Malaria is a life-threatening, protozoan parasitic disease transmitted by female Anopheles mosquitoes, mostly prevalent throughout the tropical and subtropical regions of the world (Box 1). Currently, around 500 million cases of malaria occur every year, with between 1 and 3 million deaths, and with huge economic and social development implications for malaria-endemic areas. Of the four species of Plasmodium causing malaria in humans, Plasmodium falciparum and P. vivax are most prevalent. P. falciparum infection can progress rapidly to coma and death, and early, accurate identification of infection with this species is vital for patient therapy. P. vivax, P. malariae and P. ovale cause considerable morbidity in endemic areas and are serious health problems. As few as 10–100 Plasmodium parasites per microlitre of blood can produce significant illness in a naïve person. There

Abstract

- Malaria causes significant mortality and morbidity worldwide and is one of the major infectious diseases affecting the health of deployed Australian Defence Force personnel.
- Malaria rapid diagnostic tests (RDTs) offer great potential for early and accurate diagnosis of malaria, especially in remote areas. Many RDTs are now commercially available; however, their sensitivities have been reported to be variable.
- In this article, we examine the advantages and limitations of current malaria RDTs, analyse possible causes of the variations in sensitivity, and summarise our investigation of the effect of parasite antigen diversity on the sensitivity of RDTs that detect Plasmodium falciparum histidine-rich protein 2 (PIHRP2).
- At parasitaemias \( \leq 250 \) parasites/\( \mu \)L (enough to make a malaria-naïve person ill), a proportion of patients may return a negative PIHRP2-based RDT result because of the infecting parasite carrying a “non-sensitive” gene encoding an HRP2 protein that is not detected by the kit at sufficiently high sensitivity. Patients with persisting symptoms should have repeated tests at a later time, and microscopy should be used if at all possible.
- When choosing RDTs, the devices should be compared between different settings or assessed against locally endemic parasites. An appreciation of all limitations is critical to the evaluation of malaria diagnostic programs, in the analyses of costs, quality assurance, benefits and applicability to particular situations, to ensure patients are not misdiagnosed and timely treatment is administered.
is currently no vaccine against malaria, and the parasite has developed resistance to most of the standard, cheap antimalarial drugs. Newer antimalarial drugs and drug combinations are efficacious against malaria, but are far more expensive. The parasite’s ability to rapidly develop drug resistance presents a serious threat to maintaining efficacious drugs into the future.

Early and accurate diagnosis of malaria is critical to ensure that lives are not put at risk from a treatable disease, to ensure expensive drugs are used rationally, and to minimise the risk of resistance developing.2 Although early, accurate diagnosis of malaria also has advantages in improving management of non-malarial febrile illness through exclusion of malaria, combating multidrug-resistant malaria has been the driving force for high-quality, accurate, and affordable rapid diagnostic methods.3

**Relevance to the Australian Defence Force**

Malaria is endemic in many of the regions to which Australian Defence Force personnel deploy, and has a significant effect on ADF capacity and health. For instance, in 1999, during the first months of service in East Timor, 267 service personnel were incapacitated by malaria, despite provision of antimalarial chemoprophylaxis. Two-thirds of cases diagnosed in East Timor were caused by *P. falciparum*, and malaria episodes reported on return to Australia were primarily due to relapses of *P. vivax*.4 Many low- and moderate-transmission areas within South-East Asia are characterised by multidrug-resistant falciparum malaria, and the incidence of *P. vivax* is increasing in some areas.5 Malaria epidemics are a particular risk in the areas of social disruption to which the ADF is increasingly deploying as a stabilisation force.

**Diagnosis of malaria**

Malaria is routinely diagnosed by thick and thin blood smear microscopy, a technique that has both benefits and limitations.3,6 Microscopy allows accurate estimation of parasite density and identification of the causative species. Diagnostic microscopy can show high sensitivity — an experienced microscopist can identify infection with a parasitaemia as low as 50 P/μL. However, in non-specialised diagnostic laboratories, sensitivity may be only around 500 P/μL, by which time
A patient may be dangerously ill if the infecting species is *P. falciparum*. Microscopy requires trained and experienced microscopists, as well as provision and maintenance of microscopes, which is difficult to establish and sustain in remote areas. Laboratories within non-endemic countries do not diagnose malaria on a regular basis, so skills can be lost if diagnostic training is not undertaken regularly. Microscopy is also time-consuming, and facilities are not always available at the place and time of patient consultation in endemic areas.

Malaria rapid diagnostic tests (RDTs) using antigen capture technology have been developed over the past 15 years in the expectation that they would provide an accurate, reliable and affordable alternative to microscopy. They are available as immunochromatographic antigen–antibody capture assays in small kits (dipsticks and cassettes), and can be easily taken into the field. Such kits may be particularly useful where the electricity supply is unreliable or non-existent. RDTs are easy to use and take 15 minutes to perform, considerably less time than microscopy (Box 2). They do not require sophisticated technology or intensive training. Thus, RDTs offer great potential to improve the diagnosis of malaria, particularly in remote areas.

