Malaria diagnostics: now and the future

PETER L. CHIODINI[1,2]*

[1] *Hospital for Tropical Diseases, London WC1E 6JB, UK*
[2] *The London School of Hygiene and Tropical Medicine, London WC1E 7HT, UK*

(*Received 18 March 2014; revised 27 July 2014; accepted 30 July 2014; first published online 12 September 2014*)

SUMMARY

Light microscopy of stained blood films is still the mainstay of malaria diagnosis in many regions, but its pre-eminence is threatened by accurate and sensitive rapid diagnostic tests (RDTs) based on immunochromatography which are now widely used in the field. In well-resourced regions, nucleic acid detection easily out performs microscopy and RDTs. This paper reviews the main *in vitro* methods for parasite detection and considers future trends in diagnostics, both for sophisticated laboratory settings and for field use.

Key words: malaria, microscopy, rapid diagnostic tests, nucleic acid detection.

INTRODUCTION

Malaria parasites of humans were first described by Alfonse Laveran (Laveran, 1880). His drawings, made using a light microscope without the benefit of staining bear testament to acute observational skills. Identification of malaria parasites was made more accurate and arguably more reproducible by Romanowsky's staining methods, developed in 1891 (Gilles, 1993). More than 100 years later, Romanowsky-stained blood films using Giemsa or Field stain are still widely deployed as first-line diagnostics for malaria. However, lack of microscopy facilities in many malaria-endemic areas and the advent of new technologies are now eroding the formerly pre-eminent position of blood film examination.

Malaria parasite species to be considered

The 'big four' species have now become 6. The zoonotic parasite *Plasmodium knowlesi* is now recognized as the cause of many human cases of malaria in the Asia-Pacific region, many of them previously misdiagnosed as *Plasmodium malariae*, which it closely resembles. Furthermore, *Plasmodium ovale* has been shown to consist of 2 sympatric and morphologically identical sub-species, *P. ovale curtisi* and *P. ovale wallikeri*.

In 2010, the World Health Organisation (WHO) stated that in all settings, clinical suspicion of malaria should be confirmed with a parasitological diagnosis (WHO, 2010). The individual methods used to achieve this goal must be appropriately selected for the clinical setting concerned and may differ considerably between locations.

* Corresponding author: Department of Clinical Parasitology, Hospital for Tropical Diseases, Mortimer Market, London WC1E 6JB, UK. E-mail: peter.chiodini@uclh.nhs.uk

METHODS CURRENTLY IN USE

Light microscopy

Examination of stained thick and thin blood films is still widely deployed in the temperate zone for the diagnosis of imported malaria in travellers or migrants, mainly by haematology laboratories. Where available, microscopy is also used extensively by national malaria control programmes, for example in India where millions of tests are performed annually (Kumar *et al*. 2007).

Once the capital outlay to purchase a good microscope has been made, the major expense of light microscopic diagnosis of malaria is labour costs, which vary by geographical location, but even in regions with high wages malaria microscopy remains a relatively inexpensive test.

There are major advantages for microscopy in the diagnosis of malaria. Within an hour of making blood smears it is possible to demonstrate the species present, assess parasitaemia (essential in the case of *Plasmodium falciparum* or *P. knowlesi* infections), distinguish asexual stages from gametocytes, and demonstrate the presence of mixed species infection. Furthermore, unlike malaria-specific diagnostic methods, light microscopy can show the presence of additional parasites (e.g. microfilariae) or make alternative diagnoses, for example African trypanosomiasis. Blood film examination also provides information on red blood cell and white blood cell morphology and a differential white blood cell count. The limit of sensitivity of blood film examination depends on optimal staining methods and is highly observer-dependent. A competent microscopist

Parasitology (2014), **141**, 1873–1879. © Cambridge University Press 2014
doi:10.1017/S0031182014001371

https://doi.org/10.1017/S0031182014001371 Published online by Cambridge University Press

should routinely be able to detect 50 parasites per microlitre. The limit of detection for a reference laboratory microscopist is 5 parasites per microlitre, but that level of sensitivity is unlikely to be achieved on every slide and most microscopists would struggle routinely to achieve a limit of detection of below 20 parasites per microlitre.

