivity of this method can be excellent, with detection of malaria parasite densities as low as 5 to 10 parasites/ μ l of blood (approximately 0.0001% parasitemia)² but most laboratories achieve a lower sensitivity of around 500 parasites/ μ l. Microscopy permits determination of the infecting species based on parasite morphology, shape and size of infected red blood cells as well as the stage of the circulating parasites. Moreover, circulating parasite density can be determined, which may help in the prognosis, and serial examinations can monitor the parasitological response to chemotherapy. Examining an individual sample is relatively inexpensive. Finally, malaria smears provide a permanent record for quality assessment of the microscopy diagnosis. Despite these strengths, microscopy possesses a number of limitations. The procedure is labor-intensive and time-consuming. Variability in stains and in techniques used to collect

and process blood affects slide interpretation. Importantly, accurate microscopic diagnosis is a skill still learned with extended training and experience, whether in countries where malaria is endemic or in countries with imported malaria. Routine clinical microscopy cannot reliably detect very low parasitemias (<5 to 10 parasites/ μ l) or sequestered parasites.

Microscopy diagnostic errors are noted more commonly for low-density parasitemias (10 to 100 parasites/ μ l of blood). In addition, mixed infections are often missed, especially when *Plasmodium malariae* or *Plasmodium ovale* parasites are present, as their densities are often low in comparison to that of *Plasmodium falciparum*. Fluorescence microscopy techniques based on dyes with affinity for parasite nucleic acids have also been applied as diagnostic assays.³ A commonly used dye is acridine orange (AO), which when excited by UV light of the correct wavelength fluoresces strongly. Difficulty in discriminating between fluorescence-stained parasites and other nucleic acid-containing cellular debris has limited the sensitivity of AO techniques to >100 parasites/ μ l. Although processing time is reduced over routine microscopy, there is a requirement for special equipment. Species differentiation is often difficult and requires confirmation by alternative methods. For these reasons, fluorescent methods offer little, if any, improvement over standard staining techniques.

Despite their continued application as key diagnostic tests, microscopic techniques have several key limitations which render them inappropriate for universal or targeted donor screening. Specifically, they lack the required sensitivity to detect all infectious units, are too time-consuming (generally requiring an hour or more for preparation and thorough examination), and require significant expertise and specialized equipment when fluorescence methods are used.

Antigen detection

These tests are based on the detection of antigens derived from malarial parasites in lysed red blood cells, using immunochromatographic methods. Most of them employ a dipstick or test strip bearing monoclonal antibodies directed against the target antigens. The tests can be performed in 15 minutes. Characteristics required of a rapid diagnostic test (RDT) vary based on regional

malaria epidemiology and the goals of a malaria control program. The critical characteristics needed for a diagnostic method in order to reduce mortality from malaria in sub-Saharan Africa include high sensitivity for detecting *P. falciparum* and rapid availability of test results. RDTs should require minimal operator training and yield highly reproducible test interpretations. The results of the test should be rapidly available while the physician is actively managing patients, typically in less than 1 hour. The ideal test should be able to detect a response to therapy, including detection of a recrudescence or relapse. The stability of RDT components should be such that refrigeration and a cold supply chain are not needed. Storage shelf life should be of sufficiently long duration that the logistical burden of resupply is minimized.

