

Rapid Diagnostic Tests for Malaria Parasites

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INTRODUCTION	66
ROUTINE LABORATORY DIAGNOSIS	67
Stained Blood Films	67
Thick blood film	67
Thin blood film.....	67
Sensitivity of thick blood film	67
Estimation of Parasitemia by Using Blood Films.....	67
Examination of thick blood films	67
Examination of thin blood films	68
DIAGNOSIS USING FLUORESCENCE MICROSCOPY.....	68
DETECTION OF SPECIFIC NUCLEIC-ACID SEQUENCES	69
NONMICROSCOPIC RAPID DIAGNOSTIC TESTS.....	70
IMMUNOCHROMATOGRAPHIC TESTS.....	71
Principle.....	71
Malaria Antigens Suitable as Targets for Rapid Diagnostic Tests.....	71
HRP	71
pLDH and aldolase.....	71
IMMUNOCHROMATOGRAPHIC DIPSTICK ASSAYS USED FOR DIAGNOSIS	71
Assays for Detection of HRP-2	72
ParaSight F.....	72
ICT <i>Pf</i> and PATH Falciparum Malaria IC.....	72
Self-diagnosis.....	72
Limitations.....	72
Persistence of HRP-2.....	73
Assays for Detection of pLDH	73
OptiMAL	73
OTHER CANDIDATE ENZYMES WITH THE POTENTIAL FOR USE AS INDICATORS OF INFECTION.....	75
Aldolase	75
DISCUSSION	75
REFERENCES	77

INTRODUCTION

Malaria presents a diagnostic challenge to laboratories in most countries. This paper attempts to review the current methodology and approach to the diagnosis of malaria in a practical and helpful way for the laboratory and for the physician caring for the patient. The urgency and importance of obtaining results quickly from the examination of blood samples from patients with suspected acute malaria render some of the more sensitive methods for malaria diagnosis impractical for routine laboratory use. For the purposes of this review, laboratory methods that require more than 1 h to provide a clear diagnosis of malaria are not considered rapid tests, although they may be considered reference procedures.

Endemic malaria, population movements, and foreign travel all contribute to the malaria diagnostic problems faced by the laboratory that may not have appropriate microscopy expertise available. Changing patterns of accepted morphological appearances of malaria species, possibly due to drug pressure,

strain variation, or approaches to blood collection, have created diagnostic problems that cannot easily be resolved merely by reference to an atlas of parasitology. Fortunately, new technology provides additional diagnostic options, which can be reviewed and compared to more traditional methods. Concurrently the World Health Organization (WHO) has begun a dialogue with scientists, clinicians, and manufacturers of malaria diagnostic test devices regarding the realistic possibilities for developing accurate, sensitive, and cost-effective rapid diagnostic tests for malaria. Stipulations for these rapid tests include the capability to detect 100 parasites/ μ l from all *Plasmodium* spp. and the ability to perform semiquantitative measurements for monitoring drug treatment results. Also, these new technologies must be compared against accepted “gold standard” methods.

The majority of malaria cases are found in countries where cost-effectiveness is an important factor and ease of diagnostic test performance and training of personnel are also major considerations. Most new technology for malaria diagnosis incorporates immunochromatographic capture procedures, with conjugated monoclonal antibodies providing the indicator of infection. Preferred targeted antigens are those which are abundant in all asexual and sexual stages of the parasite; cur-

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rently interest is focused on the detection of histidine-rich protein 2 (HRP-2) from *Plasmodium falciparum* and parasite-specific lactate dehydrogenase (pLDH) or *Plasmodium* aldolase from the parasite glycolytic pathway found in all species. Clinical studies provide effective comparisons between different test formats, as well as clarification on the feasibility and clinical relevance of using nonmicroscopic methods.

ROUTINE LABORATORY DIAGNOSIS

Stained Blood Films

The accepted laboratory practice for the diagnosis of malaria is the preparation and microscopic examination of blood films stained with Giemsa, Wright's, or Field's stain (69). Blood obtained by pricking a finger or earlobe is the ideal sample because the density of developed trophozoites or schizonts is greater in blood from this capillary-rich area (17). Blood obtained by venipuncture collected in heparin or Sequestrene (EDTA) anticoagulant-coated tubes is acceptable if used shortly after being drawn to prevent alteration in the morphology of white blood cells (WBC) and malaria parasites. Both thick and thin blood films should be prepared.

Thick blood film. The thick blood film concentrates the layers of red blood cells (RBC) on a small surface by a factor of 20 to 30 and is stained as an unfixed preparation using Field's stain or diluted Wright's or Giemsa stain. The thick blood film provides enhanced sensitivity of the blood film technique and is much better than the thin film for detection of low levels of parasitemia and reappearance of circulating parasites during infection recrudescence or relapse. The lysis of the RBC during the staining process can make the process of scanning for parasites more difficult until experience is gained in finding the parasites among the WBC and platelets.

Thin blood film. The thin blood film is methanol fixed and stained with diluted Giemsa or Wright's stain using buffered water at pH 7.2 to emphasize the parasite inclusions in the RBC. Because of the fixed monolayer of RBC available in this procedure, the morphological identification of the parasite to the species level is much easier and provides greater specificity than the thick-film examination. The thin blood film is often preferred for routine estimation of the parasitemia because the organisms are easier to see and count. The ability to count parasites in sequential blood films enables the response to therapy to be monitored, particularly for *P. falciparum* infections.

Sensitivity of thick blood film. The expected sensitivity that can be achieved by an experienced microscopist for the examination of the thick blood film procedure is about 50 parasites/ μl of blood (assuming a total RBC count of $5 \times 10^6/\mu\text{l}$ of blood), which is equivalent to 0.001% of RBC infected. Milne et al. (48) found that most routine diagnostic laboratories generally achieved a lower sensitivity of detection (average, 0.01% RBC infected, 500 parasites/ μl) in an examination of results from British laboratories submitted to the Malaria Reference Laboratory.

The blood film technique has undergone very little improvement since its development in the early 1900s. Although more stable stains are now available, the staining process may take up to 60 min of preparation time to produce a stained thin or thick film and is labor-intensive.

Interpretation requires considerable expertise, particularly at low levels of parasitemia (69). In addition, patients with *P. falciparum* malaria may have parasites sequestered in the deep capillaries (spleen, liver, bone marrow), and organisms may not be seen in equivalent numbers in circulating peripheral blood drawn by venipuncture or fingerstick. Thus, a *P. falciparum* infection may easily be missed because there are insufficient numbers of parasites for detection in the blood films. Posttreatment examination of blood films can result in the observation of a number of circulating parasites; however, the organisms may be dead and have not yet been cleared by the host, thus prolonging the assumed positivity of the patient and extending treatment (65).

Estimation of Parasitemia by Using Blood Films

One of the most important aspects of reporting malaria infections from laboratory diagnostic methods is the information gained from estimating the level of parasitemia present in a blood film. A morphological assessment of the parasites is also critical to accurate interpretation, particularly noting development stages and the presence of hemozoin pigment-containing asexual parasites when reporting *P. falciparum* infections. This information may indicate the possibility of a more severe clinical situation, particularly cerebral involvement, owing to the release into the peripheral blood circulation of developing schizont stages of the parasites sequestered in the capillaries. Under these circumstances, modification of the method of drug administration from the oral route to the intravenous route may need to be considered.

Several methods for estimation of parasitemia have been described (*WHO Basic Malaria Microscopy Training Manual*, WHO, Geneva, Switzerland) using either the thin or thick blood film. It is important that every positive blood film be assessed and the parasitemia reported in exactly the same way on posttreatment specimens as on the initial patient specimen.

Examination of thick blood films. A recognized way of estimating the number of parasites present in $1 \mu\text{l}$ of blood is to use a standard value for the WBC count (8,000 WBC/ μl). Counting the number of parasites present until 200 WBC have been seen and then multiplying the parasites counted by 40 will give the parasite count per microliter of blood. If an accurate WBC count is known, this can be used to give a more accurate figure with appropriate adjustment of the multiplication factor.

The most accurate counts are obtained by counting the total parasites seen in a measured volume of blood. The blood volume should not be less than $2 \mu\text{l}$ and is spread over an area of 1 cm^2 . However, this method is time-consuming, and a count of parasites against 200 WBC in a thick film, if an accurate total white count is known, gives satisfactory accuracy for most clinical purposes.

The major disadvantage of the thick blood film is the lysis of the RBCs during the staining process, making the slide more difficult to read with the absence of RBC features and irregularities in the thickness of the film. Using the thick film to estimate the intensity of the parasite infection, the examiner should, in theory, only count parasites in thick-film fields containing no more than 20 WBC/100 \times oil immersion field. In reality, fields containing 20 WBC are often too thick to count whereas the fields that are easiest to read are those that con-

tain only 5 or 6 WBC. It has been suggested that prior to staining, the microscopist should be able to read print through a well-prepared thick blood film. Transfer of parasites from positive slides to a negative one is a distinct possibility, and care should be taken when bulk staining thick blood film slides. The use of 50× or 60× oil immersion objectives may be insufficient to identify some smaller ring forms seen on the thick film, and 10× eyepieces for the microscope are essential in these circumstances.

Warhurst and Williams (69) reported that examination of thin blood films is only 1/10 as sensitive as examination of thick blood films for the quantification of malarial parasites, although morphological identification of the *Plasmodium* species present is much easier using thin films. Therefore, most laboratories involved in the quantification and identification of malarial parasites by microscopy produce both thick and thin blood films. It is highly recommended that both thick and thin films be prepared and examined each time blood film examination for parasites is requested.