Ensuring accuracy and consistency in malaria microscopy requires a well-maintained microscope, standard operating procedures for preparation and staining of blood films and internal quality control (e.g. of stain quality and re-examination of selected blood films by a second microscopist). The British Committee for Standards in Haematology Guidelines for the laboratory diagnosis of malaria recently revised (Bailey *et al*. 2013) provide the detailed methodology for preparation and staining of blood films. They are intended for use in the UK, but are relevant to laboratory practice in other non-endemic areas.

External Quality Assessment of a laboratory's performance is essential, both to reassure users of the service and to provide information to the Laboratory Director on the strengths and weakness of the service provided, which feeds into continuous quality improvement. The WHO Regional Office for Africa in conjunction with the National Institute for Communicable Diseases, National Health Laboratory Service, South Africa includes malaria in its Microbiology External Quality Assessment (EQA) Programme for national reference laboratories in Africa and the WHO malaria microscopy quality assurance manual (available at http://www.who.int/malaria/publications/malaria_microscopy_QA_manual.pdf) provides detailed information to assist national malaria control programmes in providing sustainable malaria microscopy programmes.

How good is malaria microscopy in the UK? The UK National EQA Scheme for Blood Parasitology began in 1986 in response to concerns about poor performance in a pilot Parasitology EQA survey conducted in the early 1980s among UK diagnostic laboratories (Ridley, unpublished). In the current established scheme, 8 distributions, each containing 1 or 2 stained blood films are despatched each year. Participants are given a numerical score for their reports. Analysis of results for the first 15 years showed a statistically significant rise in performance in laboratories taking part since the start of the scheme (Kettelhut *et al*. 2003). Performance on slides containing more than 1 malaria species was poor, with only 13–27% of reports showing correct identification of both malaria species present in mixed infections. Detection of *P. falciparum* was good, but many participants failed to calculate the parasitaemia and for those who did, 13–39% (depending on the sample) failed to do so correctly. Writing a decade later, the same EQA scheme reported that for slide distributions containing *P. falciparum*, between 4 and 54% (mean 23% and median 21·5%) failed to estimate the parasitaemia; between 9 and 35% (mean 18% and median 15%) overestimated; and between 0 and 25% (mean 6% and median 4%) underestimated (Manser *et al*. 2013). This suggests that performance in this area has reached a plateau and it will be difficult to make further improvements without much greater exposure to positive malaria specimens against a background of declining numbers of global malaria cases. A further challenge will come as new non-microscopic methods for malaria diagnosis risk deskilling laboratory staff in malaria microscopy.

Rapid diagnostic tests (RDTs)

RDTs, sometimes referred to as dipsticks, are lateral flow devices which use immunochromatography to detect soluble malarial antigens. The principle of RDT action is summarized in Fig. 1 (WHO, 2012).

One of the earliest reports of RDT use was at ICOPA 7 in 1990 and the first RDT product to be marketed was ParaSight F in 1995. Unfortunately, it was not commercially successful, because at that time it was cheaper to give a treatment course of chloroquine, then in extensive use in the tropics, than to make a parasite-based diagnosis of malaria with an RDT. Thus, ParaSight F was taken off the market in 2000 (see http://nihrecord.od.nih.gov/newsletters/2010/06_25_2010/story7.htm).

With the advent of artemisinin-based combination therapy for malaria, it became cost-effective as well as clinically safer, to base a treatment decision on the result of a properly performed test using a good quality RDT. As a result, the global market in RDTs rose substantially, reaching 205 million in 2012.

A variety of products is available. Most are in the form of a cassette, but dipstick; card; cassette and dipstick; and card and cassette formats are also marketed.

The principal target antigens for RDTs are histidine-rich protein 2 (HRP2) a water-soluble antigen produced by *P. falciparum* only; the glycolytic enzyme plasmodial lactate dehydrogenase (pLDH) whose isotypes can be used to detect *P. falciparum* or *Plasmodium* genus; or another glycolytic enzyme, plasmodial aldolase, used to detect *Plasmodium* genus.

