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A Review of Malaria Diagnostic Tools: Microscopy and Rapid Diagnostic Test (RDT)

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Abstract. The absolute necessity for rational therapy in the face of rampant drug resistance places increasing importance on the accuracy of malaria diagnosis. Giemsa microscopy and rapid diagnostic tests (RDTs) represent the two diagnostics most likely to have the largest impact on malaria control today. These two methods, each with characteristic strengths and limitations, together represent the best hope for accurate diagnosis as a key component of successful malaria control. This review addresses the quality issues with current malaria diagnostics and presents data from recent rapid diagnostic test trials. Reduction of malaria morbidity and drug resistance intensity plus the associated economic loss of these two factors require urgent scaling up of the quality of parasite-based diagnostic methods. An investment in anti-malarial drug development or malaria vaccine development should be accompanied by a parallel commitment to improve diagnostic tools and their availability to people living in malarious areas.

INTRODUCTION

The wide range of 200 million in the frequently quoted "300–500 million cases per year" in itself reflects the lack of precision of current malaria statistics. Any attempt to estimate the number of malaria cases globally is likely to become subject to argument.[1,2] Had accurate malaria diagnosis been achieved together with an improved public health data reporting system and healthcare access, such a conjecture would be lessened.

Clinical diagnosis is imprecise but remains the basis of therapeutic care for the majority of febrile patients in malaria endemic areas, where laboratory support is often out of reach. Scientific quantification or interpretation of the effects of malaria misdiagnosis on the treatment decision, epidemiologic records, or clinical studies has not been adequately investigated. Despite an obvious need for improvement, malaria diagnosis is the most neglected area of malaria research, accounting for less than 0.25% ($700,000) of the U.S.$323 million investment in research and development in 2004.[3]

Rational therapy of malaria is essential to avoid non-target effects, to delay the advent of resistance, and to save cost on alternative drugs. Accurate diagnosis is the only way of effecting rational therapy. Confirmatory diagnosis before treatment initiation recently regained attention, partly influenced by the spread of drug resistance and thus the requirement of more expensive drugs unaffordable to resource-poor countries.[4] This review focuses on microscopy and rapid diagnostic tests (RDTs), the two malaria diagnostics that are likely to have the largest impact on malaria control today.

MALARIA DIAGNOSTIC METHODS

Clinical diagnosis. Clinical diagnosis is the least expensive, most commonly used method and is the basis for self-treatment. However, the overlapping of malaria symptoms with other tropical diseases impairs its specificity and therefore encourages the indiscriminate use of anti-malarials for managing febrile conditions in endemic areas. Although highly debatable, this practice was understandable in the past when inexpensive and well-tolerated anti-malarials were still effective.[5,6]

Accuracy of a clinical diagnosis varies with the level of endemicity, malaria season, and age group. No single clinical algorithm is a universal predictor.[7,8] Studies of fever cases in populations with different malaria-attributable proportions from Philippines, Sri Lanka, Thailand, Mali, Chad, Tanzania, and Kenya have shown a wide range of percentages (40–80%) of malaria over-diagnosis and its associated potential for economic loss.[7–13]

Only in children in high-transmission areas can clinical diagnosis determine the treatment decision.[14,15] In this situation, a majority of the population is chronically parasitemic; malaria may be concomitant but not the responsible agent of the febrile illness.

Biologic diagnosis. In 1904, Gustav Giemsa introduced a mixture of methylene blue and eosin stains.[16] Microscopic examination of Giemsa-stained blood smears has subsequently become the gold standard of malaria diagnosis.