Examination of thin blood films. The parasitemia may be estimated by examination of a well-stained thin blood film. This is usually accomplished by noting the number of parasitized RBC (not individual parasites) seen in 10,000 RBC (equal to approximately 40 monolayer cell fields of a standard microscope using the 100× oil immersion objective; however, microscopists are advised to calculate the average number of cells per microscope field of view for their own microscopes) and expressing the number of parasitized cells seen as a percentage. The approximate numbers of parasites present in 1 μ l of blood can be calculated by assuming that 1 μ l of blood contains 5×10^6 RBC; therefore, a 1% parasitemia will contain 1 parasite/100 RBC or 50,000 parasites/ μ l of blood. Similarly, a 0.1% parasitemia will contain 5,000 parasites/ μ l of blood. This may be corrected to exact counts if the total RBC count per microliter is known.

This method of expression of the parasitemia as a percentage infection of RBC is the method of choice for routine practice in areas where infection is not endemic, where parasitemias are usually low. Counting parasites in a limited area of the thick film (10 fields) is acceptable when larger numbers of parasites are encountered in areas of endemic infection.

Microscopic examination of blood films is accepted as the current universal "gold standard" for the diagnosis of malaria; however, there is no accepted single standard method in current use by all investigators for the quantification of parasites. Hanscheid (19) discussed the problems that can arise when different methods for the diagnosis of malaria are compared and no consistent format is used for the estimation of parasitemia against which the comparison of sensitivity for detection of parasites can be made. Nevertheless, the blood film is still the only widely available result against which the newer methods for diagnosis of malaria can be compared, although a consistent method for enumeration of parasites from thick or thin blood films should also be used for comparability.

DIAGNOSIS USING FLUORESCENCE MICROSCOPY

In an attempt to enhance the detection of malaria parasites in blood films, alternative methods have been introduced. Certain fluorescent dyes have an affinity for the nucleic acid in the

parasite nucleus and will attach to the nuclei. When excited by UV light at an appropriate wavelength, the nucleus will fluoresce strongly. Two fluorochromes have frequently been used for this purpose, acridine orange (AO) and benzothioicarbonyl-purine (BCP), which are both excited at 490 nm and exhibit apple green or yellow fluorescence. Rhodamine-123 is also useful for assessing the viable state of parasites, since its uptake relies on an intact, working parasitic membrane.

Several methods have been published in which AO is used either as a direct-staining technique or combined with a concentration method such as a thick blood film (10, 15). The centrifugal quantitative buffy coat or QBC II (QBC) (Becton Dickinson, Franklin Lakes, N.J.) combines an AO-coated capillary tube and an internal float to separate layers of WBC and platelets using centrifugation. Parasites concentrate below this layer of cells, appearing in the upper layer of RBC but also sometimes appearing within the layers of platelets and WBC. Parasites can be viewed through the capillary tube using a special long-focal-length objective (paralens) with a fluorescence microscope (Fig. 1) (1, 6, 9, 16). The simpler Kawamoto technique employs an excitation filter mounted in the pathway of the transmitted light beam and allows the excitation wavelength of AO (470 to 490 nm) to pass to the stained film. A second filter (510 nm) is placed in the ocular for viewing the fluorescing parasites stained with AO. This method can make use of strong sunlight (if a shield to surround the observer's eyes is used) or a quartz halogen source as the exciting wavelength source (20, 21, 32, 33).

Clinical trials using AO techniques (1, 4, 5, 6, 9, 15, 37, 39, 74) have been performed under laboratory and field conditions. The QBC fluorescence method is the more technically demanding and requires specialized equipment to separate the cell layers by centrifugation and a good fluorescence microscope with a high-intensity mercury vapor or quartz halogen lamp to provide the excitation wavelength.

Although AO is a very intense fluorescent stain, it is non-specific and stains nucleic acids from all cell types. Consequently, the microscopist using AO must learn to distinguish fluorescence-stained parasites from other cells and cellular debris containing nucleic acids (10). Particular care is needed when there are Howell-Jolly bodies in the field in blood from patients with hemolytic anemia. The sensitivity of AO staining for detection of malaria parasites in infections with parasite levels of <100 parasites/ μ l (0.002% parasitemia) has been reported to range from 41 to 93% (73). The specificity for infections with *P. falciparum* is excellent (>93%) (16), with most observers recognizing the small ring forms. Ring forms or early trophozoites of other species may result in misdiagnosis, particularly in the early phase of the asexual cycle, when only ring forms may be present. For *P. vivax* and other non-*P. falciparum* infections, particularly during later stages of development, the specificity when using AO staining has been found to be lower (52%), particularly for the QBC centrifugal method, where the denser late-stage parasites may be hidden in the separated mononuclear cell layer.

An alternative fluorochrome procedure uses a solution of BCP (7, 8, 40). BCP can be applied directly to a lysed blood suspension or to an unfixed but dry thick blood film and stains

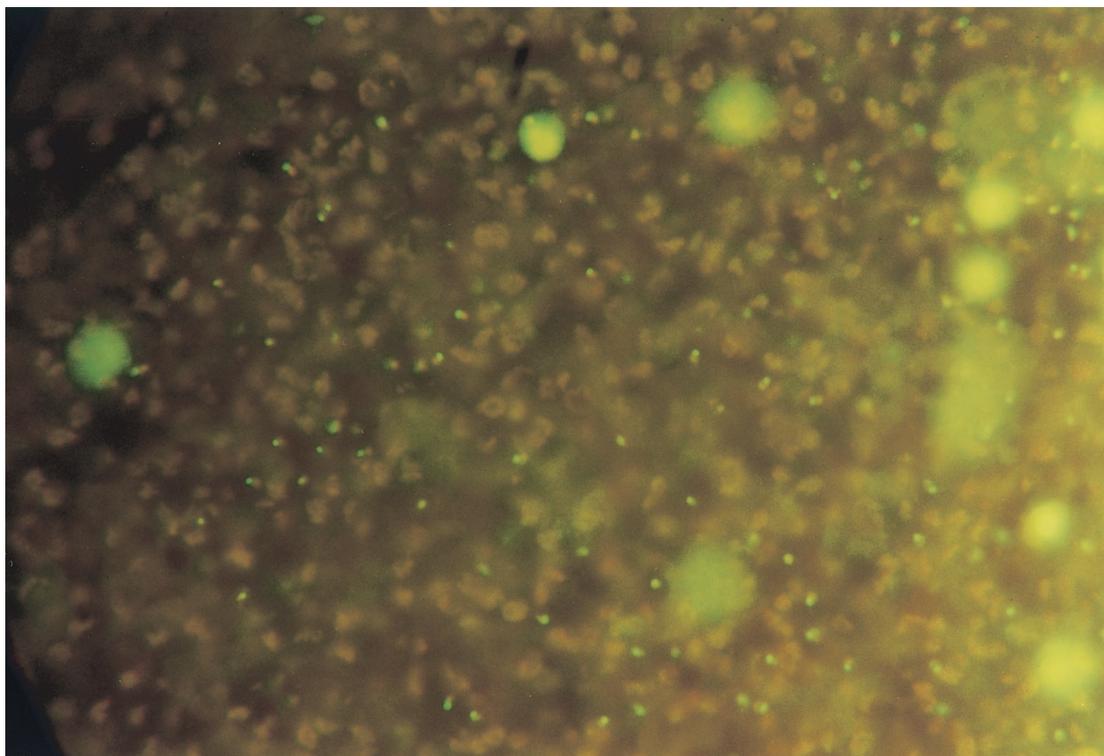


FIG. 1. Trophozoites of *P. falciparum* stained with AO in the QBC UV fluorescence method.

the nucleic acid of viable *P. falciparum* parasites intensely. In contrast, RBC inclusions and the nuclei of leukocytes are poorly stained (Fig. 2). This method overcomes some of the problems inherent in some fluorescence systems, such as the need for rapid examination to prevent fading or precipitation of the dye. Several reports on the use of this compound have found it to be a sensitive and rapid diagnostic method, comparable to Giemsa staining, with a reported sensitivity and specificity of >95% for *P. falciparum*. The non-*P. falciparum* parasites are more easily distinguished in the thick-film preparation than in the buffy coat of the QBC.

With experience, workers using methods involving fluorochrome compounds are able to detect parasites rapidly and accurately. However, an important limitation of methods involving both AO and BCP is their inability to easily differentiate among *Plasmodium* spp. With experience, the various stages of malaria seen in the peripheral blood may be located in defined areas of the centrifuged buffy coat (QBC), but specific identification of species remains difficult without seeing the RBC morphology and parasite inclusions. In spite of some reservations about the use of fluorescence methods for malaria detection (10), including the requirements for special training and expensive equipment and supplies, fluorescence microscopy remains a viable and rapid alternative to Romanowsky staining. There will still be a problem in certain areas of the world where fluorescence microscopes or adequate training in their use is not available. In these areas, alternative nonmicroscopic tests for the diagnosis of malaria will be of potential benefit (41).

DETECTION OF SPECIFIC NUCLEIC-ACID SEQUENCES

PCR cannot strictly be considered a rapid technique for the initial diagnosis of malaria. Its value lies in its sensitivity, with the ability to detect five parasites or less/ μ l of blood (64). 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 (2, 27, 34, 61, 62, 64, 70, 71). These assays have been used for the initial diagnosis, following the response to treatment, and as sensitive standards against which other nonmolecular 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 (2, 34, 54, 63, 64, 69, 70, 71). The large-subunit RNA gene is extensively conserved among *Plasmodium* spp. and is also suitable as a genus-specific DNA target region (29), with the amplified target sequence being detected by internal probes or analyzed by gel electrophoresis.