RDTs utilizing immunochromatographic lateral-flow-strip technology were introduced in the early 1990s. Immunochromatographic technology remains the common basis for all practical RDTs under consideration at this time. The World Health Organization (WHO) has recommended a minimal standard of 95% sensitivity for *P. falciparum* densities of 100/μl and a specificity of 95%.⁴ This technology has been employed for a variety of other diagnostic assays, including pregnancy tests. In these assays, the clinical sample migrates as a liquid across the surface of a nitrocellulose membrane by means of capillary action. For a targeted parasite antigen, two sets of antibodies, a capture antibody and a detection antibody, are used. Either of these antibodies can be monoclonal or polyclonal. Monoclonal antibodies can be very specific but less sensitive, while polyclonal antibodies can be more sensitive, but less specific. Also, the source of antigen used to induce the RDT antibodies (purified native protein, recombinant proteins, or peptides) can make significant differences in the performance characteristics of the final assay. Even monoclonal antibodies directed against the same antigen, if they target different epitopes of that antigen, may exhibit quite different sensitivities and specificities. The capture antibodies are sprayed as a stripe by machine onto the nitrocellulose membrane and bind to the membrane in an immobile phase. These fixed antibodies serve to extract and bind parasite antigens from the migrating liquid sample. The second set of antibodies is conjugated to an indicator, typically gold particles, in a mobile phase. These antibody-indicator complexes bind to the parasite antigen that has been captured by the immobile antibody on the membrane, producing a visible line if the targeted antigen is present in the clinical sample.

The characteristics of the malaria antigen target and the detection antibodies are paramount to understanding the assay performance. Malaria antigens currently used as diagnostic targets are either specific to a *Plasmodium* species or conserved across the human malaria. *P. falciparum*-specific *P. falciparum* monoclonal antibodies have been developed for histidine-rich protein 2 (HRP-2) and *Plasmodium* lactate dehydrogenase (pLDH), while *P. vivax*-specific monoclonal antibodies have undergone limited evaluation.⁵

The first antigen used in a commercial assay was HRP-2, a-soluble protein unique to *P. falciparum*, which is localized in the parasite cytoplasm and on the parasitized erythrocyte membrane.⁶ The HRP-2 concentration increases as the parasite develops from

the ring stage to the late trophozoite. This antigen is found predominantly in the asexual stages but is also found in young *P. falciparum* gametocytes therefore it can detect only *P. falciparum* infection. This test has shown an overall average sensitivity of 77 to 98% when >100 parasites/μl are present (0.002%), with a specificity of 83 to 98 % for *P. falciparum* compared with thick blood smear microscopy.⁷ There is evidence that certain individuals may actually have a gene deletion for the production of HRP-2 and so will never give a positive result with these tests. HRP-2 sequences were observed, prompting a prediction that only 84% of *P. falciparum* infections with low parasite densities (<250/μl) could be detected in the Asia-Pacific region. Other limitations of tests for this antigen relate specifically to technical aspects of the HRP-2 test system. Several reports have been made that the monoclonal IgG antibody used cross-reacts with serum rheumatoid factor, causing false-positive results.⁸

Parasite enzymes comprise the other primary antigen diagnostic targets. pLDH, the terminal enzyme in the malaria parasites glycolytic pathway, is also an antigen target for detection of viable sexual and asexual malaria parasites. It has been found in all four human malaria species. Monoclonal antibodies have now been developed that can target a conserved element of pLDH on all human malaria species (panmalarial) or specific regions unique to *P. falciparum* or *P. vivax*.⁹ Three monoclonal antibodies are used in the RDT immunochromatographic dipstick test (OptiMAL). Two of the monoclonal antibodies are panspecific, recognizing all four species of malaria; a third monoclonal antibody is specific only for *P. falciparum* LDH. One panspecific antibody (6C9), conjugated to gold particles as the indicator, is used to capture all the malaria pLDH antigen present from a blood sample. The other two monoclonal antibodies act as separate immobilized capture sites on an immunochromatographic dipstick. One of the monoclonal antibodies (17E4) is specific for the capture of *P. falciparum* pLDH, and the other (19G7) is a panspecific pLDH antibody. The malaria antigen/labeled-antibody complex will be captured by either or both of the immobilized capture lines (*P. falciparum* spp.) or by the panspecific line only (non-*P. falciparum* spp.). The gold-conjugated antigen-antibody complex builds as a purple line at the capture stripe. The presence of a goat anti-mouse monoclonal antibody capture control line indicates a successful test. In the presence of mixed infections of *P. falciparum* and a non-falciparum species, the results would indicate *P. falciparum*.² The monoclonal antibodies used in the test have been exhaustively tested for cross-reactivity with LDH from other blood protozoa such as Leishmania, Babesia, and pathogenic bacteria or fungi, and no evidence of such cross-reactivity has been found. The sensitivity obtained with this test for *P. falciparum* and *P. vivax* was 94 and 88%, respectively, with a specificity of 100 and 99% respectively. Samples found positive by microscopy, but negative by the test (3%) had < 100 parasites/μl of blood (0.002% parasitemia).¹⁰