RDT products are thus available to detect single species, for example *P. falciparum* or *Plasmodium vivax; P. falciparum* and pan malaria species; *P. falciparum* and *P. vivax*, their selection for use depending on the species type likely to be encountered in the country of use. At present, there are no RDTs specifically able to identify *P. knowlesi*. HRP2-based tests detect *P. falciparum* alone, so cannot be expected to detect *P. knowlesi*, whereas aldolase-based tests can do so, but may miss low parasitaemias and are non-specific, indicating the presence of

https://doi.org/10.1017/S0031182014001371 Published online by Cambridge University Press

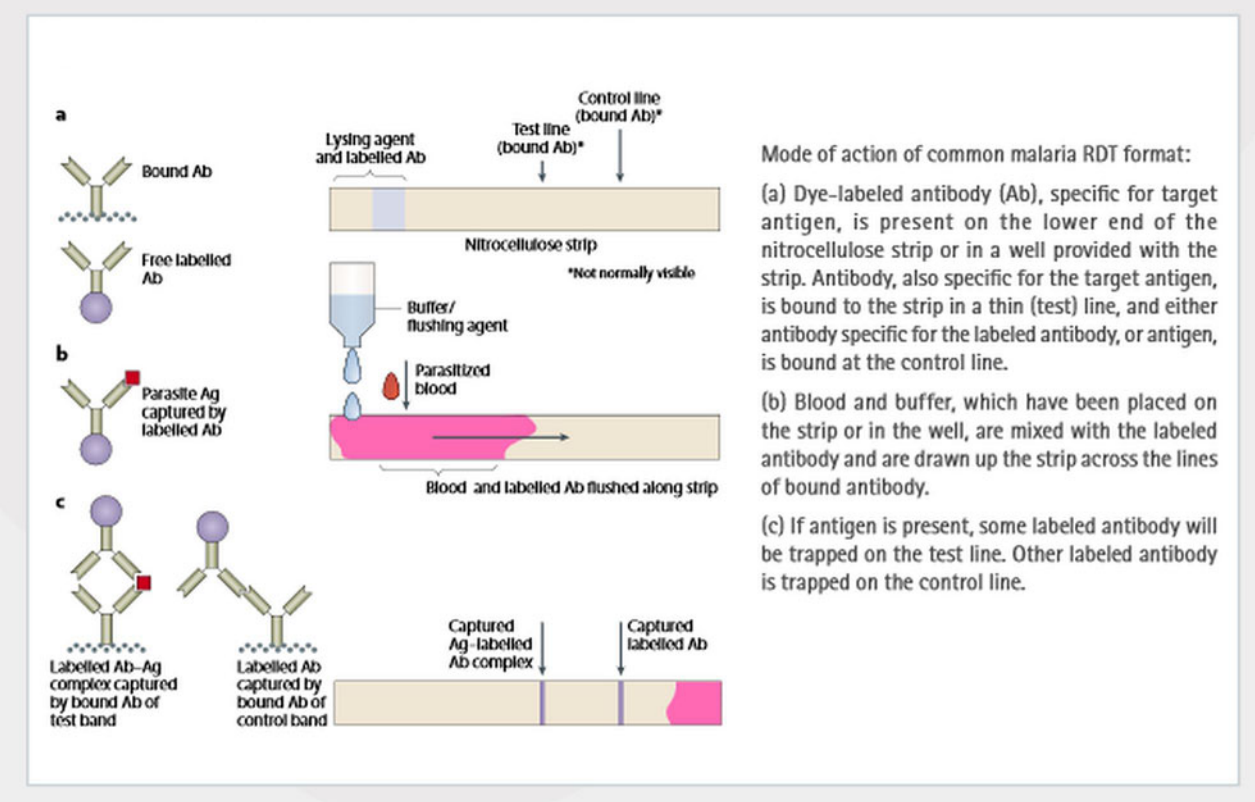


Fig. 1. Mode of action of antigen-detecting malaria RDTs. Reproduced from: World Health Organisation (2012) *Malaria RDT Performance*. Results of WHO product testing of malaria RDTs: 4 (2012). WHO, Geneva, Switzerland.

malaria parasites but not naming the species (Bronner *et al*. 2009; Van Hellemond *et al*. 2009); pLDH-based tests can also detect *P. knowlesi*, but cannot identify it from a mixed infection with both *Plasmodium vivax* and *P. falciparum* (McCutchan *et al*. 2008) and cross-reactivity with the falciparum test line led to mostly '*P. falciparum*' results in *P. knowlesi* samples tested with 1 particular pLDH-based RDT as noted by Foster *et al*. (2014). The same authors reported '*P. vivax*' results using another brand of RDT on polymerase chain reaction (PCR)-confirmed *P. knowlesi* specimens. There is a clear need for field-ready new diagnostics for *P. knowlesi* (Jeremiah *et al*. 2014).