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*. Sethabutr et al. (62), using two amplification systems, K1-14 (DNA) and CS, used PCR to monitor the response to therapy in 33 patients with *P. falciparum* infections. In 103 specimens

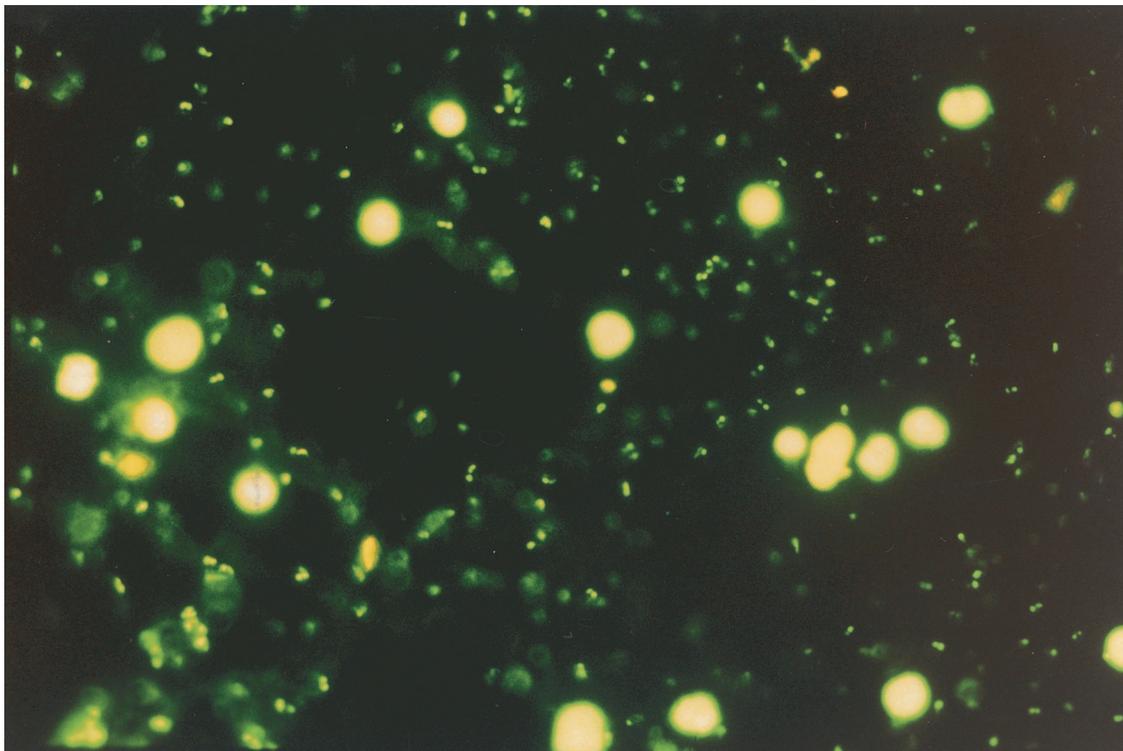


FIG. 2. Trophozoites of *P. falciparum* stained with BCP in the fluorescence method.

examined overall, the K1-14 and CS systems identified 95 and 93% of microscopically positive cases, respectively. On day 1 following treatment, PCR detection had fallen to 50% (K1-14) and 20% (CS) of the cases, and on day 4 following treatment, only 3 and 0% were detected by PCR. The use of two independent PCR systems demonstrated that *P. falciparum* DNA was present transiently in the blood of infected patients at a time when parasites could no longer be detected microscopically.

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 equal specificity (34, 64). 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.

Several workers have investigated DNA persistence in blood following treatment. Kain et al. (30) investigated the disappearance of parasites of *P. falciparum* during treatment and found that the PCR remained positive for a median of 144 h compared to 66 h for microscopy. The authors also reported that if PCR yielded positive results for 5 to 8 days after treatment, therapeutic failure, possibly due to drug resistance, might be predicted. However, Srinivasan et al. (65) noted that PCR detected DNA from circulating nonviable parasites after successful therapy, which could lead to clinical confusion.

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 (69). PCR-based methods are particularly useful for studies on strain variation, mutations, and studies of parasite genes involved in drug resistance. The ability to detect all species of *Plasmodium* in nested or multiplex assays and the enhanced sensitivity make them ideal procedures for the diagnosis of malaria. Progress in rapid DNA extraction methods and in thermocycler development, possibly using the newly developed light cyclers, may enable the amplification of malaria parasite DNA to be performed within a time frame that is clinically relevant for acute diagnosis in both field and laboratory settings. This is not the current situation, and microscopy remains the mainstay for diagnosis for the majority of clinical diagnostic centers.

NONMICROSCOPIC RAPID DIAGNOSTIC TESTS

A recent congress of the WHO produced a document entitled *New Perspectives in Malaria Diagnosis* (WHO/MAL/2000.1091) (74). Certain recommendations were presented on what non-microscopic rapid diagnostic tests (RDT) should provide. The document concluded that results from these test devices should be at least as accurate as results derived from microscopy performed by an average technician under routine field conditions. Other criteria included the sensitivity, which should be above 95% compared to microscopy, and the detection of parasitemia, such that levels of 100 parasites/ μl (0.002% par-

asitemia) should be detected reliably with a sensitivity of 100%. Quantitative or semiquantitative information on parasite densities in circulating blood was considered essential. Other essential criteria suggested were the ability to distinguish viable parasites from parasite products such as antigens or nucleic acids not associated with viable organisms and also to indicate the prediction of treatment outcomes or resistance to common antimalarial drugs.

These objectives are demanding for scientists and manufacturers alike, and the extent to which they have been or can be achieved, along with economic considerations, will provide the scientists involved in the development of these RDT for malaria with a number of exciting challenges.

Although alternative methods to stained blood films for the diagnosis of malaria have been around for a considerable time (65), the early methods based on detection of fluorescing malaria parasites or using fluorescein-labeled antibodies to *P. falciparum* were limited by the need for complex apparatus, a lack of sensitivity, and an inability to distinguish active from prior infections. The new immunochromatographic antigen capture tests are capable of detecting >100 parasites/ μ l (0.002% parasitemia) and of giving rapid results (15 to 20 min). They are commercially available in kit form with all the necessary reagents, and the ease of performance of the procedures does not require extensive training or equipment to perform or to interpret the results (53, 63).

IMMUNOCHROMATOGRAPHIC TESTS

Principle

Immunochromatography relies on the migration of liquid across the surface of a nitrocellulose membrane. Immunochromatographic tests are based on the capture of parasite antigen from peripheral blood using monoclonal antibodies prepared against a malaria antigen target and conjugated to either a liposome containing selenium dye or gold particles in a mobile phase. A second or third capture monoclonal antibody applied to a strip of nitrocellulose acts as the immobile phase. The migration of the antigen-antibody complex in the mobile phase along the strip enables the labeled antigen to be captured by the monoclonal antibody of the immobile phase, thus producing a visible colored line. Incorporation of a labeled goat antibody capture ensures that the system is controlled for migration (56). Migration depends on several physical characteristics of the component reagents, primarily the porosity of the membrane controlling the flow rate and the components of the buffer solution used to transport the labeled antigen-antibody complex in the lysed blood sample (Fig. 3).

Malaria Antigens Suitable as Targets for Rapid Diagnostic Tests

Malaria antigens currently targeted by RDT are HRP-2, pLDH, and *Plasmodium* aldolase.

HRP. Rock et al. (59) described how *P. falciparum*-infected RBC (IRBC) synthesize three histidine-rich proteins, HRP-1 (the knob-associated HRP), HRP-2, and HRP-3. HRP-1 (M_r 80,000 to 115,000) was identified in all knob-positive *P. falciparum* parasites but small amounts only were present in Gambian isolates and in several culture-adapted strains. HRP-2 (M_r

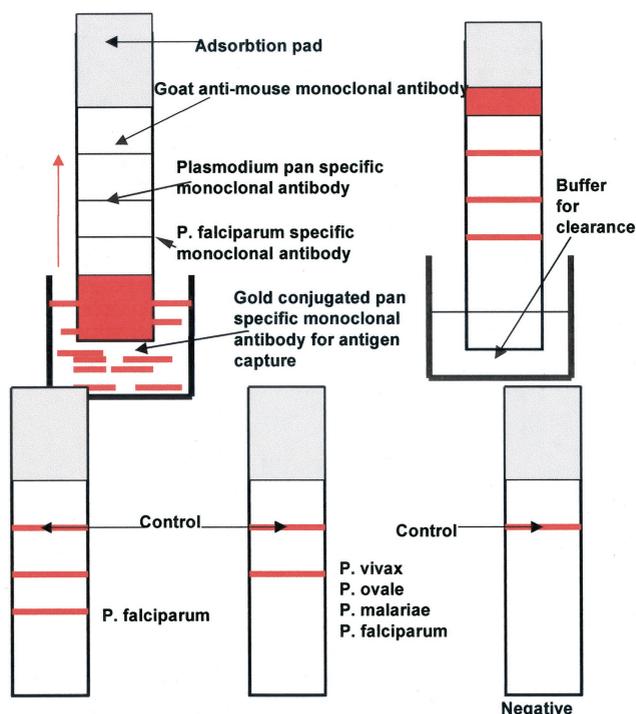


FIG. 3. Principle of immunochromatographic RDT for malaria.

60,000 to 105,000) was identified in all *P. falciparum* parasites regardless of the knob phenotype and was recovered from culture supernatants as a secreted water-soluble protein. HRP-2 was shown to be a surface-exposed protein complex of several close bands. HRP-3 (M_r 40,000 to 55,000) was present at the lowest abundance compared to HRP-1 and HRP-2. Neither HRP-1 nor HRP-2 was found in a range of other knob-positive and -negative strains of non-*P. falciparum* malaria.

HRP-2 is a water-soluble protein produced by asexual stages and young gametocytes of *P. falciparum* (59). It is expressed on the RBC membrane surface, and because of its abundance in *P. falciparum*, it was the first antigen to be used to develop an RDT for its detection.

pLDH and aldolase. pLDH, an enzyme found in the glycolytic pathway of the malaria parasite, is produced by sexual and asexual stages of the parasite (44). Different isomers of pLDH for each of the four *Plasmodium* spp. infecting humans exist, and their detection constitutes a second approach to RDT development. Several other enzymes of the malaria parasite glycolytic pathway, notably aldolase (45), have been suggested as target antigens for RDT for species other than *P. falciparum*.

