Malaria rapid diagnostic tests: challenges and prospects

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In the last decade, there has been an upsurge of interest in developing malaria rapid diagnostic test (RDT) kits for the detection of *Plasmodium* species. Three antigens – *Plasmodium falciparum* histidine-rich protein 2 (PfHRP2), plasmodial aldolase and plasmodial lactate dehydrogenase (pLDH) – are currently used for RDTs. Tests targeting HRP2 contribute to more than 90% of the malaria RDTs in current use. However, the specificities, sensitivities, numbers of false positives, numbers of false negatives and temperature tolerances of these tests vary considerably, illustrating the difficulties and challenges facing current RDTs. This paper describes recent developments in malaria RDTs, reviewing RDTs detecting PfHRP2, pLDH and plasmodial aldolase. The difficulties associated with RDTs, such as genetic variability in the *Pfhrp2* gene and the persistence of antigens in the bloodstream following the elimination of parasites, are discussed. The prospect of overcoming the problems associated with current RDTs with a new generation of alternative malaria antigen targets is also described.

Introduction

Malaria remains a serious human health issue and is particularly prevalent in developing countries. It is estimated that 3.3 billion people worldwide are at risk of malaria, with 90% of cases occurring in Africa south of the Sahara (WHO, 2011a). In 2010, the World Health Organization (WHO) reported 216 million malaria cases with an estimated 655,000 deaths, principally among children (WHO, 2011a). The high morbidity and mortality are attributed to the development of resistance of the parasite to antimalarial drugs and of the mosquito vector to currently available insecticides. There is no malaria vaccine at present.

Malaria is transmitted to humans by mosquitoes of the genus *Anopheles*. Malaria is known to be caused by four plasmodia species, namely *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae*, with *P. falciparum* being the most lethal. There are increasing reports of a fifth human-infecting species, *Plasmodium knowlesi*, which has been described in south-east Asian countries (Van den Eede et al., 2009; Lee et al., 2009, 2011; Singh & Daneshvar, 2010). Diagnosis is complicated by *P. knowlesi* and *P. malariae* having similar morphology, and it is difficult to differentiate *P. falciparum*, *P. malariae* and *P. knowlesi* by microscopy (Chin et al., 1968; Lee et al., 2009; Ong et al., 2009). For efficient treatment and management of malaria, rapid and accurate diagnostic testing is imperative. The lack of proper diagnostics results in a waste of already scarce resources and impacts negatively on the prompt treatment of malaria. Various techniques are available for malaria diagnosis. Patients presenting with a febrile illness in endemic areas are likely to be diagnosed with malaria (Wilson, 2013). Microscopy has been in use for over 100 years and is inexpensive, rapid and relatively sensitive when used appropriately (Laveran, 1891). Microscopy is regarded as the ‘gold standard’ for malaria diagnosis (WHO, 1999). However, the lack of skilled technologists in medical facilities in affected areas often leads to poor interpretation of data. Furthermore, microscopy is time consuming and labour intensive, cannot detect sequestered *P. falciparum* parasites (Leke et al., 1999) and is less reliable at low-density parasitaemia (<50 parasites (µl blood)^−1^) (Kilian et al., 2000; Bell et al., 2005). None the less, a good microscopist can differentiate species with microscopy and microscopy is used to follow treatment (Kilian et al., 2000; Mayxay et al., 2004; Lee et al., 2009). Parasite DNA can be stained with acridine orange and other fluorescent dyes and detected with a fluorescence microscope or by flow cytometry (Moody, 2002). Amplification of parasite DNA with PCR is specific and can detect low concentrations of parasites but takes time and requires specialized equipment, and is thus not suitable in most field settings. A modification of PCR, loop-mediated isothermal amplification (LAMP), is a technique with interesting potential (reviewed by Han, 2013). A commercial LAMP test has
recently been evaluated in a UK reference laboratory and a rural clinic in Uganda, with promising results (Hopkins et al., 2013; Polley et al., 2013).

To address the limitations of microscopy and PCR-based techniques, other methods are being explored. To be validated, these methods must be benchmarked against microscopy or PCR analysis and against reference strains. For example, rapid diagnostic tests (RDTs), according to the WHO (1999), must be able to reliably detect 100 parasites µl⁻¹, equivalent to 0.002 % parasitaemia, and must have a minimum sensitivity of 95 %, compared with microscopy, and a minimum specificity of at least 90 % for all malaria species. Malaria RDTs were developed in the mid-1990s (Dietze et al., 1995; Palmer et al., 1998). These RDTs use immunochromatography, whereby a coloured detecting antibody binds to lysed parasite antigen and is carried by capillary action on a nitrocellulose strip and arrested by a capture antibody, resulting in a coloured band on a test strip. RDTs are simple and easy to use with little expertise required for interpretation, although some training improves interpretation. RDTs are generally available in easily transportable strips and do not require a source of electricity; thus, they are suitable for field tests and for use by travellers or tourists. A result is obtained within a few minutes. Although not suitable, at present, for qualitative outcomes, RDTs are very useful in endemic areas where many people can be screened in a short period of time. However, caution should be exercised in purchasing and using these kits, as not all commercial kits have the same performance; RDTs are produced by many companies and with variable quality control. The WHO, together with the Foundation for Innovated New Diagnostics (FIND), has established and offers a testing programme to evaluate the performance of RDTs (WHO, 2009, 2010, 2011b). The commonly used RDTs target \( P. falciparum \) histidine-rich protein 2 ( PfHRP2) and two enzymes in the \( Plasmodium \) parasite glycolytic pathways, namely plasmodial lactate dehydrogenase (pLDH) and aldolase. This paper describes malaria RDTs, discusses the challenges associated with the tests and elucidates the utilization of new potential antigen targets for malaria RDTs.

Malaria diagnosis using rapid diagnostic tests

PfHRP2

Three \( PfHRP \), \( PfHRP1 \), \( PfHRP2 \) and \( PfHRP3 \), have been identified (Howard et al., 1986; Rock et al., 1987; Parra et al., 1991). \( PfHRP1 \) is expressed in all knob-positive \( P. falciparum \) species and is associated with the cytoskeleton of infected red blood cells. \( PfHRP2 \) has been identified in all \( P. falciparum \)-infected red blood cells regardless of knob phenotype. \( PfHRP3 \), like \( PfHRP2 \), has histidine-rich amino-acid repeats regions, is secreted by \( P. falciparum \) parasites and is recognized by some of the monoclonal antibodies detecting \( PfHRP2 \) in RDTs (Baker et al., 2010a; Gamboa et al., 2010; Lee et al., 2006a; Maltha et al., 2012). This review focuses on \( PfHRP2 \), which is a good marker for \( P. falciparum \) infection (WHO, 2003).