RDT issues in practice

In 1999, a WHO informal consultation on malaria diagnostics set a target sensitivity for malaria RDTs as 95% at 100 parasites per microlitre (WHO, 2000).

As RDTs which had performed well in early studies came into use on a larger scale, reports from the field began to appear describing sensitivity for parasite detection significantly lower than that required for operational use (summarized in Chiodini *et al*. 2007). The explanations for this differ between reports, but include operator error; antigenic polymorphism of the analyte to be detected; the operational difficulties of working in remote areas; and damage to the RDT during either or both storage and transport under field conditions.

Poorly performing RDTs which miss a diagnosis of malaria are not only a hazard to patient safety, but also risk bringing the concept of non-microscopic parasite-based diagnosis of malaria in regions where there is no microscopy into disrepute, thus threatening the programme of Artemisinin – based Combination Therapy (ACT)-based treatment directed by RDT results.

Good data on the performance of individual RDT products were thus required to support procurement decisions made by national malaria programmes. Therefore in 2006, the WHO and Foundation for Innovative New Diagnostics began an evaluation programme to assess malaria RDT performance against a standard set of samples, which would permit the direct comparison of commercially available products. Not only would this assist those making procurement decisions, it would also encourage improvements in manufacturing. The Round 1 product testing report was published in 2009 (WHO, 2009) and the results of Round 5 are expected before the end of 2014.

WHO product testing assesses RDT performance on the following sample types: 20 culture line *P. falciparum* at 200 and 2000 parasites per microlitre; 99 wild-type *P. falciparum* at 200 and 2000 or 5000 parasites per microlitre; 35 wild-type *P. vivax*

https://doi.org/10.1017/S0031182014001371 Published online by Cambridge University Press

at 200 and 2000 or 5000 parasites per microlitre, all PCR-confirmed as single-species infections. Results are expressed in percentages as a panel detection score. To look for false positives and cross-reactivity with non-malarial diseases, RDTs are also tested against samples from healthy volunteers; and panels of sera which are Antinuclear Antibody (ANA) positive, anti-mouse antibody positive, rheumatoid factor positive, RPR positive, *Trypanosoma cruzi* antibody positive, dengue antibody positive, *Leishmania* antibody positive and *Schistosoma* antibody positive representing some of the main infections which might mimic malaria (WHO, 2009).

In order to be placed on the WHO procurement list, an RDT must achieve a *P. falciparum* panel detection score of 75% or more at 200 parasites per microlitre; a *P. vivax* panel detection score of 75% or more at 200 parasites per microlitre; a false-positive rate of <10%; and an invalid rate of <5% (http://www.wpro.who.int/malaria/NR/rdonlyres/3B0EEC88-A85A-4C66-BED8-46F13F47D087/0/RDT_selection_criteria2012.pdf).

As at September 2013, Rounds 1–4 inclusive had evaluated 168 RDTs (124 unique products) and 53 products had been placed on the WHO procurement list: 25 *P. falciparum*; 26 combo; 1 pan; and 1 *P. vivax* detecting RDTs (http://www.wpro.who.int/sites/rdt/home.htm).

Whilst RDTs have control lines which indicate whether the device has performed properly, they do not contain positive controls. As RDTs are used extensively in the field, it is highly desirable to be able to check their performance on known malaria antigen-positive samples at regular intervals in the diagnostic setting. In that way, end-users will have reassurance that their RDTs are still capable of detecting malaria antigen and thus have greater confidence to act clinically on the results. Positive control wells, which themselves must be stable under field conditions, are under development. Various formats are possible, but a disposable tube containing a panel of freeze-dried recombinant antigens which reflects the target epitopes of the RDTs in use and could be reconstituted for single use would be stable and readily made in large numbers.

RDTs are very unlikely to replace microscopy completely, as current formats do not measure parasitaemia, do not distinguish asexual from sexual stages, and do not make species-specific diagnosis of *P. ovale, P. malariae*, or *P. knowlesi* infections, but their considerable strength is in bringing reliable diagnosis of malaria to locations where previously there was none and providing hitherto unavailable public health data. In that context RDT use can result in a substantial reduction in antimalarial prescribing if the end-user acts on the test results (Odaga *et al*. 2014).