IMMUNOCHROMATOGRAPHIC DIPSTICK ASSAYS USED FOR DIAGNOSIS

RDT dipstick format kits for the detection of malaria antigens are now commercially available. Many field and laboratory studies have been completed that compare immunochromatographic methods with results obtained by conventional microscopy, fluorescence microscopy, and PCR (5a, 9, 14a, 16, 21, 22, 23, 23a, 54, 65, 71a, 72).

Assays for Detection of HRP-2

Extensive trials on three commercial dipstick formats for HRP-2 detection (ParaSight F [Becton Dickinson, Franklin Lakes, N.J.], ICT *Pf* or *Pf/Pv* [Amrad ICT, Sydney, Australia], and PATH Falciparum Malaria IC test [PATH, Seattle, Wash.]) have been performed, although there are now several other manufacturers of tests for HRP-2 for which published data are not available.

ParaSight F. Laboratory trials of the ParaSight F immunochromatographic test for HRP-2 for the detection of *P. falciparum* in blood samples have shown an overall average sensitivity of 77 to 98% when >100 parasites/ μ l are present (0.002% parasitemia), with a specificity of 83 to 98% for *P. falciparum* compared with thick blood film microscopy (3, 5a, 11, 35, 36, 57, 64). The lower sensitivity range obtained from different studies probably reflects the inability of the observer to detect parasites at densities as low as 200 parasites/ μ l by microscopy (many laboratories examine only 10 fields of a thick film as a routine) or the failure to read faint positive lines from the test strip. The low level or absence of HRP-2 secretion by sexual forms may explain the negative results in some cases (5a).

Studies by Humar et al. (22) and Pieroni et al. (54) investigated the sensitivity and specificity of the ParaSight F antigen capture test for *P. falciparum* compared with PCR. They demonstrated a sensitivity and specificity for the ParaSight F test of 88 and 97%, respectively. The reduced sensitivity was indicative of the greater ability of PCR to detect low levels of parasitemia.

ICT *Pf* and PATH Falciparum Malaria IC. Experience with the AMRAD ICT *Pf* and PATH Falciparum Malaria test strip is similar to that found with the ParaSight F test (16). Mills et al. (47) compared results of tests of blood from 148 overseas travelers by using the PATH Falciparum Malaria IC Strip impregnated with Immunoglobulin M (IgM) monoclonal anti HRP-2, with results obtained using microscopy and PCR. They obtained a sensitivity and specificity of 96 and 99% with HRP-2 detection for *P. falciparum*, with discrepant results having <100 parasites/ μ l (0.002% parasitemia).

Wongsrichanalai et al. (73) assessed the ICT *Pf* test for detection of asexual *P. falciparum* parasitemia in 551 subjects in three groups in Thailand: (i) symptomatic patients self-referring for diagnosis, (ii) villagers in a screening survey, and (iii) patients recently treated for *P. falciparum* malaria. Expert light microscopy was the reference standard. They found that the ICT test performance was similar for diagnostic and screening modes. Four findings emerged: (i) test sensitivity correlated directly with parasite density, (ii) test band intensity correlated directly with parasite density, (iii) persistent test positivity after parasite clearance precluded its use for monitoring early therapeutic responses, and (iv) a false-negative test at 18,000 parasites/ μ l was unexplained. They concluded that a strong positive ICT test was highly predictive of *P. falciparum* asexual parasitemia for the diagnosis of new cases of *P. falciparum* malaria in Thailand but that a negative test result was inadequate to exclude parasitemia of <300 parasites/ μ l (0.006% parasitemia) and, in some instances, an even higher parasitemia.

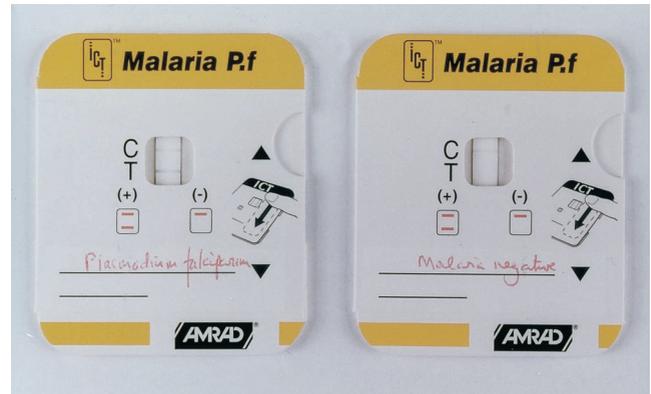


FIG. 4. Amrad ICT *Pf* immunochromatographic test device for detection of *P. falciparum* HRP-2.

Self-diagnosis. Kits for self-diagnosis by travelers in remote areas have been advocated. Symptomatic patients aged 16 to 60 years who presented at the Hospital for Tropical Diseases, London, United Kingdom, between September 1997 and July 1999 requesting malaria testing were invited to take part in a study of malaria self-diagnosis; a total of 153 patients were enrolled in the study. ICT *Pf* malaria cards were used. Whitty et al. reported that the patients' test kit results were compared with microscopy (thick and thin blood films) (71a). Of the patients, 115 (75%) indicated that the instructions were easy to follow and 128 (84%) said the tests were easy to read. Of the 16 failures resulting in invalid tests, 4 patients were unable to use the lancet, 3 obtained insufficient blood, 4 could not read the card, 1 forgot to add the reagent, and the others gave various reasons for failure. Compared with microscopy, the specificity and sensitivity for self-testing were 97 and 95%, respectively. Although these results are encouraging, technical problems and test sensitivity need to be addressed by additional field testing. Currently, this technology will not serve as a substitute for seeking proper medical help as rapidly as possible if malaria is suspected (71a).

The application of the ICT *Pf* and *Pf/Pv* card format (Fig. 4) has generally been found more user friendly than are tests that require several steps or use a well containing the conjugated antibody in which the strip is placed. More recently, the use of a cassette format has provided a more satisfactory device for safety and manipulation.

Limitations. Although HRP-2-based immunochromatographic tests permit rapid diagnosis of *P. falciparum* malaria, their clinical usefulness for the diagnosis of other *Plasmodium* spp. and for monitoring of the therapeutic response is limited. Since HRP-2 is expressed only by *P. falciparum*, these tests will give negative results with samples containing only *P. vivax*, *P. ovale*, or *P. malariae*; many cases of non-*falciparum* malaria may therefore be misdiagnosed as malaria negative. The increase in the number of geographical areas where *P. falciparum* is the dominant or only species present, particularly with its life-threatening potential, is of great importance and makes the development of tests for this parasite most important. However, awareness of the economic importance of other species to countries where they are endemic and other implications such as imported malaria to areas where the infection is

not endemic are important issues that require inclusion of these parasites within the diagnostic workup.

Persistence of HRP-2

HRP-2 has been shown to persist and is detectable after the clinical symptoms of malaria have disappeared and the parasites have apparently been cleared from the host (38, 46). Humar et al. (22) detected circulating HRP-2 antigen in 68% of treated patients on day 7, and in 27% it was still present on day 28. The reason for the persistence of the HRP-2 antigen is not well understood and may reflect the presence of latent, viable parasites (possibly the result of treatment failure or circulating antigen-antibody complexes) (68). The action of antimalarial therapy on the parasite may influence the persistence of HRP-2. Schiff et al. (60) noted that 10% of patients treated with Fansidar had detectable HRP-2 antigen on day 14. Karbwang et al. (31) also detected persistent HRP-2 antigen during and after artemether therapy, acknowledging that the HRP-2 signal was of no value during the first week of treatment but appeared to be a precise indicator of treatment failure under field conditions, when it was detected on day 14 posttreatment.

Low-level parasitemias seen in areas of endemic infection because of constant exposure to the malarial parasites may result in positive results with doubtful clinical significance (67). Also, 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.

Other limitations of tests for this antigen relate specifically to technical aspects of the HRP-2 test system. Several reports that the monoclonal IgG antibody used in the ParaSight F cross-reacts with serum rheumatoid factor, causing false-positive results, have been made (25, 38). The Amrad ICT *Pf* and PATH Falciparum Malaria tests use a monoclonal IgM antibody, and reports of false-positive reactions occurring with rheumatoid factor are less frequent (26).

Assays for Detection of pLDH

pLDH is a soluble glycolytic enzyme expressed at high levels in asexual stages of malaria parasites. It has been found in all four human malaria species (55, 56). pLDH activity is correlated with the level of parasitemia found in *in vitro* cultures of malaria and in the plasma of infected patients as determined by microscopy (42, 43, 49, 56).

Specific measurement of pLDH from *Plasmodium* spp. in the presence of human host LDH can be measured by using the substrate 3-acetylpyridine adenine dinucleotide (APAD), an analogue of NAD, in an immunocapture assay (IC assay) (55). Although the Michaelis-Menten constants are similar, the turnover number of the pLDH in the presence of APAD is much greater than that of the human enzyme with the same cofactor (19). This characteristic is due in part to conformational changes that occur in the enzyme, which is involved in the rate-limiting step for the oxidation-reduction (13, 18). Piper et al. (55) initially compared the use of two diagnostic assays based on the specific detection of pLDH activity. The ICpLDH assay detected the enzyme in the blood in a wet assay using APAD as substrate. The authors emphasized the importance of findings showing that pLDH activity was detected

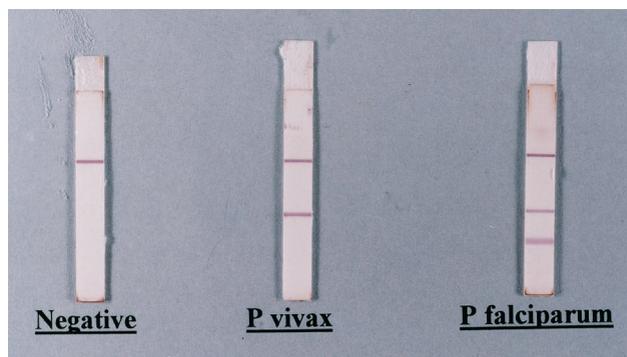


FIG. 5. OptiMAL immunochromatographic test device for the detection of pLDH.

from viable sexual and asexual parasites of *P. falciparum* and *P. vivax* infections.

