

**RECENT ADVANCES IN THE LABORATORY DIAGNOSIS OF MALARIA**<sup>1</sup>Onile, B. A., <sup>2</sup>Taiwo, S. S.

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Malaria is a global health problem with about 2-4 billion people at risk, about 200-300 million cases occurring annually resulting in about 1 million death, 90% of which occur in the sub-Saharan Africa. Prompt and accurate diagnosis is the key to effective disease management, one of the main interventions of the Global Malaria Control Strategy. The "gold standard" test for the diagnosis of malaria, blood film microscopy, has in recent times come under some criticism. Apart from being cumbersome and time consuming, the reliability of the test depends on the competence of the microscopist and the test is not sensitive when parasitaemia is less than 100 parasites/ $\mu$ L of blood, a situation usually seen in non-immune subjects. Several new innovative malaria diagnostic tests called Rapid Diagnostic Tests (RDT) have been developed to circumvent these limitations. The application of these new techniques in clinical laboratories is limited by cost, variable sensitivities, spill-over antigenaemia and false positive reactions in some cases. Although the polymerase chain reaction (PCR) assays for malaria diagnosis is extremely sensitive and specific, and has been suggested to replace blood film microscopy as the gold standard, the long time, high cost and technical expertise required is limiting the usefulness of these techniques, especially in Africa. This communication provides information on the available malaria diagnostics and the recent advances in the laboratory diagnosis of malaria.

**Key words:** Recent advances, malaria diagnostics**INTRODUCTION**

Malaria is a global health problem with about 2-4 billion people at risk. Worldwide, about 200-300 million cases occur annually with about 1 million deaths, 90% of which occur in the sub-Saharan Africa (1). Prompt and accurate diagnosis is the key to effective disease management, one of the main interventions of the Global Malaria Control Strategy (2):

Unfortunately, poor diagnosis has continued to hinder effective malaria control especially in the developing countries where high prevalence of asymptomatic infection and non specific clinical presentation occurs, and where resources are lacking and access to health care providers and health facilities are insufficient, thereby encouraging widespread practice of self-treatment for clinically suspected malaria.

The "gold standard" for the laboratory diagnosis of malaria is the microscopic examination of stained blood film for malaria parasites (2, 3). Although this is a sensitive and specific method of diagnosis when correctly performed, it can be unreliable and wasteful when poorly executed. It is also labour intensive and time-consuming taking at least 60 minutes for result to be available, a situation that may make clinicians take decisions on treatment without the benefits of the result.

These limitations have led to the development of alternative methods of diagnosis such as the nucleic acid detection and the Rapid Diagnostic Tests (RDTs) over the past 15 years. Most of the RDTs for malaria use immunochromatographic technique to detect malaria parasite antigens in peripheral blood, many of which have been tested in clinical and field

situations with comparable sensitivities, specificities and accuracies with the blood film microscopy.

This review highlights the various diagnostic methods available for diagnosis of malaria and discusses the benefits and limitations of the RDTs and other newer methods in developing countries.

#### **BLOOD FILM MICROSCOPY**

The conventional light microscopy examination of the stained blood film is the "gold standard" method with which other diagnostic methods are compared. Samples that can be analyzed by microscopy include peripheral blood (venous), finger prick blood (capillary), buffy coat, bone marrow aspirates, cord blood or placenta impression smear and postmortem smears of grey matters (capillary/post capillary venous blood). Examination is done usually under the oil immersion light microscope

##### **Thick blood film**

The thick film concentrates malaria parasites and makes this method very sensitive. It is mainly used for making a diagnosis of malaria and estimation of parasite density. When examination is done by a competent microscopist, the sensitivity approaches 5-10 parasites/ $\mu\text{L}$  of blood or 0.0001-0.0002% parasitaemia (3), but under general field conditions, the detection capabilities may be realistically placed at 100 parasites/ $\mu\text{L}$  of blood (4). 3% Geimsa stain is the commonly employed stain without any need for fixation, but Field stains (A and B) can equally be used.