The water-soluble, heat-stable, 30 kDa \( PfHRP2 \) protein is synthesized only by \( P. falciparum \) parasites (Stahl et al., 1985; Howard et al., 1986; Wellens & Howard, 1986; Rock et al., 1987). This protein is very stable, with a positive correlation between blood concentration of the protein and parasite biomass (Desakorn et al., 2005; Dondorp et al., 2005). A laboratory strain of \( P. falciparum \) lacking \( PfHRP2 \) has been characterized (Scherf & Mattei, 1992), as well as a laboratory-induced mutant (Wellens et al., 1988). In addition, some isolates from Africa and South America have been found lacking the \( PfHRP2 \) and -3 antigens (Baker et al., 2005, 2010b; Gamboa et al., 2010; Houzé et al., 2011). Recently, Kumar et al. (2013) reported on Indian \( Plasmodium \) field isolates lacking both the \( Pfhrp2 \) and \( Pfhrp3 \) genes. \( PfHRP2 \) is expressed in gametocytes (Hayward et al., 2000) and all erythrocytic stages of \( P. falciparum \). The protein is released upon schizont rupture and is thus found in the supernatants of cultured parasites and in the blood of parasite-infected individuals (Howard et al., 1986; Rock et al., 1987; Parra et al., 1991). This enables the detection of \( PfHRP2 \) when sequestered parasites cannot be detected by microscopy. As \( P. falciparum \) parasites develop in the infected red blood cells from ring stage to trophozoites, they disappear from the peripheral circulation and cytoadhere to various organs in the host (MacPherson et al., 1985; Goldring et al., 1992; Goldring & Hommel, 1993; Goldring, 2004). \( PfHRP2 \) is found in the parasitophorous vacuole or in the parasite cytoplasm and the protein actively facilitates the polymerization of toxic haem, resulting from the degradation of host haemoglobin, to form a malaria pigment, haemozoin, which is no longer toxic (Sullivan et al., 1996). \( PfHRP2 \) contains 35 % histidine, 40 % alanine and 12 % aspartate, but the percentages vary depending on the isolate expressing \( PfHRP2 \) (Wellens & Howard, 1986). \( PfHRP2 \) has multiple repeats of AHHAAD, with AH and AHHA and the presence of repetitive B-cell epitopes; this enables the detection of the protein by multiple antibodies, increasing the sensitivity of methods detecting the protein (Wellens & Howard, 1986; Panton et al., 1989). Commercial assays that detect \( PfHRP2 \) include Paracheck-Pf (Orchid Biomedical Systems), ICT Diagnostics Malaria Pf (ICT Diagnostics) and ParaSight F (Becton Dickinson). A full list of commercial assays that have been evaluated is available from the WHO (2011b). \( PfHRP2 \)-based RDTs are the most widely used of the RDTs and have been utilized in low- and high-density malaria zones in both pregnant women and non-pregnant individuals for the diagnosis of mild and paediatric severe malaria (Leke et al., 1999; Mayxay et al., 2001; Tjitra et al., 2001; Moody, 2002; Mueller et al., 2007; Hopkins et al., 2007, 2008; Houzé et al., 2009; Laurent et al., 2010; Abba et al., 2011; Hendriksen et al., 2011; Kattenberg et al., 2011, 2012b; Kyabayanize et al., 2011; Aguilar et al., 2012). The \( PfHRP2 \) RDT is not useful for the prediction of parasite responses to therapy because of the persistence of the antigen in the peripheral blood circulation after parasite clearance (Tjitra et al., 2001; Houzé et al., 2009).
Placental malaria infection has been reported as one of the main causes of low child birth weight, leading to high numbers of stillbirths and premature births (Okoko et al., 2002). During pregnancy, malaria parasites sequester in placental tissue and when the parasites are sequestered they cannot be detected by microscopy in peripheral blood. RDTs have been explored for the possible diagnosis of malaria in pregnancy. A study undertaken on pregnant women in Cameroon demonstrated 89.1% *P. falciparum* infection using the ICT Diagnostics Malaria Pf, a *PfHRP2*-based RDT; in combination with microscopy, 93.8% of women were accurately diagnosed with the presence of the parasite (Leke et al., 1999). Similarly, *P. falciparum* placental infection in pregnant women in an endemic area in Uganda was studied using both microscopy and a *PfHRP2* RDT (named diagnosticks; SSA Diagnostics & Biotech Systems; Kyabayinze et al., 2011). The study reported a sensitivity of 96.8% and specificity of 73.5% in febrile women, which, although not better than microscopy, meant the RDT was considered a useful tool for determining malaria in pregnancy.

To better understand the role of *PfHRP2* RDTs for the diagnosis of malaria in pregnant women, a comprehensive systematic review was published by Kattenberg et al. (2011). In this meta-analysis, the majority of studies described the detection of *P. falciparum*, with 72, 81 and 94% of cases detected by microscopy, RDT and PCR, respectively. The authors concluded that more studies with placental histology as a reference test are urgently required to reliably determine the accuracy of RDTs for the diagnosis of placental malaria. More recently, Aguilar et al. (2012) compared microscopy and the ICT Diagnostics Malaria Pf RDT in detecting placental malaria in placental blood. They found an agreement of 82.9% between both tests, with differences between them at either low parasitaemias (<500 parasites μl⁻¹) or negative with microscopy. They concluded that the ICT Diagnostics Malaria Pf RDT is a good alternative to microscopy for diagnosing placental malaria at delivery. These results also confirmed *PfHRP2* as a good target for malaria detection in pregnant women compared with RDTs detecting *P. falciparum* LDH (Kattenberg et al., 2012b).