In the past 50 years, alternative methods became available (e.g., detection of malaria antibodies by indirect immunofluorescence antibody assay [IFA] and enzyme-linked immunosorbent assays [ELISA]).[17,18] Later, scientists developed methods to detect malaria antigens, the most significant being the immunochromatographic assay, which forms the basis of commercial malaria RDTs available today.[19,20]

Molecular methods, namely, DNA probes and polymerase chain reaction (PCR) were introduced in the 1980s–1990s.[21,22] Methods for detecting malaria parasites by fluorescent staining also emerged (e.g., by the quantitative buffy coat [QBC] analysis, interference filter system for acridine orange-stained thin blood smear, and flow cytometry).[23–26] Detection of malaria pigments by depolarized laser light and mass spectrometry showed limited success.[27,28]

Giemsa microscopy. In spite of a variation in the basic targets of malaria control from elimination of mortality and minimizing morbidity to reducing prevalence or eradication, all malarious countries share a common need for reliable laboratory-diagnostic services to ensure early and rational treatment, reliable epidemiologic information, and epidemic preparedness. Giemsa microscopy is regarded as the most suitable diagnostic instrument for malaria control because it is inexpensive to perform, able to differentiate malaria species,

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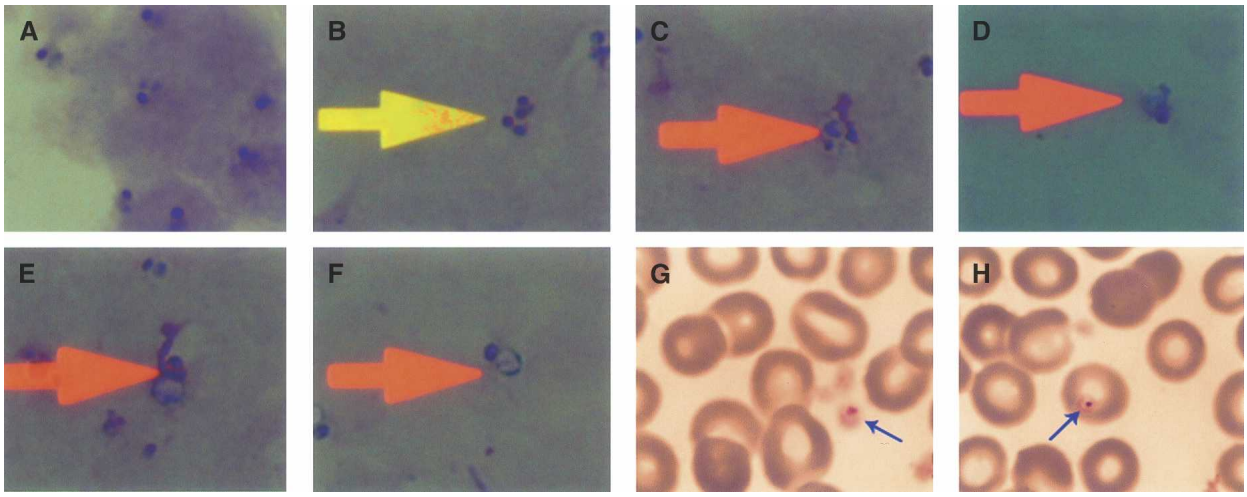

FIGURE 1. Giemsa-stained thick blood films showing early trophozoites (ring form, $N = 7$) of *P. falciparum* in a specimen with high parasite density (**A**), two ring forms that look like artifacts (**B**), and various artifacts that resemble *P. falciparum* trophozoites on thick (**C–F**) and thin (**G–H**) films. (Courtesy of J. K. Baird.)

and quantify parasites. In the age of high-quality light-emitting diode (LED) illumination and solar battery chargers, microscopy has become more feasible in remote areas. However, microscopy requires well-trained, competent microscopists and rigorous maintenance of functional infrastructures plus effective quality control (QC) and quality assurance (QA).

During the Malaria Eradication (ME) era, microscopy was the mainstay of malaria diagnosis. Despite the organizational and logistic challenges, numerous countries accounting for 50% of the residents of originally malarious areas successfully eliminated the disease.[29] Many countries, particularly in southern Asia and the Americas, however, did not succeed in creating or maintaining the system and eventually diagnostic services were given little attention. ME was never considered a feasible objective in tropical Africa.