OptiMAL. A panel of monoclonal antibodies that can bind to active pLDH was developed from *P. falciparum*-infected erythrocytes. Three of these monoclonal antibodies are used in an RDT immunochromatographic dipstick test (OptiMAL; Flow Inc., Portland, Oreg.). 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 nonfalciparum species, the results would indicate *P. falciparum* (Fig. 5). The monoclonal antibodies used in the OptiMAL test have been exhaustively tested for cross-reactivity with LDH from other blood protozoa such as *Leishmania*, *Babesia*, and pathogenic bacteria or fungi; no evidence of such cross-reactivity has been found (42).

Clinical trials using OptiMAL have been undertaken in many centers (14a, 23, 23a, 24, 27, 41, 49, 50, 51, 52, 56, 58). Of note, in several studies the decline in pLDH activity has also been shown to parallel the decline of viable parasites during therapy (42, 49, 50, 56). Therefore, this assay may be used to monitor patient progress during therapy and serves as an indication of recrudescence and possible drug-resistant infections, assuming that pLDH levels persist in these conditions.

Palmer et al. (51) examined the ability of OptiMAL to detect *P. vivax* and *P. falciparum* during an outbreak of malaria in Honduras. Results obtained with the OptiMAL tests were compared to those obtained with Giemsa-stained thick blood films from the same sample. Whole-blood samples were obtained from 202 patients suspected of having malaria; 96 (48%)

TABLE 1. Results of routine blood samples examined for malaria by microscopy compared to OptiMAL rapid dipstick test^a

No. of samples and species present by microscopy	No. OptiMAL positive	Sensitivity (%)
<i>Plasmodium falciparum</i>		
54 with parasitemia of >1% (50,000 parasites/μl)	54	100
103 with parasitemia of 0.1–1% (5,000 parasites/μl)	103	100
72 with parasitemia of 0.01–0.09% (500 parasites/μl)	72	100
32 with parasitemia of 0.001–0.009% (50 parasites/μl)	23	72
11 with parasitemia of 0.0001–0.0009% (5 parasites/μl)	8	73
5 with gametocytes only	4	80
<i>Plasmodium vivax</i>		
110 (all asexual stages)	105	96
<i>Plasmodium malariae</i>		
17 (late trophozoites)	8	47
Negative		
214 (no malaria parasites found)	0	100

^a Adapted from reference 49 with permission of the publisher.

of the samples were positive by microscopic examination, and 91 (45%) were positive with the OptiMAL test. Of the films positive by microscopy, 82% were found to contain *P. vivax* parasites and 18% contained *P. falciparum*. OptiMAL detected *P. vivax* and *P. falciparum* in 81 and 19% of positive cases, respectively. The overall sensitivity obtained with the OptiMAL test for *P. falciparum* and *P. vivax* in this series was 94 and 88%, respectively, with a specificity of 100 and 99%, respectively. Samples found positive by microscopy but negative by OptiMAL (3%) had <100 parasites/μl of blood (0.002% parasitemia).

In another field study of symptomatic hospital patients and asymptomatic volunteers that was conducted by Quitana et al. (58) for hospitalized patients, *P. falciparum* and *P. vivax* parasites were detected by thick-film microscopy and PCR with a mean parasite density of 590/mm³. When OptiMAL dipstick results for the same samples were compared to the results obtained by microscopy and PCR, OptiMAL had a sensitivity of 100% and a specificity of 95% for samples containing *P. falciparum*. Although the sensitivity and specificity of the dipstick in this study were similar to those of thick-film microscopy for *P. vivax*, when compared with PCR the dipstick test was unable to correctly identify mixed infections owing to the common capture obtained with the panspecific antibody band.

Further studies in India by John et al. (28) and in The Gambia by Hunt-Cooke et al. (23) with *P. falciparum* and *P. vivax*-positive samples from a cohort of malaria-exposed semi-immune individuals confirmed the sensitivity and specificity found by other workers for OptiMAL. Iqbal et al. (24) looked at pLDH detection compared to microscopy and PCR in 550 immigrants from malaria-endemic areas who were entering Kuwait, where malaria is not endemic. They concluded that for parasite levels of >100 parasites/μl (0.002% parasitemia), the sensitivity obtained for OptiMAL was 97%. However, because half of the samples with <50 parasites/μl (0.001% parasitemia)

detected by microscopy were not detected, the authors recommended that the test should be used with great caution and should not replace conventional microscopy in the diagnosis of malaria. In another study by Iqbal et al., the performance of the pLDH assay was comparable to that of microscopy to detect *P. falciparum* infection at a parasitemia of >100/μl. The pLDH assay offers an advantage over the ICT Pf assay for HRP-2 since samples infected with *P. vivax* are easily distinguished from those infected with *P. falciparum*; mixed infections with both *P. falciparum* and *P. vivax* can also be detected (23a). However, blood film examination remains the standard method for diagnosing malaria since it detects all *Plasmodium* spp. and allows visualization of parasite growth stages, which is essential for making therapeutic decisions.

Jelinek et al. (27), in a multicenter trial of 231 patients examined for malaria by using dipsticks for HRP-2 and pLDH, found that 53 patients gave positive results for *P. falciparum* infections. Based on the specificity and sensitivity obtained, they concluded that dipsticks have the potential of enhancing the specific accuracy of the diagnosis of *P. falciparum* malaria if nonspecialized laboratories are involved. Moody et al. (49), investigating the possibility of replacing microscopic examination of routine blood films for malaria parasites with OptiMAL in a study with a cohort of patients with worldwide travel history, obtained a much lower sensitivity for non-*P. falciparum* malaria other than *P. vivax* (Table 1).

In a study of the OptiMAL assay for detection and identification of malaria infections in asymptomatic residents in Indonesia, Fryauff et al. (14a) found an 88 to 92% sensitivity for detecting infections of 500 to 1,000 parasites/μl, a range covering the mean parasitemia of primary symptomatic *P. falciparum* infections in malaria-naïve Indonesian transmigrants. However, the system was markedly less sensitive than was expert microscopy for discriminating between malaria species.

Several workers have noted that during therapy the clearance of parasites from blood films and decreased pLDH levels parallel each other (49, 50, 65). Moody et al. (49) monitored this event in 60 patients undergoing treatment for *P. falciparum* at the Hospital for Tropical Diseases, London, United Kingdom and noted that the samples positive by OptiMAL paralleled the daily decline in parasitemia as seen by microscopy (Table 2).

TABLE 2. Results obtained by microscopy and OptiMAL from sequential blood samples from 60 patients receiving therapy for *P. falciparum* malaria^a

Sample collection time	No. of patients with positive microscopic parasitemia	Mean parasitemia (%)	No. (%) OptiMAL positive	Sensitivity (%) of OptiMAL at this parasitemia from initial samples
Day of diagnosis (Pretreatment)	60	2.43	58 (98)	100
Day 1 posttreatment	51	1.96	39 (76)	100
Day 2 posttreatment	44	0.525	27 (61)	100
Day 3 posttreatment	29	0.11	18 (62)	100
Day 4 posttreatment	16	0.031	11 (68)	100
Day 5 posttreatment	8	0.0022	3 (38)	72

^a Adapted from reference 49 with permission of the publisher.

Srinivasan et al. (65) monitored the course of 17 patients with *P. falciparum* who were undergoing treatment with quinine, an antimalarial drug that attacks the membrane of the parasite, using rhodamine-123 (R123) a dye previously used to show parasite viability in in vitro cultures (26, 66). R123 is a cationic permeant dye which selectively accumulates inside the malaria parasite due to its high membrane potential (inside negative), which is maintained by an electrogenic H⁺-ATPase pump located in the membrane. Thus, R123 is a vital dye that stains only viable parasites. They were able to demonstrate the continued presence of circulating but nonviable parasites and concluded that these parasites may contribute to prolonged microscopic parasitemias beyond apparent clinical cure. This advocates the possible use of tests measuring pLDH as valuable tools in monitoring antimalarial therapy (14a, 49, 50, 56) particularly in areas where microscopic examination may not be readily available.

OTHER CANDIDATE ENZYMES WITH THE POTENTIAL FOR USE AS INDICATORS OF INFECTION

Aldolase

Other enzymes within the glycolytic pathway of the malaria parasite have been recognized and considered as targets for rapid malaria diagnostic tests. The energy metabolism of the blood stages of the human malaria lacks a functional citric acid cycle, and generation of ATP depends fully on the glycolytic cycle. Aldolase is a key enzyme in this pathway. In an experiment to examine stage-specific expression of aldolase isoenzymes in the rodent malaria parasite *Plasmodium berghei*, Meier et al. (45) cloned two genes encoding the glycolytic enzyme aldolase (aldo-1 and aldo-2). aldo-1 was found to be virtually identical to *P. falciparum* aldolase, whereas aldo-2 had a 13% sequence diversity. Specific antibody probes were able to detect aldo-1 in the sporozoite stage and aldo-2 in asexual stages of malaria parasites found in the blood.

Monoclonal antibodies produced against *Plasmodium* aldolase are panspecific in their reaction and have been used in a combination test with HRP-2 to detect *P. vivax* as well as *P. falciparum* in blood. Tjitra et al. (67) evaluated the combined Pf/Pv immunochromatographic test (ICT Pf/Pv) formatted with capture stripes for both HRP-2 and aldolase. They reported that the specificity and negative predictive values for the diagnosis of *P. vivax* were 94.8 and 98.2%, respectively. The overall sensitivity of 75% and positive predictive value of 50% for *P. vivax* malaria were less than desirable in their situation. The sensitivity obtained for >500 parasites/μl (0.01% parasitemia) was 96%, but for values below this level it was only 29%.