The level of parasitaemia is essential in *Plasmodium falciparum* infection and this is better achieved with a thick film, which is usually in excess of 50,000 parasites/ $\mu\text{L}$  of blood. A number of methods can be used to

estimate parasite density. The most reliable is counting the malaria parasites in relation to number of white blood cells (WBC), usually 200 WBC (or 500 WBC when the number of parasites is less than 10 per 200 WBC counted) and multiplying this by the average of the total WBC count in such individual (5). An average of 8,000 leucocytes per  $\mu\text{L}$  is usually taken as standard WBC count but in our environment; a value of 6,000 leucocytes per  $\mu\text{L}$  appears more appropriate (6, 7).

Another method by Earle and Perez (5) employed counting asexual parasites per known volume (usually 5  $\mu\text{L}$ ) of blood spread as thick film. This is however time consuming and generally employed only in research studies. The 'plus' system is an alternative but less precise method commonly used in our environment.

Because the stains destroy the red blood cells, it is difficult to identify the species of plasmodium in thick film.

##### **Thin blood film**

The thin film allows the observation of details about the species of plasmodium and the blood film morphology, which enable speciation of parasites, hence, it is used mainly for speciating the parasites. The blood film preparation is carried out on moisture and grease free slides and staining is better done with 10% Geimsa stain following an initial 1-2 minutes methanol fixing step, to preserve the red cells. The modified Field stain is rapid but does not stain Schuffner's dots and is useful only as a screening method. The Leishman's stain is methanol based and is useful only in thin film. It is however superior to other stains in demonstrating details of malaria parasites, therefore aiding better speciation.

Examination of thin film is preferably done in the lower third of the smear where erythrocytes are just overlapping but the tail of the film demonstrate better the matured *Plasmodium ovale*, *P. vivax* and gametocytes of *P. falciparum*. A good film should reveal erythrocytes as pale straw or light grey and the leucocytes as cell with dark blue or purple nuclei with lighter cytoplasm and platelets as blue or purple. The size of the erythrocytes and presence of stipplings as well as the size and number of the different forms of the parasite's life cycle are important in arriving at the species of the parasite.

In *P. falciparum* infection, the erythrocytes are usually of normal size. The ring forms (early trophozoites) of the parasites are usually small and multiple (> 2) in the erythrocytes while the late trophozoite forms have moderate, compact and granular pigment with irregular large, red mauve dots called Maurer's cleft. Schizonts of *P. falciparum* are rare in peripheral circulation as most of them are sequestered in the microcirculation because of their adhesive nature. The gametocytes are crescent (boat shaped) with single nucleus.

In *P. vivax* infection, the erythrocytes are usually larger than normal; the ring trophozoites are large and < 2 within the erythrocytes while the late trophozoites are large amoeboid with fine pigment and the red cells have numerous fine stipplings called Schuffner's dots. The erythrocytes in *P. ovale* infection are also larger than normal; the ring form appear compact and < 2 per cell and the late trophozoites are small, oval, non-amoeboid

with coarse pigments with some erythrocytes containing numerous stipplings called James' dots. In *P. malariae* infection, the erythrocytes are smaller than normal; the ring forms are compact and < 2 per cell while the late trophozoites are small, compact (and band like) with coarse pigments but erythrocyte usually show no stippling. The gametocytes of *P. vivax*, *P. ovale* and *P. malariae* appear spherical and compact with single nucleus, which is diffuse and coarse, though in *P. vivax* and *P. malariae*, this appear smaller and less diffuse.