Countries with well-run ME programs had the advantage of a time buffer between the collection and examination of blood samples from suspected malaria cases, afforded by the then still effective "presumptive treatment" at the time of blood sampling. In contrast, the countries where ME failed became increasingly affected by drug resistance and the majority of them were unprepared for dealing with the new situation caused by resistance of *P. falciparum* to 4-aminoquinolines and antifolates. Artemisinin-based combination therapy (ACT) either already is or will soon be the first-line medication for *P. falciparum* treatment in most affected countries. Accurate diagnosis is deemed essential before prescribing ACT, which may be less well-tolerated and more expensive.

Laboratory and field accuracy. The detection threshold in Giemsa-stained thick blood film has been estimated to be 4–20 parasites/mcL.[21,30,31] Under field conditions, a threshold of about 50–100 parasites/mcL blood is more realistic.[32,33] In remote settings with less skilled microscopists and poor equipment, a still higher threshold is likely.

Poor microscopy has long been recognized in practice and is a function of multiple factors, including training and skills maintenance, slide preparation techniques, workload, condition of the microscope, and quality of essential laboratory supplies. Even among local laboratories with similar equipment and equal training and among reputed experts, abilities vary significantly.[34,35] This variability combined with the risk of untreated malaria in the face of safe, inexpensive therapy in the past led clinicians to treat febrile patients without regard to the laboratory results.[12,36,37]

Even in developed countries, expert malaria microscopists are scarce and impaired microscopy-based diagnosis in hospital laboratories is common.[33,38–40]

False positive. In comparison to expert microscopy, a wide range of poor specificity of local microscopy is reported.[41,42] Poor blood film preparation generates artifacts commonly mistaken for malaria parasites, including bacteria, fungi, stain precipitation, and dirt and cell debris (Figures 1–3).[43] Normal blood components such as platelets also confound diagnosis. Improved training and higher quality of smear preparation and staining are required to reduce false positive reading.

False negative. The chance of false negative results increases with decreasing parasite densities.[35,42] Greater microscopist experience and increased examination time/number of microscopic fields examined reduce such an error.[31,44] Recommended numbers of fields on a thick blood film required for examination before declaring a slide negative vary from 100–400.[39,44,45]

Errors in species identification. A well-trained, proficient microscopist should be able to recognize the *Plasmodium* species correctly in thick blood films at relatively low parasite density. Sometimes it may be necessary to check the thin film for morphologic, differential-diagnostic details such as erythrocyte size, shape, and crenation, characteristic dots in the erythrocyte stroma, pigment structure and color, as well as

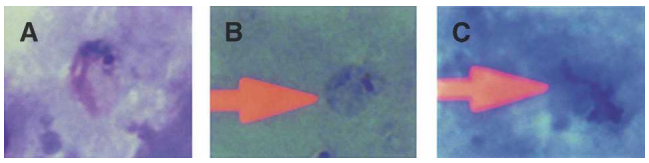

FIGURE 2. Giemsa-stained thick blood films showing a growing trophozoite of *P. vivax* (**A**) and artifacts that could be mistaken for *P. vivax* trophozoites (**B and C**). (Courtesy of J. K. Baird.)

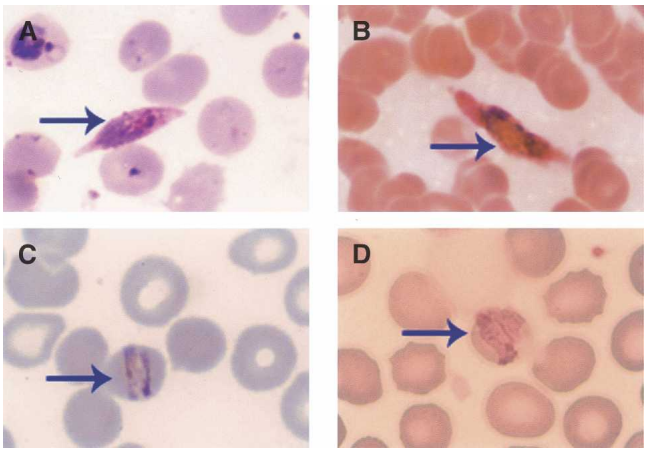

FIGURE 3. Errors in diagnosis are more common when Giemsa staining is poor. Compare the *P. falciparum* gametocyte on a thin film in (**A**) to the poorly stained, elongate-shaped artifact that looks like a *P. falciparum* gametocyte in (**B**); similarly, the well-stained band-form of *P. malariae* in (**C**) to what appears to be a band-form but is not, in (**D**). Images of artifacts were taken from blood smears of healthy donors.