Problems associated with *PfHRP2*-based rapid diagnostic tests

Variability in both the specificity and sensitivity of RDTs is generally associated with the manufacturing process of the kits (WHO, 2011b). Diagnostic sensitivity is defined as ‘the percentage of positive tests among the total number of positive samples’. Diagnostic specificity is ‘the percentage of negative tests among the total number of negative samples’. The total number of positive/negative samples is the number detected by either microscopy in blood slides or PCR (adapted from Saah & Hoover, 1997).

A key concern is the genetic variation in the *PfHRP2* amino acid sequence among parasites isolated from geographically different locations, leading to false-negative results from RDTs. In the mid-2000s, Baker et al. (2005) revealed a significant *PfHRP2* sequence difference within and between 75 isolates of *P. falciparum* from 19 countries. The effect of *PfHRP2* sequence variation on the binding of specific mAbs was analysed by Lee et al. (2006a). They found that various isolates had different numbers of repeats and combinations of repeat amino acid sequences, which affected the sensitivity of RDTs. A larger study with *PfHRP2* DNA obtained from isolates from African and South American countries showed extreme sequence variation, but concluded that diversity of the protein was not a major cause of the varying sensitivities of RDTs (Baker et al., 2010b). A study conducted in the Amazon region of Peru revealed isolates that lacked the *Pfhrp2* gene, indicating the need to use microscopy or other RDTs for *P. falciparum* detection in areas where deletion of the gene is suspected (Gamboa et al., 2010; Maltha et al., 2012). The absence of the *PfHRP2* antigen for detection by *PfHRP2*-based RDTs was confirmed as the cause of diagnostic failure in a French visitor who returned from France with malaria from the Brazilian Amazon region, where *P. falciparum* is present (Houzé et al., 2011). The protein has also been reported to not be expressed in some isolates in India (Kumar et al., 2012, 2013). Parasites that fail to produce *PfHRP2* can cause patient bloodstream infections and false-negative results, as reported in Mali by Koita et al. (2012). The presence of polymorphisms in the *PfHRP2* antigen (Mariette et al., 2008), or the absence of the gene and thus the targeted antigen sequence, means that alternative RDTs other than a *PfHRP2*-based RDT need to be considered for the diagnosis of a malaria infection in areas where deletions of the *Pfhrp2* gene have been detected. False-negative findings can be explained by the absence of bands on an RDT either from excess antibodies or antigens – a phenomenon called the prozone effect, which appears to be restricted to *PfHRP2*-based RDTs (Gillet et al., 2009; Luchavez et al., 2011).

The type of immunoglobulin used for antigen capture influences the outcome of the RDT. RDTs are coated with either IgM or IgG mAbs. For example, the ICT Diagnostics Malaria Pf RDT is coated with IgM (Parra et al., 1991), whilst ParaSight F uses mAb IgG1, a subclass of IgG (Beadle et al., 1994). IgM is the initial class of antibody produced once a person or animal is exposed to viruses, bacteria or toxins. IgM has a very short lifespan, disappearing a few days after infection and being replaced by IgG in body fluids (i.e. blood, lymph and exudates). *PfHRP2*-based RDTs have given false-positive results and there has been some debate as to the cause of these false positives. Mishra et al. (1999) suggested that IgG-based *PfHRP2* RDTs cross-react with rheumatoid factors whilst IgM-based tests do not. The conclusion that IgM is incapable of binding to rheumatoid factors was questioned by Grobusch et al. (1999). Laferi et al. (1997) reported that eight out of 12 patients with rheumatoid factor tested positive. Iqbal et al. (2000) found that samples from...
patients with rheumatoid factor without malaria tested false positive; when rheumatoid factor was absorbed out, the samples tested negative with the RDT. False positives have been recorded due to infection with Schistosoma mekongi (Leshem et al., 2011). False positives are also caused by the persistence of PfHRP2 in the blood for long periods after parasite clearance, as determined by microscopy (Mharakurwa & Shiff, 1997; Mayxay et al., 2001; Huong et al., 1997; Iqbal et al., 2002, 2004; Moody, 2002; Grobusch et al., 2003; Mueller et al., 2007; Houze et al., 2009; Kyabayinze et al., 2011). This reduces not only the sensitivity of the test but also the chance of targeting this protein for drug-susceptibility testing. In 2001, Mayxay et al. (2001) found a positive correlation between PfHRP2 persistence and malaria parasite density. Moreover, antigen persistence was found to be likely to be influenced by gametocyttaemia (Hayward et al., 2000; Tjitra et al., 2001). Studies have shown this antigen in the blood circulation 28 days after parasite clearance, resulting in a 27% false-positive rate using the ParaSight F test (Humar et al., 1997; Moody, 2002). A study on pregnant women with malaria in Nanoro, Burkina Faso, revealed the persistence of PfHRP2 for up to 28 days after artemisinin-based combination therapy (Kattenberg et al., 2012b). Using Paracheck-Pf, an RDT that specifically detects PfHRP2, Swarthout et al. (2007) obtained positive results on patients’ blood more than 5 weeks after antimalarial treatment and parasite clearance was confirmed by microscopy. The protein is also present in gametocytes (Tjitra et al., 2001). Overall, persistence of PfHRP2 after malaria treatment has been reported in many situations, suggesting that PfHRP2 is unsuitable as a target for drug-susceptibility testing. Thus, a positive result with a PfHRP2-based RDT may indicate a previous infection and should be confirmed using microscopy, PCR or other RDT targets, for example P. falciparum LDH, which has a short half-life and is only produced by live parasites (Makler et al., 1993; Mtove et al., 2011).

Patient age, malaria transmission intensity and lack of symptoms have been demonstrated to influence the specificity and sensitivity of RDTs, which can in turn result in under- or overdiagnosis of the disease (Fryauff et al., 1997; Reyburn et al., 2007; Swarthout et al., 2007; Abeku et al., 2008; Hopkins et al., 2008; Rakotonirina et al., 2008; Harris et al., 2010; Laurent et al., 2010; Mtove et al., 2011). Working in Jaya, a malaria-endemic area in Iran, Fryauff et al. (1997) found a significant difference in the sensitivity of the ParaSight F test in residents older and younger than 10 years. A decrease in sensitivity of the ParaCheck-Pf RDT in older age groups has been reported in Tanzania, although sensitivity was uniform in Ugandan and Kenyan studies (Abeku et al., 2008; Laurent et al., 2010). False-negative results have been registered in Tanzanian children with high parasitaemias and could be explained by ‘flooding’ of RDT capture sites (Reyburn et al., 2007). Specificity appears to decrease with an increase in prevalence (Swarthout et al., 2007; Abeku et al., 2008; Laurent et al., 2010; Mtove et al., 2011).