Conventional blood film light microscopy offers several advantages. It is sensitive, detecting between 5-10 parasites/ $\mu$ L of blood (4) when done by a competent microscopist and about 100 parasites/ $\mu$ L under general field conditions. It gives precise information about the species of plasmodium involved and quantification can be done to demonstrate hyperparasitaemia associated with severe malaria and to assess parasitological response to chemotherapy. It is also relatively inexpensive with estimated cost in endemic areas ranging between US \$ 0.12 – US \$ 0.40 per slide examined (8). This cost per test however increases with low utilization or if microscopy in the health facility is used only for malaria diagnosis. Blood film microscopy also provides permanent record (smears) of the diagnostic findings and can be subjected to quality control.

However, it is labour intensive and time consuming, normally requiring at least one hour from specimen collection to result availability. It is exacting and depends absolutely on good technique, reagents,

microscopes and most importantly well trained and supervised technicians. Unfortunately, these conditions are often not met, particularly at the more peripheral levels of the health care systems and in some developed countries where competent microscopists may not be available. In these circumstances, microscopic diagnosis risks becoming an unreliable tool that use up resources for doubtful results. These shortcomings have led to the development of alternative rapid and robust methods of diagnosis.

#### **Fluorescent microscopy**

Fluorescent microscopy on blood films can be achieved by using fluorochromes such as acridine orange either on the blood smears (9) or on centrifuged blood specimens called quantitative buffy coat (QBC) technique (10). Although this is more sensitive than conventional microscopy, it is expensive and requires special equipment and supplies such as centrifuge; centrifuge tubes, ultra-violet light source and filters, and is therefore not commonly used for routine diagnostic purposes but for research.

#### **MALARIA RAPID DIAGNOSTIC TESTS (RDTs)**

Rapid Diagnostic Tests (RDTs) for the diagnosis of malaria are based on the detection of antigens derived from malaria parasites in lysed blood using immunochromatographic or enzyme-linked immunosorbent assay methods. The results of the tests are available within 5-15 minutes of specimen collection. The introduction of the first RDTs was based on the work of Howard *et al* (11) who described the production of histidine-rich protein II (HRP II), a water soluble protein, by trophozoites and young (but not matured)

gametocytes of *P. falciparum* and secreted from infected erythrocytes. A rapid technique, Parasight<sup>®</sup> F, (12) was then developed by Becton Dickinson Tropical Diseases Diagnostics, Sparks, Maryland, United States of America.

Today, several commercial test kits are available with most frequently employing dipsticks bearing monoclonal antibodies directed against the parasite target antigens. Antigens targeted include, histidine-rich protein II antigen (11) which is available from only *P. falciparum*, and parasite lactate dehydrogenase (pLDH) (13) enzyme produced by asexual and sexual (gametocytes) stages of malaria parasites. Available kits detect pLDH from all four human plasmodium species and can distinguish *P. falciparum* from non-falciparum species but cannot reliably distinguish between *P. vivax*, *P. ovale* and *P. malariae*. Other antigens that are present in all four plasmodia species are also targeted in kits that combine HRP II antigen of *P. falciparum* together with that of an, as yet unspecified, pan-malarial antigen of other species.

#### **Principle of RDTs**

The methods that detect the HRP II or pLDH, which include several commercial immunochromatographic kits such as Parasight F (12), ImmunoChromatographic Test (ICT) malaria (14), and Diamed OptiMAL test kit (15), employ the use of a nitrocellulose or glass fibre strip. Test-specific reagents are pre-deposited on the strip during manufacture and these include a line of capture antibody specific for the antigen under investigation (several lines are used if several antigens are being investigated) and a procedural control line,

with an antibody that will capture the labeled antibody.

A finger prick (2-50  $\mu$ L) blood sample collected using a microcapillary tube or anticoagulated blood or plasma, is mixed with a buffer solution that contains a haemolysin compound and specific antibody that is labeled with a visually detectable marker such as colloidal gold or sulphorhodamine, in a well or separate test tube or on a sample pad. In some kits, the labeled antibody is pre-deposited during manufacture onto the sample pad or in the well or tube, in which case only a lysing and washing buffer are added to the blood. If the blood sample contains the antigen under investigation, antigen-antibody complex migrates up the test strip by capillary action towards test-specific reagents. A washing buffer is then placed either in a well from which it migrates up the strip or it is added directly on the strip or the entire strip is washed in a test tube of buffer solution to remove the haemoglobin and permit visualization of any coloured line on the strip.