Among pregnant women with possible *P. falciparum* malaria, placental sequestration of parasites can reduce the sensitivity of microscopic diagnosis; however, HRP-2 is still detectable in peripheral blood samples. HRP-2 RDTs show a higher sensitivity for this population than does microscopy. Notably, placental malaria

infections detected by RDTs using peripheral blood were associated with a lower median birth weight. The ability to detect placental infection by antigen detection when microscopy does not identify parasitemia could have a significant impact on maternal and fetal health care. The implications of persistent HRP-2 antigenemia for up to a month after successful therapy are unclear in this setting. An unresolved issue concerns the diagnostic testing of young children with clinical illnesses compatible with malaria in regions where *P. falciparum* greatly (>90%) predominates, such as sub-Saharan Africa. In such falciparum malaria-predominant regions, the WHO currently recommends parasitological confirmation of the diagnosis of malaria as part of malaria case management in all cases except those of children under 5 years old. It is demonstrated that RDT with 95% sensitivity for parasitemia of 500/μl and 95% specificity could avert over 100,000 malaria related deaths and about 400 million unnecessary treatments.

RDTs continue to have limitations, notably including an inability to detect mixed infections, inability to distinguish every species of *Plasmodium*, failure to detect infections with low but clinically relevant concentrations of parasites, and limited ability to monitor responses to therapy.

Molecular biology methods (PCR and multiplex real-time PCR)

The first method for molecular detection of *P. falciparum* malaria was published in 1984.¹¹ Since then, much progress has been made in DNA extraction, and detection protocols have been simplified. The four human species can be specifically identified, and the more recently developed real-time amplification techniques allow rapid processing of samples and quantification of parasite loads. Molecular assessment of point prevalence at the village level has also become feasible. Various field surveys have shown that molecular methods detected up to eight times more *Plasmodium spp.* infections than microscopy, and that mixed infections could represent up to one third of them. The main drawback of molecular detection tools is their cost and workload. Furthermore none of the published methods are adapted to large-scale analysis of thousands of samples. Therefore, most decisions for malaria control programs still rely on data collected by health services, where malaria is diagnosed using microscopy. With recent moves towards malaria elimination in a number of countries, additional methods for detecting infections and infectious reservoirs are needed.

Nested and multiplex PCR methods can give valuable information when difficult morphological problems arise during attempts to identify parasites to the species level. A number of PCR assays have been developed for the detection of malaria DNA from whole blood as either single or multiplex methods. These assays have been used for the initial diagnosis, following the response to treatment, and as sensitive standards against which other non-molecular methods have been evaluated.

The small-subunit 18S rRNA and circumsporozoite (CS) genes have been used as targets for the differentiation of *Plasmodium spp.* Methods using nested PCR and reverse transcription-PCR enable all four species to be identified. The large-subunit RNA gene is extensively conserved among *Plasmodium spp.* and is

also suitable as a genus-specific DNA target region,¹² with the amplified target sequence being detected by internal probes or analyzed by gel electrophoresis. The nested PCR method based on the 18S rRNA gene marker adapted for epidemiological studies was mostly used.¹³ After a *Plasmodium spp.* specific nested PCR with the primer pairs, the primary PCR product of *Plasmodium* positive samples were separately amplified with the four species-specific primer pairs to identify the species¹³ and PCR products of some blood samples from Thai patients can be easily observed on the gel as shown in Fig 1. Other DNA targets, such as the CS gene, have been used for species-specific regions and have been coupled with specific fluorescein or radiolabeled probes for detection of *P. vivax*.