Improving RDTs to not only be qualitatively but also quantitatively acceptable could aid in minimizing false-positive results in malaria-dense areas. Richter et al. (2004) found that the ICT Diagnostics Malaria Pf/Pv (detecting both P. falciparum and P. vivax) may have the potential for semi-quantification of parasitaemia in P. falciparum malaria with the simultaneous presence of PfHRP2 and aldolase bands in blood with >40,000 parasites µl⁻¹, compared with cohorts with <40,000 parasites µl⁻¹. However, a study conducted in malaria-endemic areas could not link the band intensity of three PfHRP2-based RDTs, namely the ICT Malaria Combo Cassette Test (ICT Diagnostics), the First Response Malaria Antigen pLDH/HRP2 Combo test (Premier Medical Corp.) and SD Bioline Pf (Standard Diagnostics), to the level of parasitaemia (Luchavez et al., 2011). Under laboratory conditions, the amount of PfHRP2 antigen released has been found to be more or less proportional to the increase in parasite development, multiplication and growth (Desakorn et al., 1997). When PfHRP2 concentrations are determined in an ELISA format, there appears to be a relationship between patient plasma PfHRP2 concentrations and severity of infection in moderate to high areas of malaria transmission (Hendriksen et al., 2013). Plasma PfHRP2 levels appear to predict disease progression to severe malaria, and thus there is a need for the development of a quantitative PfHRP2 RDT for treatment and disease management (Fox et al., 2013). It needs to be emphasized that such a tool will only be valid if parasites in the area express the PfHRP2 protein.

Concerns over batch quality variations of RDT kits have been raised and the WHO/FIND testing system aims to address these concerns (Mason et al., 2002; WHO, 2011b). A study carried out in Blantyre, Malawi, compared the results from four PfHRP2-based RDTs, namely SD Bioline Pf, First Response Malaria, Paracheck-Pf and ICT Diagnostics Malaria Pf, for detecting parasitaemia among febrile patients older than 5 years (Chinkhumba et al., 2010). The RDTs evaluated showed high sensitivity (ICT Diagnostics Malaria Pf, 90%; Paracheck-Pf, 91%; First Response Malaria, 92%; SD Bioline Pf, 97%), although three of these sensitivities are below the 95% recommended by the WHO (1999). The tests had low specificity (ICT Diagnostics Malaria Pf, 54%; Paracheck-Pf, 68%; First Response Malaria, 42%; SD Bioline Pf, 39%). A study in the eastern Democratic Republic of Congo showed that Paracheck-Pf detecting PfHRP2 was as sensitive as microscopy (100%) in detecting true malaria cases in febrile children. However, the specificity of the RDT was low (52%), revealing a high level of false-positive results (Swarthout et al., 2007). Singh et al. (2010) used five RDTs – namely Parascreen [detecting pan-species of Plasmodium and P. falciparum (pan/Pf)], Falcivax Pf/Pv and Malascan (Pf/pan) (all from Zephyr Biomedical Systems), ParaHit Total (pan/Pf; Span Diagnostics) and First Response Malaria pLDH/HRP2 Combo – to diagnose P. falciparum and non-P. falciparum species among 372 patients who
were clinically suspected of suffering from malaria. These authors recorded variations in sensitivity as well as specificity. Sensitivities of 93.2 and 94.7% were reported for Parascreen and First Response Malaria pLDH/HRP2 Combo, respectively, for the detection of *P. falciparum*. The specificities were 64.3 and 58.8%, respectively. These two RDTs also diagnose *P. vivax* infections. The sensitivities for *P. vivax* were 77.2% for Parascreen and 84.2% for First Response Malaria pLDH/HRP2 Combo, with specificities of 98.1 and 96.5%, respectively. The Malascan, ParaHit Total and Falcivax test kits performed less well. Huong *et al.* (2002) reported 95.8 and 82.6% sensitivity for *P. falciparum* testing in southern Vietnam using Paracheck-Pf and ICT Diagnostics Malaria Pf/Pv, respectively, with 100% specificity for both RDTs. They concluded that Paracheck-Pf might prove useful alongside microscopy for the diagnosis of uncomplicated *P. falciparum* malaria in southern Vietnam. These results illustrate the variations in performance by different kits. The discrepancies between results could also be due to differences between studies rather than differences between test brands, and the ability of staff to carry out and interpret the results (Wilson, 2013). Abba *et al.* (2011) produced a systematic review on the diagnosis of uncomplicated *P. falciparum* in Africa and found PfHRP2 RDTs to have a mean sensitivity and specificity (95% confidence interval) of 95.0% (93.5–96.2%) and 95.2% (93.4–99.4%), respectively, for PfHRP2 tests.

The PfHRP2-based RDTs can only identify the presence of a *P. falciparum* infection. Co-infection with both *P. falciparum* and *P. vivax* represents the most common mixed malaria infection found in humans and the two species are the most widespread causes of malaria (Mayxay *et al.*, 2001, 2004). *P. vivax* is the major cause of malaria outside Africa (Mendis *et al.*, 2001). Therefore, the combination of PfHRP2-based detection with the detection of other antigens or the use of additional tools, such as microscopy and PCR, to avoid misdiagnosis of the parasite is important, as misdiagnosis has profound consequences for malaria treatment (Mayxay *et al.*, 2004; Pakalapati *et al.*, 2013). Given the problems identified with PfHRP2 as a diagnostic target, RDTs should be used with an understanding of the limitations and performance of these tests in particular circumstances.