In a study of 13 patients with malaria in Brisbane, Australia, using the ICT Pf/Pv test, Eisen and Saul (14) also noted a low sensitivity for the detection of *P. vivax* when the parasite count was below 500 parasites/μl (0.01% parasitemia). They demonstrated the decline in panspecific line intensity with *P. falciparum* or *P. vivax* infection during treatment, unlike the HRP-2 line, which remained positive for considerable periods after microscopy became negative. The authors suggest the possibility of using the panspecific line for monitoring response to therapy. However, little evidence was presented for the sensi-

tivity of the panspecific antibody of the ICT Pf/Pv for *P. falciparum* infections, since this series showed detection of >100 parasites (0.002% parasitemia) in three samples but not in five others with higher parasite counts.

DISCUSSION

The recommended method and current gold standard used for the routine laboratory diagnosis of malaria is the microscopic examination of stained thin and thick blood films, particularly with the additional sensitivity offered by examination of thick blood films. In the most capable hands, this method can be expected to detect 50 parasites/μl (0.001% parasitemia) and to identify to the species level 98% of all parasites seen. This procedure is recognized as difficult and time-consuming, requiring considerable training to obtain the necessary skills. In the past few years, efforts to replace the traditional but tedious reading of blood films have led to techniques for the detection of malarial parasites that yield sensitivities equivalent to or better than those of microscopy. Methods using fluorescence microscopy have helped improve the sensitivity but not the specificity. PCR has proven to be a sensitive method for diagnosis of all four species of human malaria parasites and can be expected to exceed the sensitivity of microscopic examination. The detection of <5 parasites/μl and identification to the species level make this an excellent technique against which to compare the sensitivity and specificity of other nonmicroscopic methods. However, PCR is an impractical standard against which to measure routine acute malaria diagnosis because of the time involved and the technical experience required. The detection of DNA from samples taken at times well removed from the infection (28) may give confusing evidence about recrudescence or drug resistance.

Immunochromatographic dipsticks offer the possibility of more rapid, nonmicroscopic methods for malaria diagnosis, thereby saving on training and time. These tests are easy to perform and require little training to interpret the results. However, there are a number of issues to which the WHO document *New Perspectives for Malaria Diagnosis* draws attention when considering the current status of development of the RDT for malaria. These are discussed below.

Sensitivity for RDT remains a problem, particularly for non-immune populations. Parasite densities above 100 parasites/μl (0.002% parasitemia) should be detected with confidence (the average parasitemia seen in patients attending the Hospital for Tropical Diseases is between 5,000 parasites/μl [0.1% parasitemia] and 50 parasites/μl [0.001% parasitemia]). While this sensitivity is a reasonable target to expect from dipsticks for *P. falciparum* diagnosis, it is at the lower end of the capability of most devices involving capture methods for HRP-2 or pLDH. Available test devices for non-*P. falciparum* malaria (ICT Pf/Pv and OptiMAL), have a sensitivity for the diagnosis of *P. vivax* of 90 to 96% (OptiMAL) and 75 to 95% (ICT Pf/Pv). Levels of parasitemia encountered for this parasite rarely exceed 1%, and a much lower figure is usually encountered.

Reports on the detection of *P. ovale* and *P. malariae* antigen (49) indicate that the panspecific monoclonal antibodies developed from *P. falciparum* in OptiMAL have a lower affinity for these antigens, with the added problem that fewer parasites are encountered than for *P. vivax*. The possibility is that several

TABLE 3. Comparison of methods for diagnosing *Plasmodium* infection in blood

Parameter	Microscopy	PCR	Fluorescence	Dipstick HRP-2	Dipstick pLDH, ICT <i>Pf/Pv</i>
Sensitivity (parasites/ μ l)	50	5	50	>100	>100
Specificity	All species	All species	<i>P. falciparum</i> good, others difficult	<i>P. falciparum</i> only	<i>P. falciparum</i> and <i>P. vivax</i> good, and <i>P. ovale</i> and <i>P. malariae</i> only with pLDH
Parasite density or parasitemia	Yes	No	No	Crude estimation	Crude estimation
Time for result	30–60 min	24 h	30–60 min	20 min	20 min
Skill level	High	High	Moderate	Low	Low
Equipment	Microscope	PCR apparatus	QBC apparatus or direct fluorescence microscope	Kit only	Kit only
Cost/test	Low	High	Moderate/low	Moderate	Moderate

isomers of these parasites exist, accounting for some of the failures of detection. It is foreseeable that a more sensitive capture monoclonal antibody for *P. ovale* may be available to improve detection (M. T. Makler, Flow Inc., personal communication). Dyer et al. (12) report on the failure of the panmalaria antibody of the ICT *Pf/Pv* immunochromatographic test to detect symptomatic *P. malariae* infection in East Timor and Papua New Guinea, even with a parasitemia of 4,080 parasites/ μ l. There are no reports of experience with this test format for *P. ovale*.

The clinical and epidemiological significance of dipsticks recognizing gametocytes of *Plasmodium* is important. In areas of nontransmission of malaria, the fact that a test will not detect all gametocytes is of less importance than in areas of high transmission. HRP-2 from sexual stages of *P. falciparum* is more readily detected than pLDH, which appears to be active in young forms but not so readily in later ones.

A negative RDT cannot at present be accepted at face value and will need to be confirmed by microscopic examination. The possibility of gene deletion isolates that do not express HRP-2 has been postulated, although the same evidence for pLDH has not yet been discovered. Further investigation for these and other nonexpressed antigens should be considered.

Several workers report the usefulness of RDT as a means of monitoring the course of a parasitemia during therapy (49, 50). Srinivasan et al. (65) investigated the significance of parasites that remain in peripheral blood after several days of therapy, as detected by microscopy. When R123, a cationic dye requiring an intact membrane found in viable parasites, is used, the dye crosses the membrane and stains the parasite. This study concluded that the microscopically observed parasites remaining at the latter end of a course of therapy are nonviable parasites, lacking pLDH enzyme activity but not yet cleared from the blood. This may influence the interpretation of various performances of different RDT methods against microscopy as the gold standard when sequential samples are taken during treatment. The persistence of HRP-2 antigenemia beyond the clearance of peripheral parasitemia in certain cases reduces the usefulness of these assays for monitoring the response to therapy.

Although several workers report quantitative or semiquantitative data from currently formatted RDT, at present it is not possible to obtain readings by eye that are linear for the parasitemia without some form of reader for the captured gold-

conjugated antibody. Quantitative measurement will enable prognostic assessment and will provide information indicating drug resistance. RDT currently on the market are easy to use; most are in cassette format with single-application areas for the blood sample and clearing buffer. Most evaluation trials have included temperature and time stability for at least 1 year at 40°C. At present, none of the commercial formats have Food and Drug Administration (FDA)-approved production in the United States, since the most urgent need during their development was for use in countries with limited resources. However, particularly with versions detecting non-*P. falciparum* malaria (ICT *Pf/Pv* and OptiMAL), the U.S. military has conducted FDA approval trials because of possible use of these devices in the field. Final production arrangements in the United States may be possible in the future. Commercial interest in producing dipsticks at a cost that many of the poorer countries could afford is a subject of ongoing debate, and even with differential pricing for various countries, the lower cost required will be difficult to achieve. The manufacturing of RDT is currently under review by the larger companies (AMRAD ICT and Becton Dickinson), and possible commercial changes can be expected in the near future.

The new generation of RDT offers a realistic practical chance to move the diagnosis of malaria away from the laboratory and nearer to the patient. A sensitivity of >100 parasites/ μ l (0.002% parasitemia) obtainable for *P. falciparum* diagnosis for both HRP-2 and enzyme-based assays is as good as most clinical laboratory staff in nonspecialized laboratories could expect to attain microscopically with limited exposure to malaria cases (47). The added assurance that life-threatening parasitemia with *P. falciparum* will not be missed is welcome, particularly for inexperienced laboratory staff during night calls. The ability to detect the majority of the non-*falciparum* malaria cases also makes these tests ideally suited as major backup procedures for malaria diagnosis.

There are many considerations to be taken into account when reviewing the methods for laboratory diagnosis of malaria (Table 3), not the least of which are the important factors of availability and cost. The present debate on the introduction of tests based on new technology is welcomed. However, it does not avoid the necessity of reviewing correctly stained thick and thin blood films as the standard operating procedure when malaria is suspected or of replacing a current training program for the identification of the *Plasmodium* species and

for detection of parasitemia below the present threshold of detection by RDT.