schizonts. Most documented species errors probably involve differentiating between *P. vivax* and *P. ovale* or recognizing occasional human infections with simian plasmodia such as *P. knowlesi*.[46] However, even failure to differentiate *P. falciparum* from *P. vivax*, the two most common species, can be quite frequent in routine microscopy but is underreported.[33,42] Underreporting of mixed-species infections is also common.[40]

Errors in the estimation of parasite density. Parasite enumeration provides useful clinical management guidance (e.g., as an indication to initiate exchange transfusion) as well as for clinical trials and epidemiologic studies.[23] Several methods of estimation exist.[23,30,47–50] No standard procedure exists for counting parasites on a thick film.

Variability in blood film preparation techniques and reading rules account for much of the variability in parasite counts.[31,51] Counting against white blood cells on a thick film and against red blood cells on a thin film, for example, could yield a large difference.[52] Such variability could significantly affect research outcomes.

Use of microscopy in research. Microscopy remains the gold standard and the only U.S. Food and Drug Administration (FDA)-approved endpoint for assessing the outcomes of drug and vaccine trials, and for serving as a reference standard in the evaluation of new tools for malaria diagnosis. In clinical trials, false positive diagnoses lower the apparent efficacy of anti-malarial agents, subjecting potentially effective drugs or vaccines to unjustified discarding.[53] False negative results could lead to overly optimistic outcomes of interventions (unless a follow-up blood smear is possible) or underestimation of the specificity of new diagnostics under evaluation.

Improving the current practice of microscopy. Some isolated efforts to improve malaria microscopy exist in the developed world. An on-line, self-test for competency in malaria microscopy now exists.[54] The Malaria Research and Reference Reagent Resource Center (MR4) in Manassas, VA, makes available on loan sets of thick and thin malaria smears with validated parasite content (http//www.malaria.mr4.org).

National malaria control programs train local microscopists with variable degrees of success. WHO training materials are still widely used, although an update is necessary.[45,55] Improving diagnostic accuracy in malaria control systems can be both technically and financially challenging.[56] Continued supervision and support are essential to ensure sustainability of accurate diagnosis and thereby appropriate treatment.

An effective QC/QA system engaging different organizational levels is needed. This involves standardization of procedures and establishment of national-level diagnostics cen-