**LDH**

LDH is an essential energy-producing enzyme and is the last enzyme in the parasite glycolytic pathway. It is soluble and is produced by the sexual and asexual stages of parasites, including the mature gametocytes of all four human *Plasmodium* species (Makler & Hinrichs, 1993; Piper *et al.*, 1999; Brown *et al.*, 2004). The parasite and erythrocyte cells (human host) lack a complete citric acid cycle for mitochondrial ATP production and depend on anaerobic glucose metabolism, making pLDH an important enzyme for energy production in the parasite (Lang-Unnasch & Murphy, 1998; Brown *et al.*, 2004; Vaidya & Mather, 2009). pLDH, despite having 26% amino acid sequence identity with human LDH, has conserved catalytic residues for enzyme activity and shares >90% amino acid identity among all plasmodial species (Brown *et al.*, 2004; Turgut-Balik *et al.*, 2004). All malarial pLDH sequences share common epitopes (Hurdayal *et al.*, 2010) and therefore pLDH-based RDTs using mAbs against a common epitope can detect all human *Plasmodium* species, including mixed infections in circulating blood (Mayxay *et al.*, 2004). RDTs based on ‘pan-malarial’ LDH probably use mAbs against common epitopes. In addition, there are sufficient differences between the *P. falciparum* and *P. vivax* LDH amino acid sequences to prepare antibodies directed against specific peptide epitopes to differentiate between the two species and a panel of mAbs can differentiate all species of parasites infecting humans, including *P. knowlesi* (McCutchan *et al.*, 2008; Hurdayal *et al.*, 2010, Piper *et al.*, 2011). Detecting all species on an RDT reduces the possibility of negative results due to a non-*P. falciparum* malaria species (Piper *et al.*, 2011). Following the WHO’s evaluation of the performance of RDTs, multiple pLDH-based RDTs performed well in the assessment (WHO, 2009, 2010, 2011b). Several studies on malaria diagnosis with pLDH assays have been carried out (Piper *et al.*, 1999, 2011; Gasser *et al.*, 2000; Iqbal *et al.*, 2001, 2002; Huong *et al.*, 2002; Moody, 2002; Hopkins *et al.*, 2007, 2008; Ashley *et al.*, 2009; Gerstl *et al.*, 2010; Singh & Daneshvar, 2010; Singh *et al.*, 2010; Hendriksen *et al.*, 2011; Heutmers *et al.*, 2012a,b). The assays can either be specific to *P. vivax* (Pv-pLDH) or *P. falciparum* (Pf-pLDH), or can be three-line tests for Pv-pLDH, Pf-pLDH and PfHRP2 detection or four-line tests that combine PfHRP2, pan-pLDH and Pv-pLDH detection and a control.

The pLDH test appears to perform poorly at low parasite densities (Ashley *et al.*, 2009; Abba *et al.*, 2011; Kattenberg *et al.*, 2011). Piper *et al.* (1999) and Makler and Hinrichs (1993) found a positive correlation between the level of pLDH and parasitaemia. In areas with low parasite density, pLDH-based RDTs appear to perform less well than PfHRP2 RDTs. For example, in Beira, Mozambique, a low malaria transmission area, a three-line pLDH-based RDT, OptiMAL-IT (DiaMed), had a specificity of 88.3% and sensitivity of 88.0% overall, but sensitivity decreased to 45.7% when parasite counts were less than 1000 µL⁻¹. In comparison, Paracheck-Pf, which detects PfHRP2, had a sensitivity of 70.9% and specificity of 94%, with sensitivity dropping to 69.9% with lower parasitaemia (Hendriksen *et al.*, 2011). Studies in diverse malaria transmission areas in Uganda using pLDH-Parabank (Zephyr Biomedical Systems) reported sensitivity of 88% compared with microscopy or 77% when corrected by PCR (Hopkins *et al.*, 2008). A comparison of three pLDH-based tests [CareStart two- and three-line tests (Access Bio) and the OptiMal-IT three-line test] in Myanmar found that none of the tests detected non-*P. falciparum* malaria at low parasitaemias with high sensitivity (Ashley *et al.*, 2009). A
retrospective evaluation of 498 outpatient samples with the CareStart (three-line) pLDH test found a sensitivity of 97% at parasite densities of more than 1000 µl⁻¹, decreasing to 58.3% when parasite density was 100 µl⁻¹ or less (Heutmekers et al., 2012b). Sensitivity of more than 95% was recorded for both pLDH (OptiMAL-IT) and PfHRP2 (ICT Malaria Pf) tests at parasitaemias of more than 100 µl⁻¹ in imported malaria cases in Kuwait, although sensitivity dropped to 76% and 84%, respectively, at lower parasitaemias (Iqbal et al., 2001). CareStart (three-line) was found to be highly sensitive (99.4%) and specific (96.0%) in diagnosing P. falciparum in children living in a malaria-dense transmission area in Sierra Leone, and sensitivity did not change with a decrease in parasitaemia (Gerstl et al., 2010).

RDTs detecting pLDH, or combinations of PfHRP2, pLDH and/or aldolase, are capable of detecting mixed infections (Moody, 2002; Wongsrichanalai et al., 2007; Maltha et al., 2013) The OptiMAL-IT test was found to detect both P. falciparum and P. vivax with more than 90% sensitivity, whilst the CareStart two-line test (pan) had better sensitivity for P. falciparum (90.5%) than for P. vivax (78.9%) in a Myanmar study (Ashley et al., 2009). The CareStart RDT had a sensitivity of 85.6% for P. falciparum and 85% for P. vivax infections in Ethiopia (Ashton et al., 2010). A First Response pLDH/PfHRP2 RDT had 94.7 and 84% sensitivity for P. falciparum and P. vivax infections, respectively, and more than 80% sensitivity for P. falciparum and P. vivax at parasitaemias of 100 parasites µl⁻¹ or more (Singh et al., 2010). A recent study in Korea evaluating the CareStart, SD Bioline, NanoSign Malaria (Biland) and Asan Easy (Asan Pharmaceuticals) RDTs detected P. vivax malaria with sensitivities of 94.4, 98.8, 93.0 and 94.7%, respectively (Kim et al., 2013). Heutmekers et al. (2012b) evaluated the sensitivity of two three-line pLDH (Pf/pan)-based RDTs, CareStart and OptiMAL-IT, for the diagnosis of cryopreserved and fresh infected blood with parasites pre-identified by an expert in microscopic diagnosis of malaria. OptiMAL-IT testing was restricted to fresh blood. CareStart was sensitive for P. falciparum (>90%), more so than for P. vivax (74.3%), and was poor for P. ovale (31.9%) and P. malariae (25%) detection. OptiMAL-IT was excellent for the diagnosis of P. vivax (100%) and detected the single P. ovale infection, but exhibited low sensitivity for P. falciparum (80%). A comparison of the OptiMAL-IT, SD Bioline Malaria Ag Pf/Pan and Humasis Pf/Pan RDTs for the detection of P. vivax malaria found a relationship between levels of pLDH protein and parasitaemia, and reported that the RDTs lost sensitivity below 1600 parasites µl⁻¹ (Jang et al., 2013).