REFERENCES

- Baird, J. K., Purnomo, and T. R. Jones. 1992. Diagnosis of malaria in the field by fluorescence microscopy of QBC capillary tubes. *Trans. R. Soc. Trop. Med. Hyg.* **86**:3–5.
- Barker, R. H., N. T. Banchongaksorn, M. M. Courval, W. Suwonkerd, K. Rimpungtragoon, and D. R. Wirth. 1992. A simple method to detect *Plasmodium falciparum* infection in human patients: a comparison of the DNA probe method to microscopic diagnosis. *Am. J. Trop. Med. Hyg.* **41**:266–272.
- Beadle, C., G. W. Long, W. R. Weiss, P. D. Meelroy, S. M. Maret, A. J. Oloo, and S. L. Hoffman. 1994. Diagnosis of malaria by detection of *Plasmodium falciparum* HRP-2 antigen with a rapid dipstick-capture assay. *Lancet*, **i**:564–568.
- Benito, A., J. Roche, R. A. Molina, C. Amela, and J. Altar. 1994. Application and evaluation of QBC® malaria diagnosis in a holoendemic area. *Appl. Parasitol.* **35**:266–272.
- Bosch, I., C. Bracho, and H. A. Perez. 1996. Diagnosis of malaria by acridine orange fluorescence microscopy in an endemic area of Venezuela. *Mem. Inst. Oswaldo Cruz* **91**:83–86.
- Brenier-Pinchart, M. P., C. Pinel, A. Croissonnier, J. P. Brion, O. Faure, D. Ponard, and P. Ambroise-Thomas. 2000. Diagnosis of malaria in non-endemic countries by the Parasight-F test. *Am. J. Trop. Med. Hyg.* **63**:150–152.
- Clendeman, T. E., G. W. Long, and J. K. Baird. 1995. QBC® and Giemsa stained thick blood films: diagnostic performance of laboratory technologists. *Trans. R. Soc. Trop. Med. Hyg.* **86**:378.
- Cooke, A. H., A. H. Moody, K. Lemon, P. L. Chiodini, and J. Horton. 1992. Use of the fluorochrome benzothiocarboxypurine in malaria diagnosis. *Trans. R. Soc. Trop. Med. Hyg.* **87**:549.
- Cooke, A. H., S. Morris-Jones, J. Horton, B. M. Greenwood, A. H. Moody, and P. L. Chiodini. 1993. Evaluation of benzothiocarboxypurine for malaria diagnosis in an endemic area. *Trans. R. Soc. Trop. Med. Hyg.* **87**:549.
- Craig, M., and B. L. Sharp. 1997. Comparative evaluation of four techniques for the diagnosis of *Plasmodium* infections. *Trans. R. Soc. Trop. Med. Hyg.* **91**:279–282.
- Delacollett, D., and P. Van der Stuyft. 1994. Direct acridine orange staining is not a 'miracle' solution to the problems of malaria diagnosis in the field. *Trans. R. Soc. Trop. Med. Hyg.* **88**:187–188.
- Dietz, R., M. Perkins, M. Boulos, F. Luz, B. Reller, and C. R. Corey. 1995. The diagnosis of *Plasmodium* using a new antigen detection system. *Am. J. Trop. Med. Hyg.* **52**:45–59.
- Dyer, M. E., E. Tjitra, B. J. Currie, and N. M. Anstey. 2000. Failure of 'pan malarial' antibody of the ICT® Malaria Pf/Pv immunochromatographic test to detect symptomatic *Plasmodium malariae* infection. *Trans. R. Soc. Trop. Med. Hyg.* **94**:518.
- Dunn, C. R., M. J. Banfield, J. J. Barker, C. W. Hinham, K. M. Moreton, D. T. Balik, R. L. Brady, and J. J. Holbrook. 1996. The structure of lactate dehydrogenase from *Plasmodium falciparum* reveals a new target for anti-malarial design. *Nat. Struct. Biol.* **3**:912–915.
- Eisen, D. P., and A. Saul. 2000. Disappearance of pan-malarial antigen reactivity using the ICT Malaria Pf/Pv kit parallels decline of patent parasitemia as shown by microscopy. *Trans. R. Soc. Trop. Med. Hyg.* **94**:169–170.
- Fryauff, D. J., Purnomo, M. A. Sutamihardja, I. R. S. Elyazar, I. Susanti, Krisin, B. Subianto, and H. Marwoto. 2000. Performance of the Optimal assay for detection and identification of malaria infections in asymptomatic residents of Irian Jaya, Indonesia. *Am. J. Trop. Med. Hyg.* **63**:139–145.
- Gay, F., B. Traore, J. Zanon, M. Danis, and M. Gentilini. 1996. Direct acridine orange fluorescence examination of blood slides compared to current techniques for malaria diagnosis. *Trans. R. Soc. Trop. Med. Hyg.* **90**:516–518.
- Gaye, O., M. Diouf, and S. Diallo. 1999. A comparison of thick films, QBC malaria, PCR and PATH falciparum malaria test strip in *Plasmodium falciparum* diagnosis. *Parasite* **6**:273–275.
- Gilles, H. 1993. Diagnostic methods in malaria, p. 78. In H. M. Gilles and D. A. Warrell (ed.), *Essential malariology*, 3rd ed. P. Edward Arnold, London, United Kingdom.
- Gomez, M. S., R. C. Piper, L. Hunsiker, and D. Vander Jagt. 1997. Substrate and co-factor specificity and selective inhibition of lactate dehydrogenase from the malarial parasite *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **90**:235–246.
- Hanscheid, T. 1999. Diagnosis of malaria: a review of alternatives to conventional microscopy. *Clin. Lab. Haematol.* **21**:235–245.
- Hakim, S. L., T. Furuta, A. N. Rain, Y. Normaznah, M. R. Zamri, S. Kojima, and J. W. Mak. 1993. Diagnosis of malaria using a light microscope fitted with an interference filter for the diagnosis of malaria. *Trans. R. Soc. Trop. Med. Hyg.* **87**:44–48.
- Hind, M., M. Agabani, I. A. El Hag, I. A. El Toum, M. Satti, and A. M. El Hassan. 1994. Fluorescence microscopy using a light microscope fitted with an interference filter for the diagnosis of malaria. *Trans. R. Soc. Trop. Med. Hyg.* **88**:61.
- Humar, A., C. Ohrt, M. A. Harrington, D. Pillai, and K. C. Kain. 1997. ParaSight® F test compared with the polymerase chain reaction and microscopy for the diagnosis of *Plasmodium falciparum* malaria in travellers. *Am. J. Trop. Med. Hyg.* **56**:44–48.
- Hunt-Cooke, A. H., P. L. Chiodini, T. Docherty, A. H. Moody, J. Ries, and M. Pinder. 1999. Comparison of a parasite lactate dehydrogenase-based immunochromatographic antigen detection assay (OptiMAL®) with microscopy for the detection of malaria parasites in human blood samples. *Am. J. Trop. Med. Hyg.* **60**:20–23.
- Iqbal, J., P. R. Hira, A. Sher, and A. A. Al-Enezi. 2001. Diagnosis of imported malaria by *Plasmodium* lactate dehydrogenase (pLDH) and histidine-rich protein 2 (PfHRP-2)-based immunocapture assays. *Am. J. Trop. Med. Hyg.* **64**:20–23.
- Iqbal, J., A. Sher, P. R. Hira, and R. Al-Owaish. 1999. Comparison of the OptiMAL® test with PCR for diagnosis of malaria in immigrants. *J. Clin. Microbiol.* **39**:3644–3646.
- Iqbal, J., A. Sher, and A. Rab. 2000. *Plasmodium falciparum* histidine rich protein-2 based immunocapture polymerase chain reaction to predict cross reactivity with rheumatoid factors. *J. Clin. Microbiol.* **38**:1184–1186.
- Izumo, A., K. Tanabe and M. Kato. 1987. A method for monitoring the viability of malaria parasites (*Plasmodium yoelii*) freed from the host erythrocytes. *Trans. R. Soc. Trop. Med. Hyg.* **81**:264–267.
- Jelinek, T., M. P. Grobusch, S. Schwenke, S. Steidl, F. N. E. von Sonnenburg, and T. Loscher. 1999. Sensitivity and specificity of dipstick tests for rapid diagnosing in non-immune travellers. *J. Clin. Microbiol.* **37**:721–723.
- John, S. M., A. Sundarsanam, U. Sitaram, and A. H. Moody. 1998. Evaluation of OptiMAL®, a dipstick test for the diagnosis of malaria. *Ann. Trop. Med. Parasitol.* **92**:621–622.
- Kain, K. C., A. E. Brown, L. Mirabelli, and H. K. Webster. 1993. Detection of *Plasmodium vivax* by polymerase chain reaction in a field study. *J. Infect. Dis.* **168**:1323–1326.
- Kain, K. C., D. E. Kyle, C. Wongrichanalai, and A. E. Brown. 1994. Qualitative and semi-quantitative polymerase chain reaction to predict *Plasmodium falciparum* treatment failure. *J. Infect. Dis.* **170**:1626–1630.
- Karbwang, J., O. Tasanor, T. Kanda, Y. Watanagoon, M. Ibrahim, K. Nabanchang, A. Thanavibul, and W. Roonty. 1996. ParaSight® F test for the detection of treatment failure in multidrug resistant *Plasmodium falciparum* malaria. *Trans. R. Soc. Trop. Med. Hyg.* **90**:513–515.
- Kawamoto, F. 1991. Rapid diagnosis of malaria by fluorescence microscopy with light microscopy and interference filters. *Lancet* **i**:200–202.
- Kawamoto, F. 1991. Rapid diagnosis of malaria by fluorescence microscopy. *Lancet* **i**:624–625.
- Kawamoto, F., H. Miyake, O. Kaneko, M. Kimura, T. D. Nguyen, Q. Lui, M. Zhou, D. D. Le, S. Kawai, S. Isomura, and Y. Wataya. 1996. Sequence variation in the 18S rRNA gene, a target for PCR-based malaria diagnosis, in *Plasmodium ovale* from southern Vietnam. *J. Clin. Microbiol.* **34**:2287–2289.
- Kilian, A. H. D., E. B. Mughusu, G. Kabagambe, and F. Von Sonnenburg. 1997. Comparison of two rapid HRP-2 based diagnostic tests for *Plasmodium falciparum*. *Trans. R. Soc. Trop. Med. Hyg.* **91**:666–667.
- Kodisinghe, H. M., K. L. Perera, S. D. T. Premawansa, S. Naotunne, A. R. Wickramainghe, and K. M. Mendis. 1997. The ParaSight® F test as a routine diagnostic tool for malaria in Sri Lanka. *Trans. R. Soc. Trop. Med. Hyg.* **91**:398–402.
- Kong, H. H., and D. I. Chung. 1995. Comparison of acridine orange and Giemsa stains for malaria diagnosis. *Korean J. Parasitol.* **33**:391–394.
- Laferi, H., K. Kandel, and H. Pichler. 1997. False positive dipstick test for malaria. *N. Engl. J. Med.* **337**:1635–1636.
- Lowe, B. S., N. F. Jeffa, L. New, C. Pederson, K. Engback, and K. Marsh. 1996. Acridine orange fluorescence techniques as alternatives to traditional Giemsa staining for the diagnosis of malaria in developing countries. *Trans. R. Soc. Trop. Med. Hyg.* **90**:34–36.
- Makler, M. T., L. K. Ries, J. Ries, R. J. Horton, and D. J. Hinrichs. 1991. Detection of *Plasmodium falciparum* infection with the fluorescent dye Benzothiocarboxypurine. *Am. J. Trop. Med. Hyg.* **44**:11–16.
- Makler, M. T., C. J. Palmer, and A. L. Ager. 1992. A review of practical techniques for the diagnosis of malaria. *Ann. Trop. Med. Parasitol.* **92**:419–433.
- Makler, M. T., J. M. Ries, J. A. Williams, J. E. Bancroft, R. C. Piper, B. L. Gibbins, and D. J. Hinrichs. 1993. Parasite lactate dehydrogenase as an assay for *Plasmodium falciparum* drug sensitivity. *Am. J. Trop. Med. Hyg.* **48**:739–741.
- Makler, M. T., and D. J. Hinrichs. 1993. Measurement of the lactate dehydrogenase activity of *Plasmodium falciparum* as an assessment of parasitemia. *Am. J. Trop. Med. Hyg.* **48**:205–210.
- Makler, M. T., R. C. Piper, and W. Milhous. 1998. Lactate dehydrogenase and diagnosis of malaria. *Parasitol. Today* **14**:376–377.
- Meier, B., H. Doheli, and U. Certa. 1992. Stage-specific expression of aldolase isoenzymes in the rodent malaria parasite *Plasmodium berghei*. *Mol. Biochem. Parasitol.* **52**:15–27.
- Miller, R. L., S. Ikram, G. L. Armelagos, R. Walker, W. B. Harer, C. J. Chief, D. Baggett, M. Carrigan, and S. M. Manet. 1994. Diagnosis of *Plasmodium*