Indeed, large-scale studies will be required, including areas with difficult access to health centers. Such studies require high sensitivity in order to detect asymptomatic carriage and improved species identification in order to adapt treatment. Such needs are best fulfilled by molecular methods, which furthermore would open the possibility to monitor vector control measures. The aim of this study was to improve molecular detection of the four *Plasmodium* species. Molecular detection approaches have been recently developed targeting the nuclear 18S rRNA gene (*18S rRNA*), and the mitochondrial *cytochrome b* gene (*cytb*), enabling mass screening of field samples for epidemiological studies. Both loci were chosen because of their obligate presence, their good intra-species conservation associated with appropriate interspecies variation.¹⁴

The *cytochrome b* gene is highly conserved and has mainly been used for phylogenetic studies. The gene is located on the mitochondrial genome, which occurs at an estimated number of 30-100 copies per parasite,¹⁵ and as a consequence is predicted to permit more sensitive detection than the 18S rRNA gene. The



Fig 1. Agarose gel analysis of a PCR diagnostic test for species-specific detection of *Plasmodium* DNA using 18SrRNA from blood samples. PCR was performed using nested primers of Snounou *et al.* 1993. Lane 1: DNA marker, lanes 1 and 4: *P. falciparum* (205 bp) lanes 3 and 7: *P. vivax* (120 bp), lanes 4 and 8: *P. malariae* (144 bp), lane 5: negative control.

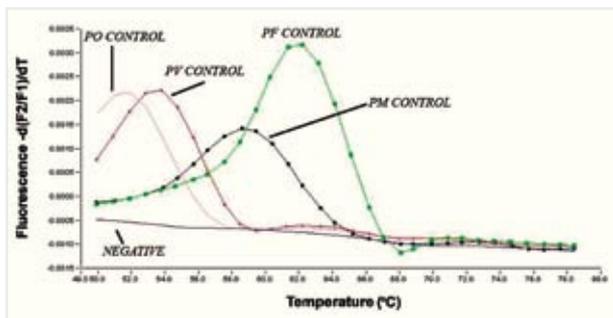


Fig 2. Melting temperature curves of positive control samples of the four species of *Plasmodium* following amplification by the LightCycler polymerase chain reaction assay. Fluorescence peaks occurring at different melt temperatures permit differentiation of the amplicons of each species.

cytochrome *b*-based method is the most sensitive, which is likely because it targets the multiple copies of the mitochondrial genome present in each parasite.¹⁴ Probably for the same reason, it also detected higher rates of *Plasmodium* spp. and *P. falciparum* infections among the field samples. Low-level parasitaemias of *P. malariae* and *P. ovale* are especially frequent in mixed infections, partly because of the species characteristics, and partly because of a density-dependant regulation mechanism. These species are, therefore, easily overlooked by microscopy, when *P. falciparum* or *P. vivax* are the major species. This also explains the large higher numbers of mixed infections detected with molecular biology tools compared to microscopic examinations.

The major advantages of using a PCR-based technique are the ability to detect malaria parasites in patients with low levels of parasitemia and identify them to the species level. Infection with five parasites or less per μl can be detected with 100% sensitivity and 100% specificity. The additional sensitivity obtained using PCR may provide positive results from subpatent infections. Although many organisms may remain sequestered in the capillary beds, these parasites may be released into circulation, but in insufficient numbers to be detected by peripheral-blood microscopy alone. Moreover, human blood samples reported as *P. malariae* infection by microscopy were positive for *P. knowlesi* by PCR. Since the appearance of *P. knowlesi* is similar to that of *P. malariae*, it is unlikely to be correctly diagnosed. Currently PCR assay and molecular characterization are the most reliable methods for detecting and diagnosing *P. knowlesi* infection.¹⁶