There are two major classes of RDTs available: those that detect *P. falciparum* only, and those that detect *P. falciparum* plus one or more other species of malaria. *P. falciparum* histidine-rich protein 2 (PfHRP2) is specific to *P. falciparum*, and is produced by the parasite 2 hours after invasion of the red blood cell. PfHRP2-detecting tests were the first type of RDT to become available, in the early 1990s, followed soon after by parasite lactate dehydrogenase (pLDH), and parasite fructose 1,6-biphosphate aldolase (aldolase) tests, which detect all four human *Plasmodium* species. Currently, the ADF uses the DiaMed OptiMAL (DiaMed, Flow Inc, Portland, Ore, USA) RDT, which is a pLDH-detecting kit.

The objectives of this article were to examine the available literature for sensitivity reported by different groups using RDTs for malaria, and to examine possible causes for observed variations. Search methods are summarised in Box 3.

### Variations in sensitivity of RDTs

Small-scale field studies of malaria RDTs initially indicated the tests had good sensitivity ranges, particularly for densities of *P. falciparum* greater than 500 P/μL. However, more recent studies have highlighted the variability in sensitivity and reliability of RDTs at both high and low levels of parasitaemia. Historically, there appear to be geographic differences in the sensitivity of PfHRP2-detecting RDTs, with reduced sensitivity particularly noted in some patients with parasitaemia < 500 P/μL. Variable sensitivity was reported for the same RDT tested in different geographic
areas, and for different RDTs tested in the same geographic area. In trials of RDTs detecting aldolase and pLDH, unsatisfactory sensitivity at relatively low parasitaemias in different geographic settings has also been reported.30,31 Some pLDH tests showed sensitivities as low as 37.5% at parasitaemias ≤ 1000 P/μL.29-32

The variability in sensitivity of these tests is of concern if early treatment intervention is to be based on diagnosis by RDT. Currently, the World Health Organization recommends a lower sensitivity limit of detection for a non-microscopic rapid diagnostic test for P. falciparum of 95% at a parasitaemia of 100 P/μL.33 This is similar to the level of sensitivity achieved by a well trained, experienced microscopist. The development of such capability presents a challenge to scientists and product manufacturers. Some recent reviews have examined the technical limitations of malaria RDTs,3,6,8,12,34 and highlighted several shortcomings of current tests.

- Only a small pool of detecting antibodies has been tested. Expert opinion has recommended a broader range of monoclonal antibodies be tested to allow a broader range of the antigen epitope availability for binding.3
- Although RDT performance approaches the sensitivity and specificity achieved by good microscopy at parasite densities greater than 500 P/μL, it rapidly declines at lower parasite densities.11,17,19,23-25,27,30 Tests that detect pLDH are reported to be less sensitive at lower parasitaemias than PfHRP2-detecting tests in some areas.32
- Most RDTs that detect multiple species do not differentiate non-P. falciparum species from each other, nor do they differentiate mixed infections of P. falciparum and non-P. falciparum from P. falciparum monoinfection.3 This is important, as treatment regimens differ for P. falciparum and the other human species of malaria.
- Current tests are essentially qualitative. Although the intensity of the results band correlates positively with parasite load, it does not quantitate the risk of developing severe complication for that patient. In many situations, a quantitative assessment of parasitaemia would be an important characteristic of a test.6,35 In humanitarian work in endemic areas, for instance, a large proportion of the population will test positive in any qualitative assay, and the clinical relevance of the parasitaemia then needs to be determined. The currently available tests also cannot distinguish between continuing asexual parasitaemia and gametocyttaemia, which is of importance to transmission and to the choice of drugs used to treat the illness.
- False-positive PfHRP2 RDT results have been reported in patients with rheumatoid-factor-positive rheumatoid arthritis.36-40 Some reports detail the false-positive results returned by tests that use an IgG antibody.37 Debate exists concerning whether fewer false-positive reactions are found with kits that use a monoclonal IgM antibody.37,40 There are also concerns about cross-reactivity with pLDH-detecting tests due to heterophile or anti-mouse antibodies or unknown factors.
- A major area for concern in the application of RDTs in tropical countries is their limited shelf life and their susceptibility to degradation by excessive heat and humidity.7,41 Prolonged exposure to temperatures greater than 30°C is likely to reduce sensitivity, necessitating cool chains where possible for transport and storage.7
- For some countries, the cost of RDTs may be prohibitive, and commercial interest and technical capability to produce RDTs at a cost that many of the poorer countries could afford are difficult to achieve.42,43 However, the high cost of newer antimalarial drugs makes it worthwhile to pay more for accurate diagnosis to prevent the use of expensive drugs in people without malaria infection.