- falciparum* infections in mummies using the rapid manual ParaSight® F test. *Trans. R. Soc. Trop. Med. Hyg.* **88**:31–32.
47. Mills, C. D., D. C. H. Burgess, H. J. Taylor, and K. C. Kain. 1999. Evaluation of a rapid and inexpensive dipstick assay for the diagnosis of *Plasmodium falciparum* malaria. *Bull. W. H. Org.* **77**:553–559.
 48. Milne, L. M., P. L. Chiodini, and D. C. Warhurst. 1994. Accuracy of routine laboratory diagnosis of malaria in the United Kingdom. *J. Clin. Pathol.* **47**:740–742.
 49. Moody, A., A. Hunt-Cooke, E. Gabbett, and P. Chiodini. 2000. Performance of the OptiMAL® malaria antigen capture dipstick for malaria diagnosis and treatment monitoring at the Hospital for Tropical Diseases, London. *Br. J. Haematol.* **109**:891–894.
 50. Oduola, A. M., G. O. Omitowoju, A. Sowunmi, M. T. Makler, C. O. Falade, D. E. Kyle, F. A. Fehintola, O. A. Ogundahunsi, R. C. Piper, B. G. Schuster, and W. K. Milhous. 1997. *Plasmodium falciparum*: evaluation of lactate dehydrogenase in monitoring therapeutic response to standard anti-malarial drugs in Nigeria. *Exp. Parasitol.* **87**:283–289.
 51. Palmer, C. J., J. F. Lindo, W. I. Klaskala, J. A. Quesada, R. Kaminsky, M. K. Baum, and A. L. Ager. 1998. Evaluation of the OptiMAL test for rapid diagnosis *Plasmodium vivax* and *Plasmodium falciparum* malaria. *J. Clin. Microbiol.* **36**:203–206.
 52. Palmer, C. J., L. Validum, J. Lindo, A. Campa, C. Validum, M. Makler, R. R. Cuadrado, and A. Ager. 1999. Field evaluation of the OptiMAL® rapid malaria test during anti malarial therapy in Guyana. *Trans. R. Soc. Trop. Med. Hyg.* **93**:517–518.
 53. Peyron, F. G., J. P. Martet, and A. Vigler. 1994. Dipstick antigen-capture assay for malaria detection. *Lancet* **i**:1502–1503.
 54. Pieroni, P., C. D. Mills, C. Ohrt, M. A. Harrington, and K. C. Kain. 1998. Comparison of the ParaSight® F and the ICT® malaria Pf test with the polymerase chain reaction for the diagnosis of *Plasmodium falciparum* malaria in travellers. *Trans. R. Soc. Trop. Med. Hyg.* **92**:166–169.
 55. Piper, R., J. Lebras, L. Wentworth, A. Hunt Cooke, S. Houze, P. Chiodini, and M. Makler. 1999. A capture diagnostic assay for malaria using *Plasmodium* lactate dehydrogenase (pLDH). *Am. J. Trop. Med. Hyg.* **60**:109–118.
 56. Piper, R. C., D. L. Vanderjagt, J. J. Holbrook, and M. Makler. 1996. Malaria lactate dehydrogenase: target for diagnosis and drug development. *Ann. Trop. Med. Parasitol.* **90**:433.
 57. Premji, Z., J. N. Minjas, and C. J. Schiff. 1994. Laboratory diagnosis of malaria by village health workers using the rapid ParaSight® F test. *Trans. R. Soc. Trop. Med. Hyg.* **88**:418.
 58. Quitana, M., R. Piper, H. Boling, M. Makler, G. E. Sherman, E. Fernandez, and S. Martin. 1998. Malaria diagnosis by dipstick assay in a Honduran population with coendemic *Plasmodium falciparum* and *vivax*. *Am. J. Trop. Med. Hyg.* **59**:868–871.
 59. Rock, E. P., K. Marsh, S. J. Saul, T. E. Wellems, D. W. Taylor, W. L. Maloy, and R. J. Howard. 1987. Comparative analysis of the *Plasmodium falciparum* histidine-rich proteins HRP1, HRP2 and HRP3 in malaria diagnosis of diverse origin. *Parasitology* **95**:209–227.
 60. Schiff, C. J., Z. Premji, and J. N. Minjas. 1993. The rapid manual ParaSight® F test. A new diagnostic tool for *Plasmodium falciparum* infection. *Trans. R. Soc. Trop. Med. Hyg.* **87**:646–648.
 61. Seesod, N., J. Lundeberg, A. Hedrum, L. Asland, A. Holder, S. Thaitong, and M. Uhlen. 1993. Immunomagnetic purification to facilitate DNA diagnosis of *Plasmodium falciparum*. *J. Clin. Microbiol.* **31**:2715–2719.
 62. Sethabutr, O., A. E. Brown, S. Panyim, K. C. Kain, K. Webster, and P. Echeverria. 1992. Detection of *Plasmodium falciparum* by polymerase chain reaction in a field study. *J. Infect. Dis.* **166**:145–148.
 63. Singh, N., M. Valencha, and V. P. Sharma. 1997. Malaria diagnosis by field workers using a immunochromatographic test. *Trans. R. Soc. Trop. Med. Hyg.* **91**:396–397.
 64. Snounou, G., S. Viriyakosol, W. Jarra, S. Thaitong, and K. N. Brown. 1993. Identification of the four human malarial species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. *Mol. Biochem. Parasitol.* **58**:283–292.
 65. Srinivasan, S., A. H. Moody, and P. L. Chiodini. 2000. Comparison of blood-film microscopy, the OptiMAL® dipstick, Rhodamine 123 and PCR for monitoring anti-malarial treatment. *Ann. Trop. Med. Parasitol.* **94**:227–232.
 66. Tanabe, K. 1983. Staining of *Plasmodium yoelii*-infected mouse erythrocytes with the fluorescent dye rhodamine 123. *J. Protozool.* **30**(4):707–710.
 67. Tjitra, E., S. Suprianto, M. Dyer, B. J. Currie, and N. M. Anstey. 1999. Field evaluation of the ICT malaria Pf/Pv immunochromatographic test for detection of *Plasmodium falciparum* and *Plasmodium vivax* in patients with presumptive clinical diagnosis of malaria in eastern Indonesia. *J. Clin. Microbiol.* **37**:2412–2417.
 68. Verle, P., L. N. Bink, T. T. Lieu, P. T. Yen, and M. Coosemans. 1996. ParaSight® F test to diagnose malaria in hypoendemic areas and epidemic prone regions of Vietnam. *Trop. Med. Int. Health* **1**:794–796.
 69. Warhurst, D. C., and J. E. Williams. 1996. Laboratory diagnosis of malaria. *J. Clin. Pathol.* **49**:533–538.
 70. Wataya, Y., M. Arai, F. Kubochi, C. Mizuloshi, T. Kakutani, N. Ohta, and A. Ishii. 1993. DNA diagnosis of *falciparum* malaria using a double PCR technique: a field trial in the Solomon Island. *Mol. Biochem. Parasitol.* **58**:65–168.
 71. Wataya, Y., F. Kubochi, C. Mizukoshi, Y. Ohya, K. Watanabe, M. Arai, A. Ishii, and A. Nakagam. 1991. DNA diagnosis of *falciparum* malaria. *Nucleic Acids Symp. Ser.* **25**:155–156.
 - 71a. Whitty, C. J. M., M. Armstrong, and R. H. Behrens. 2000. Self-testing for *falciparum* malaria with antigen-capture cards by travellers with symptoms of malaria. *Am. J. Trop. Med. Hyg.* **63**:295–297.
 72. Wongsrichanalai, C., N. Chuanak, S. Tulyayon, N. Thanosingha, A. La-boonchal, T. G. Thimasarn, and D. G. Heppner. 1999. Comparison of a rapid field immunochromatographic test to expert microscopy for the detection of *Plasmodium falciparum* asexual parasitemia in Thailand. *Acta Trop.* **73**:263–273.
 73. Wongsrichanalai, C., J. Pornsilapatip, V. Namsiriponpun, H. K. Webster, A. Luccini, P. Pansamdang, H. Wilde, and M. Prasittisuk. 1991. Acridine orange fluorescent microscopy and the detection of malaria in populations with low-density parasitemia. *Am. J. Trop. Med. Hyg.* **44**:17–20.
 74. World Health Organization. 2000. WHO/MAL/2000.1091. New perspectives in malaria diagnosis. World Health Organization, Geneva, Switzerland.