The labelled antigen-antibody complex is immobilized at the pre-deposited line of capture antibody and is visually detectable. Whether or not the blood contains antigens, the control line will become visible as labeled antibody is captured by the pre-deposited line of antibody directed against it. The complete test run time varies from 5 to 15 minutes.

While the method that detect HRP II is only available for *P. falciparum* which will show only 2 visible lines on the strip (test and control), the method that detect pLDH can differentiate *P. falciparum* from non-falciparum malaria parasites but not *P.*

*vivax* from *P. ovale* or *P. malariae*. A negative blood will therefore show visibility only at the control line on the strip, non falciparum malaria as two visible lines while pure *P. falciparum* or mixed infection will show three visible lines on the strip.

#### **Merits of RDTs**

RDTs are simple to perform and interpret, and they do not require electricity, special equipment or training to perform. Peripheral health workers and other health care providers as well as community volunteers can be taught the procedure in a matter of hours with good retention of skills over one-year period (8).

They are also relatively robust and test performance and interpretation vary relatively little among individual users and the kits can be shipped and stored under ambient conditions. This makes them especially useful in the developing countries. Because they detect circulating antigens, they may detect *P. falciparum* infection even when they are sequestered in the deep vascular compartments and thus undetectable by microscopic examination of peripheral blood smear. Also in women with placenta malaria as demonstrated by placenta impression smear, RDT have detected circulating HRP II even though blood smears were negative due to sequestration of *P. falciparum* in the placenta (16).

The test performance of RDTs has been assessed in diverse clinical settings in both malaria endemic and non-endemic countries. In Nigeria, few researchers have investigated the use of HRP II antigen based RDTs, with reported sensitivity {compared to gold standard thick blood film (TBF) microscopy} of > 80% and specificity of >

90% for parasitaemia of > 100 parasites/ $\mu$ L of blood (6, 17, 18, 19). Others have reported lower sensitivity of 70% or less (7, 20). In some of these studies (7, 20, 21), the limited number of subjects employed does not allow conclusive inferences to be drawn. However, nearly all the investigators reported a spill-over antigenaemia effect lasting for 7-14 days after appropriate malaria therapy. This has affected the use of this diagnostic kit in monitoring response of patients to antimalaria therapy and detecting resistant infections.

Detection methods based on pLDH enzymes have equally been investigated. Agomo *et al* (22) reported a sensitivity of 63.95% for "OptiMAL 1" test kit (based on pLDH) in a study of 240 subjects with clinically diagnosed malaria in Ogun State of Nigeria. Ujah *et al* (21) also reported that sensitivity of OptiMAL test kit was higher (though not statistically significant) than TBF microscopy in a study of 62 patients with clinically diagnosed malaria in Jos, Nigeria. However, these rapid tests can only become useful in the diagnosis of malaria in the tropics, where malaria is endemic, when there is correlation between parasitaemia levels (at which the kit become positive) and clinical manifestations of malaria.

Studies of RDTs in Zimbabwe (23), Kenya (14), Tanzania (12), Cameroon (16), Thailand (24), India (25, 26), Brazil (27), East Indonesia (28), Kuwait (29), Canada (30) and the United States of America (15) have reported high sensitivity and specificity of the kits for the diagnosis of malaria (when compared to TBF microscopy or polymerase chain reaction (PCR) method as gold standard); especially in returned travelers from malaria endemic areas (15, 30) and in

placenta malaria (16), but their usefulness is limited by inability to differentiate the different species of plasmodium, persistent antigenaemia and false positive reactions seen in some cases. In the assay based on pLDH, false positive reactions can occur in a situation where gametocytes, which are not the infective forms of the parasite, are the only forms present in the blood.