**Addressing parasite variation**

The reported variability in sensitivity of RDTs in the field has led WHO and others to begin examining the factors affecting the performance of the tests.44 Variations in sensitivity of PfHRP2-detecting RDTs may be partially attributable to genetic heterogeneity of the PfHRP2 protein.8 This heterogeneity is important if a proportion of the parasites produce variant alleles of PfHRP2 that lack the epitope or have fewer epitopes recognised by monoclonal antibodies. Patients infected with these parasites may be misdiagnosed as malaria-negative without concurrent microscopic examination.

In a collaborative project involving the authors, with the University of Queensland, the Queensland Institute of Medical Research, the University of the Philippines, the Research Institute for Tropical Medicine in the Philippines, and WHO, the variability of the PfHRP2 antigen in parasites from geographically diverse areas was investigated. The aim was to examine the effect of this antigen variability on the sensitivity of PfHRP2-detecting RDTs. The few published sequences previously available suggested that the protein varied between different strains of the parasite.45 We hypothesised that a significant part of the variability observed in PfHRP2 RDTs is attributable to variability in the target antigen.

We examined the genetic diversity of PfHRP2, which includes numerous amino acid repeats, in 153 P. falciparum lines and isolates originating from 25 countries, and tested a subset of parasites using two PfHRP2-detecting RDTs. We extracted DNA from parasite samples and amplified the hrp2 gene by polymerase chain reaction, then sequenced the product. For analysis, DNA sequences were translated to protein sequences. We observed extensive diversity in PfHRP2 sequences, both within and between countries.46,47 Gene deletions were not observed, except in two laboratory clones. We cultured in vitro a subset of parasites for which the sequence was complete, made serial dilutions, and tested their detection limits on two RDTs.
Logistic regression analysis indicated that certain types of repeats within the sequence of the protein were predictive of RDT sensitivity (accuracy, 88.9%), with predictions suggesting that only 77% of *P. falciparum* parasites in the Asia–Pacific region are likely to be detected at densities ≤ 250 P/μL. The proportion of parasite isolates predicted as not being identified at this parasitaemia varied between individual countries. These findings provide an alternative explanation for the variable sensitivity in field tests of malaria RDTs, one that is not due to the quality of the RDTs.

**Future directions**

It would be ideal if RDTs could detect both high and low density parasitaemias. For the ADF, sensitivity at low parasitaemia is extremely important, because most of our personnel are malaria-naïve and are most likely to develop symptoms at low parasite densities. People faced with stressful situations, such as military deployment, refugees and displaced persons, and trauma patients, while not more susceptible to infection, are at much higher risk of recrudescence of an existing malaria infection.48 Malaria parasitaemia can multiply rapidly, potentially leading to serious complications for the patient within a few days.

At the Australian Army Malaria Institute, we are continuing our investigation into the genetic variation of the parasite’s histidine-rich proteins, and our collaborators are actively examining new antibodies to overcome the issues associated with antigen diversity. We have also begun determining transcription and expression levels of the *hrp2* gene and protein. The information on PfHRP2 variation has already aided WHO in assessing areas where the sensitivity of RDTs may be impaired, and in the selection and characterisation of parasites from different areas for the testing panel of RDTs. This will assist RDT-purchasing countries to better evaluate and choose appropriate RDTs, as well as providing a starting point for research into improving the current RDTs. WHO is also investigating the heat stability of anti-PfHRP2 monoclonal antibodies in collaboration with the Queensland Institute of Medical Research and the Australian Army Malaria Institute. Assessing the variations in sensitivity of RDTs that detect parasite aldolase and pLDH is now a priority task, as the areas in which *P. vivax* is endemic are increasing.

**Conclusion**

Rapid diagnostic tests for malaria offer great potential for accurate diagnosis and timely treatment of malaria patients. However, there are limitations in the sensitivity of current RDTs. Awareness of the sensitivity variations and other limitations of malaria RDTs is important for ADF medical personnel deploying to malaria-endemic areas, and for ADF laboratory staff in Australia, so that patients are not misdiagnosed by false-negative RDT results.49

The ADF currently uses the DiaMed OptiMAL RDT. Although the sensitivity of the OptiMAL test for *P. falciparum* may be lower in some global regions than the sensitivity of PfHRP2-detecting tests, this is compensated for by the feature of the OptiMAL test in detecting both falciparum and vivax malaria. For areas in which the ADF is currently deployed, this benefit outweighs the use of a test that detects PfHRP2 alone.

A patient with clinical symptoms of malaria, either in a malaria-endemic area or recently returned from an endemic area, should be immediately investigated using both an RDT and microscopy if microscopy is available. A negative RDT where clinical symptoms persist should be investigated further with RDTs and microscopy, within 24 hours of the initial test being performed, concurrently with investigation for other diseases presenting similar symptoms to malaria, such as dengue.

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**Competing interests**

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