Malaria RDTs also have a role in non-endemic areas where malaria is seen as an imported disease which may be misdiagnosed initially and where high level expertise in malaria microscopy is a challenge to maintain in centres which might see only 1 or 2 cases per year. Chilton *et al*. (2006) surveyed the use of RDTs for diagnosis of malaria in the UK. RDTs plus or minus microscopy were found to be the preferred choice in 31·2% of laboratories during normal working hours and in 44·3% of laboratories during on-call hours. RDT use had changed the diagnosis in 12% of laboratories, which supports the view that use of RDTs in combination with microscopy enhances malaria diagnosis. Where both methods are available, microscopy should continue to be undertaken on all malaria requests even if RDTs are used and that remains the recommendation of the British Committee for Standards in Haematology (Bailey *et al*. 2013).

EQA data show that performance of malaria RDTs in British laboratories is good. For example, in a distribution of lysed blood containing *P. falciparum* at 500 parasites per microlitre, equivalent to 0·01% parasitaemia, only 2 of 299 laboratories failed to report it correctly (UK NEQAS, unpublished).

A more contentious area is whether or not RDTs should be recommended for use by individual travellers. Nothdurft *et al*. (2008) reviewed published data generated by well individuals performing the test pre-travel, febrile travellers in a malarious area, and febrile returning travellers in a resource-rich non-endemic country. They noted a significant false-negative rate on *P. falciparum*-positive samples tested with HRP2-detecting RDTs by travellers, both pre-travel and in the tropics. Febrile returning travellers did better, but they had received intensive instruction by a specialist in travel medicine and a consultant physician. Nothdurft *et al*. concluded that use of RDTs for self-diagnosis by travellers should not be recommended routinely. They should be advised only for specific situations: long-term stay; travellers far from medical assistance; and expedition-type travel, after appropriate instruction and training, including successful performance of the test procedure.

Nucleic acid detection tests (NAT)

Parasitology is becoming increasingly molecular and nucleic acid-based detection of malaria parasites is now routine in reference laboratories. Nested (Snounou *et al*. 1993), single-round multiplex (Padley *et al*. 2003) and real-time methods (Kamau *et al*. 2013) are well established, and capable of detecting malarial parasitaemia with a limit of detection at least 10-fold better than the best microscopists. Furthermore, molecular methods have permitted separation of *P. ovale* into 2 separate but sympatric species, *P. ovale curtisi* and *P. ovale wallikeri* (Sutherland *et al*. 2010) and revealed the importance of *P. knowlesi*, previously misdiagnosed

https://doi.org/10.1017/S0031182014001371 Published online by Cambridge University Press

as the morphologically very similar *P. malariae*, as a zoonotic infection of humans (Singh and Daneshvar, 2013). RNA-based detection can be even more sensitive and can distinguish asexual from sexual stages (Beurskens *et al*. 2009).

Nucleic acid-based detection does not yet provide a numerical value for percentage parasitaemia or parasites per microlitre as used by clinicians, so it is still necessary to examine peripheral blood films in order to do so. However, it is now possible to examine the efficacy of anti-malarial drugs *in vivo* using quantitative PCR measurement of parasite clearance (Beshir *et al*. 2010).