Some manufacturers of the HRP II antigen kit have attempted to incorporate a "pan malaria antigen" in the kit to differentiate falciparum from non falciparum malaria especially *P. vivax*. Although the sensitivity and specificity of these types of kits for *P. falciparum* is 85-95% and > 95% respectively, the sensitivity for *P. vivax* can be as low as 60% in some cases (29) and generally less than 80%, though the specificity approaches 100% (28, 29).

At present, the RDTs can only complement the TBF microscopy in the diagnosis of malaria in the tropic, especially in areas where microscopy may be unavailable and in certain conditions such as placenta malaria or cerebral malaria where sequestration of parasites occur in the placenta or brain micro-capillaries. Before they can gain widespread use, the cost of the kit must be drastically reduced. The cost per test at the moment varies from \$0.60 to \$2.50 and possibly more depending on the marketing area (8). The kit must also be able to differentiate the different species of plasmodium especially in areas where two or more species co-exist and issue of false positivity and spill-over antigenaemia must be addressed, so that they can be useful in disease monitoring and detecting early resistance.

## **PLASMODIUM NUCLEIC ACID DETECTION**

### **DNA hybridization method**

The earliest DNA probe described by Barker *et al* (5) was specific for *P. falciparum* and detects 20-25 parasites/ $\mu$ L of blood. The probe was a  $^{32}$ P-labelled DNA (Pf 14 DNA probe) containing 1 kb of *P. falciparum* DNA made of tandemly arranged degenerate 21 bp repeats. It is only useful for large number of samples and screening blood donors for malaria in endemic countries. It is very expensive, requiring high technical expertise and is also time consuming.

### **Polymerase chain reaction detection method**

The polymerase chain reaction (PCR) method employed the use of oligonucleotide primers with *P. falciparum* sequences. The earliest primers constructed were similar in sequence to those of DNA probes of Barker *et al* (5). Amplified products following PCR are detected by Southern blotting or dot blot hybridization with chemiluminiscent substrates following blot transfer onto hybridization membrane or nitrocellulose. Sensitivity can be as high as 10 parasites/ $\mu$ L of blood.

In recent times, the sequence of a small subunit rRNA (SSUrRNA) or 18S rRNA genes of plasmodium have been found to be highly stable and conserved (31, 32, 33). These subunits contain both genus-specific and species-specific sequences. PCR primers directed against these subunit sequences have allowed for detection of the different human plasmodium species involved in single or mixed infections (31-37) and assays to detect them have displayed no cross reactions to human DNA or other human pathogen DNA or RNA including non-human plasmodium species.

Evaluation of the traditional "gold standard" PCR assay, the nested PCR, for the detection of plasmodium species in human infection has shown a higher sensitivity and specificity than the TBF microscopy (38), especially at parasite count of  $< 100/\mu$ L. This assay is however a lengthy procedure that requires specialized and costly equipment and reagents, as well as laboratory conditions that are often, not available in the field. Because it takes a long time (about 8 hours) for the result to be available to the health care physicians, the routine use of this assay in clinical laboratory is limited. Several innovative PCR assays are now available (39, 40, 41, 42) with result made available within 2 hours of specimen collection. For example, the "Real-Time" PCR assay for detection of malaria parasites provide qualitative (39, 41) or both qualitative and quantitative (40, 42) estimates of parasitaemia and are particularly useful in returned travelers with low parasitaemia and in patients with mixed infections. The results are also available in less than 45 minutes (39) or within 2 hours (40-42).

Knowledge of several other gene sequences of the different stages of malaria parasites have been used to construct primers for identification of genetic variants of the different plasmodium species (43). Such gene sequences include cysteine protease (44) and mitochondrial cytochrome b gene sequence (45) of trophozoites and ookinete surface protein gene sequence (46).