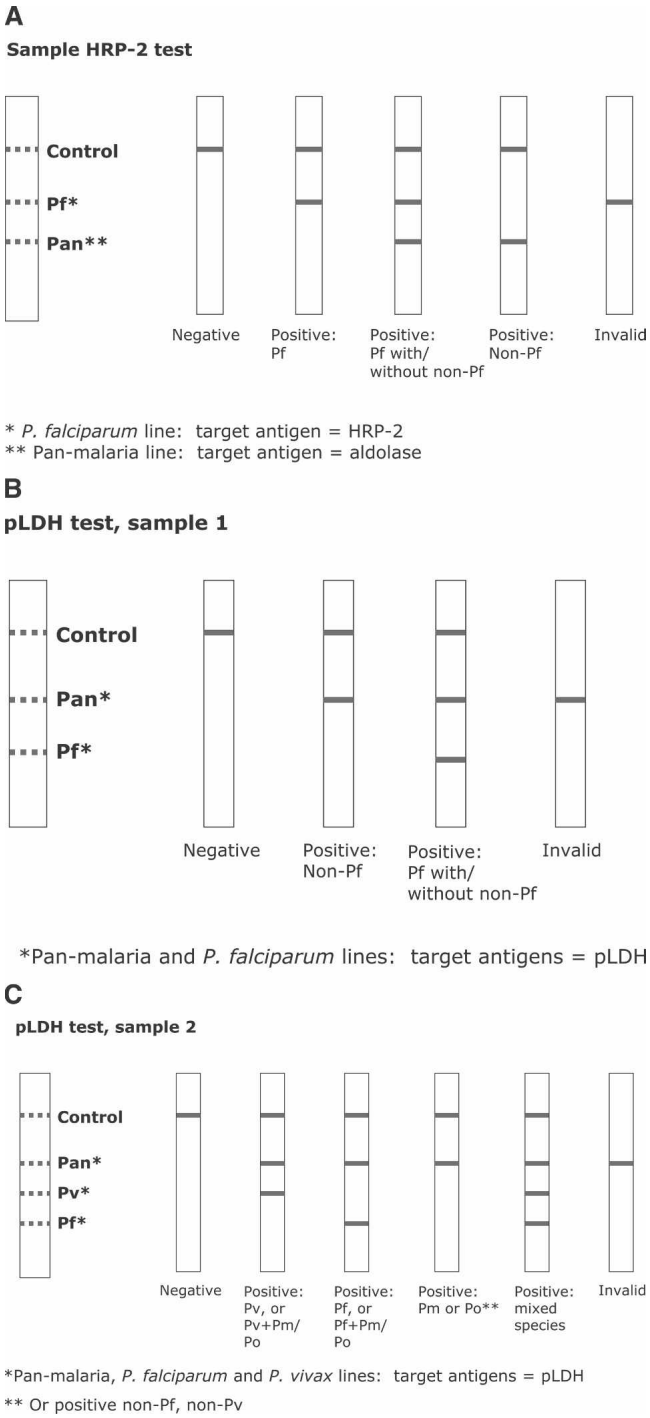

FIGURE 4. (**A–C**) Sample test line configurations of commercial RDTs and their result interpretations.

TABLE 1
Results of selected trials

First author/ publication year	RDT product name	Target antigens	Study site/Year	No. of subjects	% Malaria positive*
1. Studies in endemic countries					
Buchachart, 2004[72]	KAT™-Quick (KAT Medical, South Africa)	HRP-2	Thailand/2000	90	100% (Pf 56%)
Fernando, 2004[68]	ICT Malaria Pf/Pv (AMRAD ICT, Australia)	HRP-2 and Aldolase	Sri Lanka/2002	328	38% (Pf 19%, Pv 81%)
Forney, 2003[66]	ParaSight F + V† "FV 99-2" prototype (Becton Dickinson, U.S.A.)	HRP-2 Pv-specific‡	Thailand and Peru/1999	1,887	41%; (Pf 44%, Pv 56%)
Mboera, 2006[73]	Paracheck Pf® (Orchid Biomedical Systems, India)	HRP-2	Tanzania	1,655	23% (Pf 100%)
Pattanasin, 2003[74]	Paracheck Pf® (Orchid Biomedical Systems, India)	HRP-2	Thailand/2002	271	53% (Pf 35%, non-Pf 65%)
	OptiMAL-IT® (Diamed, Switzerland)	pLDH			
Iqbal, 2003[75]	OptiMAL (Flow, Inc., U.S.A.)	pLDH	Pakistan	930	42% (Pf 38%, Pv 55%, mixed 7%)
2. Studies of returning travelers in non-endemic countries					
Farcas, 2003[76]	NOW® ICT (Binax, U.S.A.)	HRP-2 and Aldolase	Canada/ 1999–2003	256	82% (Pf 50%, non-Pf 50%)
Grobusch, 2003[77]	ParaSightF (Becton Dickinson, U.S.A.)§	HRP-2	Germany/ 1998–2001	554	26%
	ICT Malaria P.f. (ICT Diagnostics, Australia)	HRP-2		226	23%
	ICT Malaria Pf/Pv (Binax, U.S.A.)	HRP-2		492	27%
	OptiMAL (Flow Inc., U.S.A.)	pLDH		539	24%
Palmer, 2003[78]	OptiMAL (Flow, Inc., U.S.A.)	pLDH	U.S.A.	216	20% (Pf 66%, non-Pf 34%)
Richardson, 2002[79]	Makromed® (Makro Medical, Pty. Ltd., South Africa)	HRP-2	Canada/ 1995–1997	200	75% (Pf 66%, non-Pf 34%)