Conventional PCR requires use of a thermal cycler and real-time PCR requires significant outlay for capital equipment, so isothermal methods for DNA amplification have been explored. The loop-mediated amplification (LAMP) method (Poon *et al*. 2006) uses DNA polymerase from *Bacillus stearothermophilus* which is both isothermal and has strand displacement activity. Amplification product can be detected visibly under UV fluorescence or via densitometry. Polley *et al*. (2010) examined the performance of LAMP in malaria parasite detection using newly developed mitochondrial primers. With primer MtPfID869 specific for *P. falciparum*, LAMP detected 5 parasites per microlitre in 23 out of 25 replicates and with MtPgID4, which amplifies all species of malaria, LAMP detected 2 parasites per microlitre in 22 out of 25 replicates. Amplification times were under 30 min. A study in returned travellers at the Hospital for Tropical Diseases London compared the performance of LAMP using *Plasmodium* genus and *P. falciparum* primers with that of nested PCR (which takes 16 h). With LAMP, a single staff member was routinely able to process 14 patient samples to a final result in 90 min. Using *P. falciparum* primers, LAMP showed 98·4% sensitivity and 98·1% specificity compared with nested PCR; and 97·0% sensitivity and 99·2% specificity using *Plasmodium* genus primers (Polley *et al*. 2013). The individual method of sample preparation using the LAMP Pure device reduces the chance of cross-contamination and its simplicity renders this method usable by non-parasitologists, which would make it attractive to general laboratories in industrialized countries called upon to diagnose cases of imported malaria. Such cases however, represent a tiny fraction of the global burden of malaria, so will LAMP have a place in malaria-endemic regions? Hopkins *et al*. (2013) assessed its performance in a remote clinic in Uganda. Two technicians performed the LAMP assay after 3 days of training and compared with 3-well nested PCR, sensitivity of both LAMP and single-well nested PCR was 90%. In samples containing greater than or equal to 1 parasite per microlitre, LAMP detected malarial DNA in 145 of 149 (97·3%) compared with 3-well nested PCR and even in specimens with less than 1 parasite per microlitre, sensitivity was 66% *vs* 3-well-nested PCR. This performance is greatly superior to that achievable routinely by microscopy. A high-throughput version of LAMP would be of potential benefit in seeking cases of sub-microscopic parasitaemia in malaria eradication programmes.

As more and more nucleic acid detection assays appear, it becomes highly important to be able to compare their performance objectively against known standards. Those laboratories using NAT to detect DNA should use the WHO International Standard for *P. falciparum* DNA (Padley *et al*. 2008). The standard equates to approximately 9·8% parasitaemia and contains 10^9 International Units (IU) per mL. It is available from The National Institute for Biological Standards and Control (NIBSC; http://www.nibsc.org/;NIBSC code number 04/176). Use of the International Standard both for assay development and standardization and to calibrate run controls is strongly recommended. Standards for non-falciparum species and for RNA-based nucleic acid detection will need to be developed.

Just as EQA schemes are in place for malaria microscopy and for malaria RDTs, so there is a compelling need for EQA of nucleic acid-based assays, many of which are in-house methods. UK NEQAS is in the pre-pilot phase of a Molecular EQA Scheme for Malaria DNA detection which is scheduled to despatch its first samples for testing in mid-2014.

THE FUTURE

Advances in computer-based imaging analysis have made it worthwhile to evaluate digital microscopy. If successful, it would overcome the issues of fatigue and inter-observer error inherent in light microscopy, although the ability to fine focus up and down, so valuable in distinguishing parasites from artefacts or counting merozoites in a schizont, would be a challenge to replicate.

More sensitive RDTs, using different target antigens, alternative detection formats, machines to read them and increase the ability to detect what are currently seen as faint lines, are realistic goals.

As malaria eradication programmes gain momentum, there will be an associated need to detect and understand the role of parasitaemias undetectable by microscopy and the currently available RDTs. This demand could be met by high-throughput platforms based on nucleic acid detection or by other technologies if they can match NAT sensitivity.

For the diagnosis of individual patients, good microscopy and well-performing RDTs already function safely and well in clinical use. As nucleic acid-based tests expand their detection range further, it is likely that malaria primers will be included in multiplex assays; for example a panel for investigation of fever in a returning traveller. In some

https://doi.org/10.1017/S0031182014001371 Published online by Cambridge University Press

reference laboratories, malaria genus primers have already been included in PCR assays for the detection of viral haemorrhagic fever (VHF) since malaria is the commonest single-infection diagnosis in those suspected of having VHF.

Unlike the situation with bacteriology, it is not possible to provide MIC or IC50 data for patient-derived malaria parasites in clinically useful time. However, once the appropriate molecular markers of drug resistance are validated, see for example Gaillard *et al*. 2013, it will become possible to use real-time PCR to provide data on the sensitivity profile of the parasite concerned during the first 24 h of treatment.

ACKNOWLEDGEMENTS

PLC is supported by the National Institute for Health Research UCL Hospitals Comprehensive Biomedical Research Centre Infection Theme. His laboratory receives funding from the Foundation for Innovative New Diagnostics for work on the LAMP assay.

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