At present, nucleic acid based method of laboratory diagnosis of malaria is restricted to returning travelers from endemic areas and for mixed infections. The application of this technology in Africa is

limited to large research institutes and until issues of cost and technical expertise are resolved, it will have no place in routine laboratory diagnosis in this part of the world.

#### **OTHER LABORATORY DIAGNOSTIC METHODS**

##### **Antibody detection by serology**

This detection method measures antibodies that are produced by the body following exposure to the parasite and not specifically current infections (47). Antibodies to the asexual blood stages appear a few days after malaria parasites invade erythrocytes and rise in titres over the next few weeks. They may persist for months or years in semi-immune patients in endemic areas where re-infection is frequent. However, in non-immune patient treated for a single infection, antibody levels fall more rapidly and may be undetectable in 3-6 months. Re-infection or relapse leads to secondary response with high and rapid rise in antibody titres.

Various protocols for detecting these antibodies include indirect fluorescent antibody technique (IFAT) with malaria antigen for the assay prepared from peripheral blood of infected patients (all types of plasmodia) or by continuous *in vitro* culture of *P. falciparum* or adapted growth of *P. falciparum* in primates. Enzyme linked immunosorbent assay (ELISA) for the detection of antimalaria antibody to *P. falciparum* is also available in kit forms (48). Recently, a test kit "SD Bioline" with recombinant *P. falciparum* circum-sporozoite proteins (CSP) and merozoite surface protein (MSP) as antigens, was compared with "OptiMAL 1" test kit (22) in Nigeria. The performance of the "SD Bioline" was dismal with only 54.84% sensitive and 42.9%

specific, while the "OptiMAL 1" was 63.95% sensitive and 92.2% specific when compared to TBF microscopy.

Antibody testing in endemic area is not useful and is only helpful in prospective screening of blood donors and retrospective confirmation of malaria in residents of non-endemic areas recently treated empirically overseas. It may be useful in the investigations of cases of tropical splenomegaly syndrome (TSS) now called hyper immune malaria splenomegaly (49).

##### **Depolarized monocytes/Pseudoreticulocytosis**

Some researchers have attempted the use of "Cell-Dyn 4000 Haematology Analyzer", a new generation automated instrument that has found widespread use in routine haematology laboratories in developed countries (50), to diagnose imported malaria. The principle employed is based on unexpected abnormalities in differential white blood cell plots and reticulocyte histogram seen in patients with malaria.

Monocytes that have ingested malaria breakdown product, haemozoin, can be differentiated from normal monocytes because haemozoin depolarizes laser light used for routine differentiation of eosinophils. Also, nuclear materials of intra erythrocytic malaria parasites can be detected by fluorescent nucleic acid dye used for routine quantification of reticulocytes. The presence of infected erythrocytes leads to a distinct fluorescent spike in reticulocyte histogram referred to as "pseudoreticulocytosis".

Using these two parameters, Wever *et al* (50), found 62% sensitivity for imported malaria (all species) at parasite density of  $\geq 0.5\%$  when compared to expert microscopy



and 96% sensitivity for *P. falciparum* infection alone. This novel method may be a useful addition to conventional microscopy in the diagnosis of imported malaria, especially when expert microscopists are not available. It is an expensive test however, requiring the use of expensive novel equipment and generally not applicable in the developing countries.

#### Laboratory culture of plasmodium

*In vitro* cultivation of plasmodium in the laboratory is usually not for routine diagnosis but for purposes of antigen preparation for serology and for malaria research.

#### CONCLUSION

Malaria remains a major public health problem in the tropic. Prompt diagnosis is essential for timely initiation of appropriate therapy. At present, blood film microscopy remains the "gold standard" and the most applicable method of malaria diagnosis in the tropics. Although the rapid diagnostic techniques have a role to play in certain situations and in certain clinical conditions, the high cost prohibits their widespread use. The place of the polymerase chain reaction based method in malaria diagnosis in most parts of Africa is even less certain.

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