* By Giemsa thick film, or by PCR where indicated; †Obsolete; ‡Proprietary (Becton Dickinson, U.S.A.); §Obselete.
Pf = *P. falciparum*; Pv = *P. vivax.*

ters responsible for developing training modules, training, identifying the materials needed to support microscopy QA, and improving the performance and maintaining the competence of microscopists. Allocating a small percentage of the national malaria control budget to microscopy QA could yield large benefits through targeted use of costly drugs.[57] At an international level, a comprehensive repository of malaria slides to provide external validity and certification of microscopists would be useful to both malaria research and control.[35,57]

Rapid Diagnostic Test (RDT). Rapid diagnostic test is a device that detects malaria antigen in a small amount of blood, usually 5–15 μL, by immunochromatographic assay with monoclonal antibodies directed against the target parasite antigen and impregnated on a test strip. The result, usually a colored test line, is obtained in 5–20 min. RDTs require no capital investment or electricity, are simple to perform, and are easy to interpret.

Current RDT test formats (e.g., in a plastic cassette enclosure, or attached to cardboard) promote ease-of-use and safety in comparison to the earlier assays of the early and mid-1990s. RDT consumption, especially in developing countries, has increased for the past few years. One product received U.S. FDA clearance in June 2007. Most commonly used RDTs only detect *P. falciparum;* however, RDTs that distinguish *P. falciparum* from the three non-falciparum species are available. Commercial tests are manufactured with different combinations of target antigens to suit the local malaria epidemiology (http://www.wpro.who.int/sites/rdt/documents/).[58] Histidine-Rich Protein 2 (HRP-2) is the most common malaria antigen targeted and is specific for *P. falciparum*. Some commercial tests carry both an assay for genus-specific aldolase enzyme and an HRP-2 assay thus making it capable of distinguishing an infection with non-*P. falciparum* only from that due to *P. falciparum* (with/without non-falciparum). Parasite lactate dehydrogenase (pLDH) enzymes are the other major group of targeted antigens. Monoclonal antibodies against pLDH are commercially available for the detection of *Plasmodium* spp. (pan-malaria), *P. falciparum*, and *P. vivax.* The *P. vivax*-specific assay is new and not yet adequately evaluated. Test line configuration and interpretation of RDT results vary with products (Figure 4). Products that incorporate an HRP-2 assay with a pan-malaria pLDH assay are also available.

As opposed to HRP-2, which often persists in the patient's blood for weeks after successful treatment, pLDH is a more appropriate target for treatment monitoring.[59] However, plasmodial gametocytes also produce pLDH and so a pLDH test may remain positive despite clearance of the asexual parasite forms.[60] Persistent HRP-2, on the other hand, could be an advantage in detecting low-level, fluctuating parasitemia in chronic malaria.[61] Both HRP-2 and pLDH-based tests have been used with peripheral and placental blood specimens for the detection of malaria in pregnancy with variable outcomes.[62–65]

Accuracy of RDTs. To be a useful diagnostic, RDTs must achieve greater than 95% sensitivity.[14] Most RDTs today have achieved this goal for *P. falciparum,* but not for non-*P. falciparum.* In the evaluation of an HRP-2 prototype assay in Thailand and Peru, *P. falciparum* sensitivity was found to be