Lee et al. (2011a) developed and evaluated an RDT called FMV ag, which was designed for single-species infection with P. falciparum or P. vivax and co-infection with both species. Sensitivities of 96.5% for P. falciparum, 95.3% for P. vivax and 85.7% for mixed-species infections and a specificity of 99.4% were recorded. The limit of detection for P. vivax was 250 parasites µl⁻¹, which is lower than that detected by the BioLine Malaria Pf/Pan Ag test and the same as the NanoSign Malaria Pf/Pan Ag test. This is the most sensitive RDT to date that differentiates P. falciparum and P. vivax infections. The FMV ag RDT has not yet been tested by the WHO or other laboratories.

P. knowlesi, which infects primates and has recently been found to naturally infect humans, is often mistaken for P. malariae with microscopy (Cox-Sing & Singh et al., 2008, van Hellemont et al., 2009). The OptiMAL-IT test detected P. knowlesi parasites in the blood of an individual returning to the Netherlands from Borneo (van Hellemont et al., 2009) and the RDTs OptiMAL-IT and the Entebe Malaria Cassette have detected P. knowlesi parasites in infected monkey blood (Kawai et al., 2009). This is not altogether surprising, as the tests target pLDH and epitopes common to all malaria pLDH sequences have been identified (Hurdyal et al., 2010). McCutchan et al. (2008) found that a panel of mAbs against pLDH detected all species of malaria, including P. knowlesi. They argued that by using panels of mAbs against pLDH, a P. knowlesi-specific RDT can be developed. There is currently no rapid immunological test for the detection of P. knowlesi and, therefore, more sophisticated and expensive tools such as flow cytometry, nested PCR and real-time PCR need to be employed (Co et al., 2010; Singh & Daneshvar, 2010).

An advantage of RDTs targeting pLDH is that, unlike PfHRP2, the protein does not persist in the bloodstream after parasites have been cleared (Fogg et al., 2008; Gerstl et al., 2010). The protein is used as a drug-sensitivity test for malaria and is thus a measure of viable parasites (Makler et al., 1993; Piper et al., 1996, 1999). pLDH is a good target for monitoring parasite responses to treatment and for predicting treatment failure (Drulhle et al., 2001; Iqbal et al., 2001, 2002; Tjitra et al., 2001; Huong et al., 2002; Moody, 2002; Fogg et al., 2008; Houzé et al., 2009; Gerstl et al., 2010). The pLDH amino acid structure does not undergo antigenic variation (Talman et al., 2007) and pLDH RDTs do not exhibit a prozone effect (Gillet et al., 2009).

pLDH-based RDTs are not without problems. pLDH-based tests have decreased sensitivity at low parasitaemias (Palmer et al., 1998; WHO, 2011b, Craig et al., 2002; Heutmekers et al., 2012b; Jang et al., 2013). This is probably because of the relative abundance of protein (Bozdech et al., 2003; Le Roch et al. 2004). pLDH is expressed by gametocytes and thus false positives can result from high gametocytaemia (Mueller et al., 2007). pLDH-based RDTs were found to be more temperature sensitive than PfHRP2 RDTs (Chiodini et al., 2007), but, in the third round of RDT testing by the WHO/FIND, the CareStart pLDH-based RDT performed as well as PfHRP2-based tests (WHO, 2011b). Other data support this finding (Ashton et al., 2010).

The origin of the antigen used for preparation of the antibody and the type of antibody coated on the nitrocellulose paper during the RDT manufacturing process (i.e. IgM or IgG, monor polyclonal) might account for the low sensitivity and
specificity of *P. ovale* and *P. malariae* detection (Parra *et al*., 1991; Moody, 2002; Piper *et al*., 2011; Maltha *et al*., 2013).

**Aldolase**

Aldolase is another target for malaria RDTs (WHO, 2009, 2010, 2011b). Aldolase is a glycolytic enzyme found in numerous tissues in the host and in the malaria parasite, where it catalyses the formation of dihydroxyacetone phosphate and glyceraldehyde-3 phosphate from fructose 1,6-bisphosphate. Three kinds of tissue-specific aldolase isoenzymes have been reported in higher vertebrates, as opposed to one kind in *P. falciparum* and *P. vivax* (Penhoet *et al*., 1966; Cloonan *et al*., 2001), findings similar to those pertaining to *Trypanosoma brucei* (Clayton, 1985). The *P. falciparum* aldolase shares 61–68 % sequence identity with eukaryotic aldolases. The aldolases of *P. vivax* and *P. falciparum* are both 369 aa in length and their amino acid sequences are relatively conserved (Cloonan *et al*., 2001; Lee *et al*., 2006b). A recent analysis of the sequence of the aldolase gene from 25 Korean *P. vivax* isolates found a single-nucleotide polymorphism at position 180 (Kim *et al*., 2012).