The variation in PCR results obtained in a number of different studies may reflect differences in techniques for collection and storage of the specimens, methods for DNA extraction and selection of primers, amplification conditions, and analysis of amplified product. Further advances in PCR technology may allow distinction of DNA from viable or nonviable parasites and thus facilitate the use of PCR-based procedures in the field. PCR-based methods are particularly useful for studies on strain variation, mutations, and studies of parasite genes involved in drug resistance. Highly sensitive detection of malaria parasites can be achieved. Sensitive molecular methods will prove useful in studies exploring malaria epidemiology, risk factors for symptomatic and asymptomatic infections, interaction of species and

importantly in monitoring the efficacy and effectiveness of the scaling up of malaria control efforts.

The introduction of automated multiplex real-time PCR that is rapid and does not require multiple complex procedures or skilled technologists has raised the possibility that these methods may have wider application in the sensitive and specific diagnosis of malaria. Multiplex real-time PCR assay has been developed using fluorescence resonance energy technology (FRET) and the LightCycler (Roche Applied Science, Indianapolis, IN).¹⁷ A genus-specific primer set corresponding to the 18S ribosomal RNA is used to amplify the target sequence. Fluorescence resonance energy technology hybridization probes have been designed for *P. falciparum* over a region containing basepair mismatches, which allow for differentiation of the other *Plasmodium* species including *P. vivax*, *P. malariae* and *P. ovale*. This method has the advantage of allowing amplification and detection of four species of human *Plasmodium* using one set of primers and probes for the 18S ribosomal RNA (rRNA) gene in a single run of the assay, which takes less than one hour to complete. Identification of the species present is accomplished by melting curve analysis of the amplified product. A melting temperature of $60 \pm 2.0^\circ\text{C}$ indicates *P. falciparum*, a melting temperature of $57 \pm 1.0^\circ\text{C}$ indicates *P. malariae*, a melting temperature of $51.8\text{--}55.5^\circ\text{C}$ indicates *P. vivax*, and a melting temperature of $49.5 \pm 1.0^\circ\text{C}$ indicates *P. ovale* (Fig 2). The mixed infection *P. falciparum* and *P. vivax* positive sample was detected based on their melting peaks (Fig 3).

The detection limit of microscopy is 5-20 parasites/ μl ,¹⁸ and the sensitivity of RDT has been reported to be above 90% if >100 parasites/ μl are present, whereas PCR assays can detect parasitaemias of <5 parasites/ μl and probably as low as 0.004 parasites/ μl .^{19,20} However, decades of malaria microscopy have taught practitioners that a single negative result does not automatically exclude malaria. The authoritative PCR could be useful in a quality assurance scheme in reference laboratories. Testing of samples from routine diagnostic laboratories could provide an excellent quality control for results obtained by conventional microscopy and other diagnostic methods.

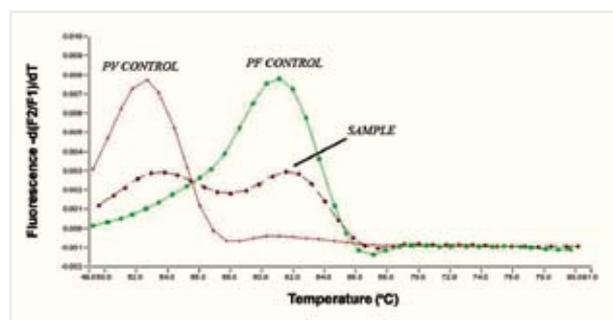


Fig 3. Melting temperature curves from one of the patients with a mixed infection due to *Plasmodium vivax* and *P. falciparum* following amplification of extracted blood by the LightCycler polymerase chain reaction assay. Two separate fluorescence peaks at different melting temperatures (53°C and 60.5°C) are present, which correspond to the peaks for the plasmid controls for each organism.

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