TABLE 1
Continued

First author/ publication year	Parasite density (parasites/mcL)	Sensitivity	Specificity	Comments
1. Studies in endemic countries				
Buchachart, 2004[72]	> 80	Pf 96%	Pf 93%	Previously diagnosed malaria patients only.
Fernando, 2004[68]	9% of Pv and 48% of Pf had ≤ 1000	Pf 100% Pv 70%	Pf 100% Pv 99%	Test line intensity and parasite density correlation noted for Pv.
Forney, 2003[66]	15% of Pf had ≤ 500 23% of Pv had ≤ 500	Pf 98% Pv 87%	Pf 93% Pv 87%	83% sensitivity for Pf ≤ 500/mcL 55% sensitivity for Pv ≤ 500/mcL
Mboera, 2006[73]	≥ 40	Pf 90%	Pf 97%	Asymptomatic individuals included. RDT storage conditions noted.
Pattanasin, 2003[74]	28% of Pf and 38% of Pv had ≤ 500	Pf 90%	Pf 96%	Recent history of falciparum malaria explained most of the false positive test results (N = 9)
		Pf 88% Non-Pf 65%	Pf 92% Non-Pf 99%	70% sensitivity for Pf and 64% for non-Pf with densities 100–500/mcL.
Iqbal, 2003[75]	12% (all species combined) had < 500	Pf 85% Pv 76%	Pf 99% Pv 99%	RDT performed better than microscopy at remote clinics.
2. Studies of returning travelers in non-endemic countries				
Farcas, 2003[76]	4% Pf and 6% Pv had ≤ 100	Pf 94% Non-Pf 84%	99% overall	Reference standard = PCR.
Grobusch, 2003[77]	Not provided	Pf 95% Pf 91% Pf 98% Pf 76%	Pf 97% Pf 99% Pf 99% Pf 100%	Only Pf was evaluated. In a cohort of 111 patients followed, the maximum number of days that RDT remains positive following parasitological cure ranged from 2 days for OptiMAL, to 34 days for ICT Malaria Pf/Pv and 42 days for ParaSight F.
Palmer, 2003[78]	Not provided	98% (Pf and Pv combined)	100%	Total 32 Pf and 11 non-Pf cases only.
Richardson, 2002[79]	8% Pf had < 100	Pf 97%	Pf 96%	94% sensitivity for Pf 100–1000/mcL. Reference standard = PCR.

100% for parasite density ≥ 500/mcL and 83% for < 500/mcL.[66] Roughly, RDT sensitivity declines at parasite densities < 500/mcL blood for *P. falciparum* and < 5,000/mcL blood for *P. vivax*. Decreased test line intensity with parasite density was also demonstrated for both an aldolase assay (pan-malaria specific) used to detect non-*P. falciparum* and an HRP-2 assay.[67,68]

In spite of over 100 published RDT trial reports, comparative assessment is difficult because (1) trials do not share common guidelines; (2) clinical and epidemiologic characteristics of the study populations, especially the parasitemia level vary; (3) reference standards are different; even among those using Giemsa microscopy, reading rules and microscopist skills vary; and (4) products of different lots may differ in quality or be damaged by extreme temperature or humidity during transportation and storage (http://www.wpro.who.int/sites/rdt/reviews_trials/).

Early published trials have been summarized elsewhere.[69–71] In Table 1, selected trials in diverse populations published in the past 4 years are listed with their findings. HRP-2 tests commonly give *P. falciparum* sensitivity of > 90% in clinical cases.[66,68,71,72,80–82] When accompanied by an aldolase assay, the non-falciparum sensitivity is usually lower.[66,68,83,84] For pLDH assays, results varied among studies and product lots and variable field stability of the test kits could not be ruled out. Sensitivity for *P. falciparum* is excellent (> 95%) in some studies and poorer (80%+) in others.[74,75,78,85,86] Recent studies suggest that the tests were less sensitive for non-*P. falciparum* than for *P. falciparum.*[74,75,87] Extremely low sensitivity had been reported earlier for both HRP-2 and pLDH tests and batch-specific problems were suspected.[88–92] Overall RDT specificity is commonly above 85%, approaching 100% when used in some groups of returning non-immune travelers.[59,76–79,93]