Fewer studies have investigated the use of antibodies against aldolase for malaria RDTs compared with *P*HRP2- and pLDH-based tests. Aldolase in conjunction with *P*HRP2 for malaria RDTs has been used for the diagnosis of *P. falciparum* and non-*P. falciparum* species, but with a poor performance for the latter group (Cho *et al*., 2001; Richter *et al*., 2004). The three-line ICT Malaria Combo dipstick (*P*HRP2/aldolase) (*Pf/Pv*) was evaluated in 674 individuals who had visited malaria-endemic areas (Richter *et al*., 2004). The sensitivity was 100 and 48.1 % for *P*HRP2 and aldolase, respectively, in identifying *P. falciparum*, although the sensitivity for *P. falciparum* aldolase improved to 80 % at high parasitaemias (>40 000 parasites μl⁻¹). The sensitivity for *P. vivax* aldolase was 37.5 %, whilst aldolase did not react with *P. ovale* or *P. malariae* parasites. Low concentrations of aldolase are released from the parasite, and thus the sensitivity level of aldolase-based RDTs is likely to be parasite-density dependent. Similarly, an ICT (*Pf/Pv*)-based RDT on a sample size of 750 patients with clinically suspected malaria attending a medical facility in Kuwait revealed sensitivities of 81 and 58 % for the detection of *P. falciparum* and *P. vivax*, respectively (Iqbal *et al*., 2002). These results decreased by 23 % at lower parasitaemias (<500 parasites μl⁻¹). In a study of 2383 samples from the Orana region of Ethiopia, sensitivity above 85 % and specificity of more than 92 % for both *P. falciparum* and *P. vivax* was obtained using the ICT Combo test targeting *P*HRP2 and aldolase (Ashton *et al*., 2010). The aldolase-based RDT sensitivity for *P. vivax* decreased from 85.7 to 66.7 % when the parasitaemias were lower than 500 μl⁻¹. This was, however, better than the results reported in the earlier study by Iqbal *et al*. (2002). The RDT did not perform well in heat-stability testing carried out by Ashton *et al*. (2010), but did perform acceptably in WHO/FIND testing (WHO, 2010). A Malascan aldolase/PfHRP2 RDT and a ParaHit aldolase/pLDH/PfHRP2 RDT had 68 and 15.8 % sensitivity, respectively, for *P. vivax* infections and 94 and 84.2 % sensitivity, respectively, for *P. falciparum* infections; the Malascan test did not lose sensitivity after a temperature-exposure test (Singh *et al*., 2010). BinaxNOW (Binax), which detects *P*HRP2 and aldolase, has an overall sensitivity of 100 % for *P. falciparum* and 83 % for *P. vivax* using fingerstick samples (Murray *et al*., 2008). The CareStart RDT has been used for comparison of a new VIKIA Malaria Ag Pf/Pv test detecting *P*HRP2 and non-*P. falciparum* aldolase. The VIKIA RDT had a sensitivity of 93.4 % for *P. falciparum* and 82.8 % for non-*P. falciparum* malaria, with specificities of 98.6 and 98.9 %, respectively (Chou *et al*., 2012). Van Hellemont *et al*. (2009) detected *P. knowlesi* using an aldolase antigen RDT (BinaxNOW) and found that the OptiMAL-IT test detected lower levels of parasites than the BinaxNOW test. Though aldolase-based RDTs appear not to have performed well in the studies noted above, aldolase remains an antigen worth considering. Recently, a panel of mAbs was developed against *P. vivax* aldolase. This panel was shown to be 99.23 % specific for *P. vivax*; it did not detect *P. malariae*, but did detect one *P. falciparum* out of 20 samples (Dzakah *et al*., 2013). The aldolase gene is highly conserved across the human malaria parasite species (Lee *et al*., 2006b). The low sensitivity of aldolase antigen detection could be either due to low expression levels of the antigen in infected erythrocytes and/or related to the RDT manufacturing process (Bozdech *et al*., 2003; Le Roch *et al*., 2004; Maltha *et al*., 2013).

**New malaria diagnostic targets**

Characteristics of the three malaria RDT target proteins are summarized in Table 1. A large number of studies have highlighted the difficulties encountered in detecting malaria parasites based on a single antigen or a combination of antigens using antibodies in an immunochromatographic method. The problems identified with the design and production of current RDTs and their performance should influence the development of and lead to new and improved diagnostic tests.

Data generated by Bozdech *et al*., 2003) and Le Roch *et al*., 2004 (2004) measuring the expression levels of mRNA for different malarial proteins, based on the expression of HRP2, LDH and aldolase as benchmarks, have identified a range of malarial antigens as potential diagnostic targets. Several alternative malarial diagnostic targets have been identified and explored. These include heat-shock protein 70 (*Hsp70*), dihydrofolate reductase (DHFR)—thymidylate synthase (TS), haem-detoxification protein (HDP), glutamate-rich protein, hypoxanthine phosphoribosyltransferase and phosphoglycerate mutase (Guirgis *et al*., 2012; Kattenberg *et al*., 2012a; Thézéna *et al*., 2013). These proteins are expressed throughout the life cycle of all plasmodia and their genes are highly conserved.

Hsps are highly conserved proteins that are found in all living organisms, including plasmodia (Morrison *et al*.,
During infection, the parasite is subjected to a wide range of temperature changes, from ambient (inside the mosquito) to temperatures above 37.5 °C (in a patient with a fever). In response to increases in body temperature (malaria fever), the parasite produces high levels of Hsps for its survival (Sharma, 1992). A 70 kDa protein, Hsp70, is the most abundant of the Hsp family of proteins induced during stress or infection. Anti-PfHsp70 IgM and anti-PfHsp90 IgG antibodies are found at high titres in the serum of malaria patients compared with malaria-free cohorts, suggesting high levels of malaria-specific Hsp70 (Minota et al., 1988; Zhang et al., 2001). The mRNA levels of PfHsp70 have been found to be higher than those of PfHRP2 and pLDH throughout the erythrocytic cycle (Le Roch et al., 2004). An early study reported a sensitivity of 84% and specificity of 90% for parasite detection using latex particle agglutination with either mAb against PfHsp70 or polyclonal antibody against P. falciparum (Polpanich et al., 2007). Although the RDT could not distinguish between malaria species, the authors suggested this method as a cheap diagnostic test if the ultimate goal is malaria parasite detection rather than parasite differentiation. A P. vivax recombinant HSP70 based ELISA detecting patient anti-PvHSP70 antibodies was shown to detect antibodies from P. falciparum and P. vivax patients, P. vivax with a sensitivity of 88.8% (Na et al., 2007). A gold nanoparticle-based fluorescence immunological assay has also been used to detect PfHsp70 antigen in the blood of P. falciparum-infected patients, although the sensitivity was very low (1000–2000 parasites µl⁻¹; Guirgis et al., 2012).

Haem is a product of the digestion of host erythrocyte haemoglobin and is toxic to the malaria parasite (Egan, 2008). Therefore, to protect itself, the parasite must detoxify the haem. HDP catalyses the conversion of haem into non-toxic haemozoin (Jani et al., 2008). HDP, expressed in all life stages of malaria parasites, has been found to be functionally conserved across the genus Plasmodium, with 60% sequence identity. It shares less than 15% identity with HDP from other species, and the amino acid sequence is highly conserved among P. falciparum isolates (Jani et al., 2008; Vinayak et al., 2009). mAbs against the PfHDP protein have detected similar quantities of antigen as HRP2 antibodies used in diagnostic tests. Some mAbs also detected P. vivax antigens (Kattenberg et al., 2012a).

Malaria parasite DHFR is the first folate enzyme to be identified in plasmodia and differs in structure from the bacterial or higher eukaryote homologue as it forms a bifunctional enzyme with TS (Sherman, 1979; Garrett et al., 1984). Both proteins are present on the same polypeptide chain, with DHFR on the N-terminal and TS on the C-terminal end, and are linked by a short junctional peptide (Ivanetich & Santi, 1990). DHFR–TS plays a crucial role in pyrimidine and DNA synthesis in all protozoa, and the production of tetrahydrofolate from dihydrofolate in plasmodia is highly dependent on the presence of DHFR–TS (Sherman, 1979). The existence of two additional sequences in the DHFR domain of all Plasmodium species has been reported. Importantly, these domains are not of equal size in all plasmodia (Ivanetich & Santi, 1990; Yuvaniyama et al., 2003) and the differences in these domains can therefore be assessed for Plasmodium species differentiation (Yuvaniyama et al., 2003). mAbs against the protein have detected P.
falciparum and P. vivax, and the protein is less persistent in blood compared with PfHRP2 (Kattenberg et al., 2012a). Kattenberg et al. (2012a) have also raised mAbs against glutamate-rich protein. This protein is unique to P. falciparum parasites and, like HRP2, has repeat regions. The protein has received attention as a malaria vaccine candidate (Jepsen et al., 2013).

A proteomic approach has identified parasite proteins in the plasma of malaria patients. Interestingly, LDH and aldolase, as well as phosphoglycerate mutase and hypoxanthine phosphoribosyltransferase, were identified. The authors concluded that hypoxanthine phosphoribosyltransferase is a promising malaria diagnostic target. Interestingly, levels of P. falciparum LDH and aldolase correlated with higher parasitaemia (Thézénas et al., 2013).

An alternative approach is to detect host markers rather than percentage parasitaemia or parasite proteins (or other metabolites).

Additional problems facing current rapid diagnostic tests

Discovery of, or research into, new RDTs may or may not solve the problems facing the current RDTs for malaria diagnostics. Technical problems include, but are not limited to: training of staff, RDT quality performance or assurance and checking, test quality and accuracy, and the packaging of the kits. The overall consequence of poor-quality RDTs, malfunction or inappropriate use of the test kit is poor diagnosis of the parasite, which directly influences the physician’s or clinician’s decision on antimalarial drug prescription. In turn, failure to provide malaria treatment can undermine the effectiveness of malaria RDTs, which are widely used for malaria diagnosis.

Quality and performance checking of RDTs is partly absent or non-existent in malaria-endemic countries. The WHO (2005) recommends that RDTs are implemented with a comprehensive quality-control strategy. The quality of testing and the accuracy of tests are also influenced by the ability of health workers to read and easily understand the instruction manual in the packaging of the kit (WHO, 2005; Harvey et al., 2008). Positive controls (Versteeg & Mens, 2009) are required to be available in field settings (Aidoo et al., 2012). Gillet et al. (2010) have reported on the external quality assessment of RDTs carried out in a non-endemic area. They found that errors in RDT performance were related mainly to RDT test-line interpretation, partly because of incorrect package insert instructions. The inclusion of control antigens would make a valuable contribution to studies comparing the performance of RDTs (Hendriksen et al., 2012; Jang et al., 2013).

Another key component of malaria RDTs and interpretation is the training of health workers. The majority of published articles state that all staff or health workers involved in the project have received adequate training. However, national policies for treating malaria differ between countries. Therefore, training must be carried out in connection with the brand and type of RDT being used. The training must also be designed to teach health workers problem-solving skills for when RDTs are not performing well (WHO, 2008; Maltha et al., 2013).

Conclusion

Parasitological confirmation of suspected malaria using microscopy, the gold standard, is cumbersome and requires trained personnel, microscopes and a source of electricity. Therefore, malaria treatment based on RDTs, which are quick and easy to perform, is becoming more attractive. PfHRP2- and pLDH-based RDTs are the most commonly used. PfHRP2 RDTs appear to be more sensitive than pLDH RDTs, particularly at low parasite densities, although there are exceptions (WHO, 2011b). To date, both PfHRP2 and pLDH RDTs are more sensitive than aldolase-based tests. PfHRP2-based tests are less specific than pLDH-based tests, regardless of the level of parasitaemia (Abba et al., 2011). A pLDH RDT should be employed when determining the efficacy of drug treatment, as PfHRP2 persists for long periods in the blood after parasites have cleared. RDTs targeting aldolase or DHFR–TS can also be employed, but are untested at present. Because of the persistence of the PfHRP2 antigen, PfHRP2 RDTs can detect antigen when P. falciparum parasites are sequestered in placental tissues or elsewhere, and thus parasites are not present in peripheral blood for detection by microscopy. Persistence of the PfHRP2 antigen after parasites decline in the blood leads to false positives. The Pfhrp2 gene undergoes antigenic variation, whilst the genes for Plasmodium pLDH and aldolase do not. The Pfhrp2 gene is deleted from isolates in the Amazon region and in some isolates from Africa and India (Baker et al., 2010b; Gamboa et al., 2010; Houzé et al., 2011; Kumar et al., 2013). The PfHRP2 RDT can have a prozone effect, which does not occur with pLDH or aldolase RDTs. The PfHRP2 protein has been used to detect malaria infections in samples from Predynastic Egyptian mummies (Cerutti et al., 1999). The concentration of PfHRP2 has the potential to predict progression to severe malaria (Fox et al., 2013), and to detect true severe malaria rather than severe non-malarial illness (Hendriksen et al., 2012).

Other biomarkers have been identified with potential for use in RDTs. The disadvantage is that the new markers have not been extensively tested, although the parameters for evaluating a new test are much better understood than previously.

An ‘ideal’ RDT would detect and differentiate between all human malaria species; distinguish low, medium and high parasitaemias; be available in a temperature-stable format; have internal controls for antigens; be easy to use; produce an unambiguous result; and remain cheap. Not all of the above characteristics are required in every setting. Current
malaria RDTs appear to have particular characteristics that should be taken into consideration when employing the test in a specific setting.

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