False positive RDT results occur in a few percent of tests. Cross-reactivity with rheumatoid factor in blood generates a false positive test line, but replacement of IgG with IgM in recent products reduces this problem.[94–96] Cross-reactivity with heterophile antibodies may also occur.[20] Occasional false negative results may be caused by deletion or mutation of the *hrp-2* gene.[97] It has been suggested that anti-HRP-2 antibodies in humans may explain why some tests were negative despite significant parasitemia.[98] Presence of an inhibitor in the patient's blood preventing development of the control line is also noted.[99]

Several factors in the manufacturing process as well as environmental conditions may affect RDT performance.[58,73] Manufacturers usually recommend 4°–30°C as the optimal temperature range. In practice, exposure of RDTs to > 70% humidity and/or > 30°C frequently occurs in the tropics. QC/QA measures are important to ensure that the purchased products meet performance expectations and that product quality is maintained through the delivery process to the periphery of the healthcare system. The recently introduced WHO initiative of RDT product testing and QA aims to standardize testing of RDTs and to assist countries and manufacturers with distribution and use.[58] Recommended guidelines for the field evaluation of malaria RDTs are available.[100]

These guidelines will allow for better comparisons between test formation and across populations.

Where and when to use RDTs. In developed countries, RDTs can be useful in screening febrile returnees from endemic areas.[59,71,101] Self-use by travelers, however, produces variable outcomes.[102,103] In developing countries, RDTs make obsolete the sole dependence on clinical diagnosis for malaria in remote areas, where good microscopy has failed or never reached. RDTs are also recommended in situations exceeding microscopy capability, such as in an outbreak or in occupationally exposed groups.[104] As RDTs improve, including in sensitivity for *P. vivax* and in ability to measure parasitemia levels, at least semi-quantitatively, the scope of RDT applications will expand. Current RDTs are not intended to replace microscopy.

Successful implementation of RDTs requires complex planning. Use of RDTs at peripheral levels such as by health workers, in informal health sectors and for self-diagnosis/self-treatment is a challenge.[58] Implementation requires new local-level algorithms for actions to be taken based on RDT results (http://www.wpro.who.int/sites/rdt/home.htm).[104]

Price and cost effectiveness. The current market price of an RDT in developing countries is about U.S.$0.55–U.S.$1.50 (depending on the number of targeted species and the order quantity), compared with microscopy at U.S.$0.12–$0.40 per malaria smear. However, in the face of the rising cost of effective anti-malarial therapy, over-diagnosis can quickly decimate pharmacy budgets. Prompt and accurate diagnosis will not only improve malaria treatment, but possibly reduce morbidity due to other febrile illnesses. Therefore RDTs should be considered as tools for the composite management of febrile illnesses.

The cost effectiveness of RDTs vary with malaria prevalence, RDT cost, cost of anti-malarial treatment, and the cost of treatment of other febrile illnesses when malaria has been ruled out. RDTs become more cost effective as the price of anti-malarials go up. A mathematical model that assists in decision making of RDT introduction in areas of high-level malaria transmission is available online (http://www.wpro.who.int/sites/rdt/Assessing+RDT+Cost-Effectiveness.htm).

CONCLUSIONS

Although a 1988 WHO report stated, "A working microscope should be available for use in the furthest periphery of the health care services," it is not until faced with a potential therapeutic impasse that an effort to scale-up microscopy (in particular, its QC/QA) is reconsidered. International health agencies and the scientific community engaged in epidemiology, drug, and vaccine work need to urgently put forth an effort to improve the global capacity to diagnose malaria. Effective malaria microscopy QA could create a culture of diagnostic excellence and professionalism among malaria laboratory technicians throughout the developing and developed world.

Quality RDT is a valuable complement to microscopy because it helps expand the coverage of parasite-based diagnosis to the periphery and minimize exclusively clinical diagnosis. The cost of improved malaria diagnosis will inevitably increase, whether by investment in microscopy or RDTs or both. However, such investment offers a more promising strategy to deal with increasing costs of therapy driven by drug resistance. Today's multi-million dollar investment in anti-malarial drug development should be accompanied by a parallel commitment to improve diagnostic tools and their availability to those living in malarious areas.

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