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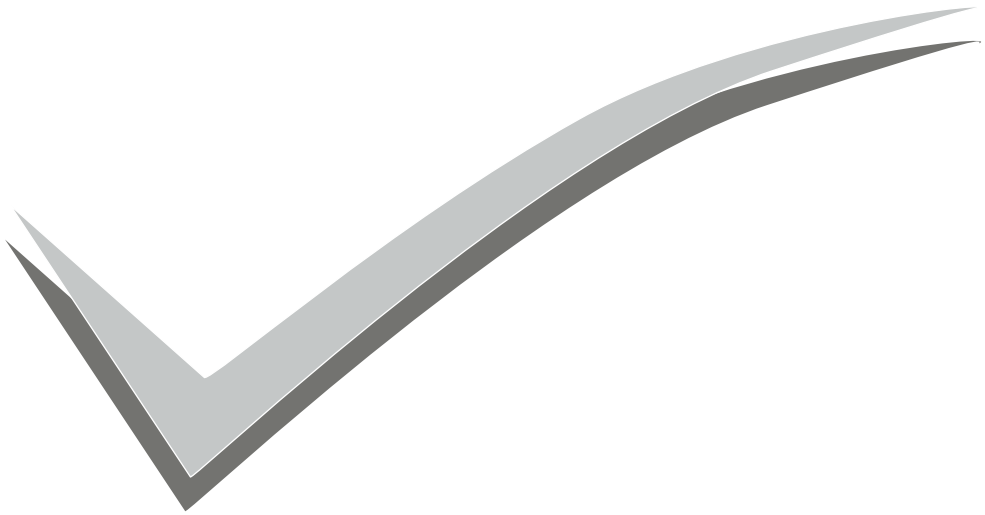

Parascreen®

Rapid test for Malaria (Pan/Pf)

Performance Evaluations

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Performance Evaluations

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EXTERNAL EVALUATIONS

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19.	Field evaluation in Jakarta
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21.	Institut Pasteur du Cambodge (IPC)

Reference Articles on Google Books

- Advances on Protozoa Research and Treatment, 2011 Edition, Scholarly Brief, Pg .18.

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Research Paper

Comparative Study of Thick Smear, Thin Smear, QBC and Antigen Card Test in Diagnosis of Malaria

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Abstract: *Rapid diagnosis is pre-requisite for institution of effective treatment and reducing mortality and morbidity of malaria. The study was taken up to compare the efficiency of various methods available, i.e. thick and thin smear, Quantitative Buffy Coat (QBC) and Antigen card test (Parascreen). In the present study, thick smear was compared with thin smear, QBC and Antigen card test for the diagnosis of malaria. A total of 406 samples were collected from patients presenting with classic symptoms of malaria. For traditional microscopy; thick and thin smear were prepared and stained with Leishman's stain, taking thick smear as a gold standard. QBC and Antigen detection were done using commercially available kits. Malaria was diagnosed in 112, 81, 98 and 110 patient by thick smear, thin smear, QBC and antigen card test respectively. In antigen card test the sensitivity 98.2%, specificity 100%, Positive predictive value (PPV) 100% and Negative predictive value (NPV) 99.3% were observed. Although the antigen card test is superior than thin smear and QBC. Antigen card test has its advantages in terms of speed, sensitivity and specificity especially in an endemic area. Therefore we recommended antigen card test which was simple, reliable and effective for the diagnosis of malaria in remote and rural areas of our country.*

Keywords: Malaria, thick smear, thin smear, QBC, Antigen card test.

1. Introduction:

Malaria presents a diagnostic challenge to the medical community worldwide. Its occurrence is noted in more than 90 countries. Malaria remains the most important parasite disease globally causing over 170 million cases usually of who over a million die in every year [1]. It is a serious, some time fatal, parasitic disease posing a major public health problem in India [2]. There is problem of diagnosis and control of malaria but the treatment has to be started immediately in order to avoid complications. The keen interest in this field will surely leads to early detection improvement in treatment regimen and implement better techniques of prevention [3].

Malaria is a mosquito- borne infectious disease of human and other animals caused by sporozoites of genus Plasmodium. It begins with a bite from an infected female Anopheles mosquito, which introduces the sporozoites through saliva into the circulatory system. In the blood, the sporozoites travel to the liver to mature and reproduce. Malaria causes symptoms that typically include fever and headache, which in severe cases can progress to coma and death. The disease is widespread in tropical and subtropical regions in a broad band around the equator, including which of sub-Saharan Africa, Asia and Americans [4].

Microscopic Examination of the peripheral blood smears (PBS) are the commonly employed method for diagnosis of malaria. Since its introduction 1903 thick PBS allows identification of the plasmodial parasite and its stages, the technique is labor intensive, time consuming and its interpretation at low level of parasitemia request considerable experienced microscopist for accurate identification [5].

In recent years newer, simple and rapid techniques like QBC (Quantitative Buffy Coat), antigen and enzyme detection have been developed to replace the conventional microscopic methods. The antigen detected is histidine rich protein-2 (HRP-2) and enzyme detected is plasmodium lactate dehydrogenase (pLDH) and Pan- specific aldolase all these techniques vary in there sensitivity, specificity, positive predictive value and negative predictive values [6]. Keeping in mind the seriousness of the condition and the current availability of diagnostic facilities across India we decided to conduct a comparative study of the thick smear with thin smear, QBC and antigen card test (parascreen).

2. Materials and Methods:

This prospective study was conducted in the department of pathology, Father Muller Medical College Hospital, Mangalore, Karnataka, India. The study was conducted from May 2011 to August 2012. This study was done in 406 cases of patients presenting pyrexia with chills, rigor and other suggestive symptoms of malaria. They were admitted in wards or attending the outpatient department of Father Muller Medical college Hospital, Mangalore, Karnataka, India.

2.1 Sample Collection:

Oral consent was taken from the patients prior to the collection of specimens. In this study 5ml of EDTA anticoagulated blood was taken and smear were prepared within an hour of collection of blood. All the sample was collected in Vacuntainer. The age group of patients varied from 4 to 80 years.

2.2 Thick and Thin Blood Smears:

Thick and thin blood smears were prepared as per the standard method. The smears were stained with Leishman’s stain [7].

2.3 Quantitative Buffy Coat Technique (QBC):

The QBC is a high precision glass haematocrit tube, pre coated internally with acridine orange stain. This tube was filled with 55 – 60 *m*l of EDTA blood. A clear plastic closer was applied. A cylindrical float, designed to be suspended in a packed red cell was inserted. This tube was centrifuged at 12000 rpm for 5 minutes. This tube is examined under light microscope with standard white light, equipped with accessory illuminated microscope objective and fluorescence. Approximately 10-20 fields were examined over 2-5 minutes.

The principle of QBC technique is based on the fact that on centrifugation at high speed. The whole blood separates into plasma, buffy coat and packed red cell layer. The component of buffy coat separated according to their densities, forming discreat bands. The topmost area of the red cell band is enlarged 10 times more than normal, this area has red cells with parasites. Due to the high buoyancy of the infected cells they appear in the upper most part of the column. Due to acridine orange the malarial parasite are seen as fluorescent bodies standing at different level of the sedimentation column depending on the stage and species of the parasite [8, 9].

2.4 Antigen Detection using Parascreen:

Commercially available antigen detection kit Parascreen (Zephyr Biomedical Systems, lot: 101051) that detects the Histidine-rich protein 2 antigen (HRP II) of P.falciparum and the lactate dehydrogenase of Plasmodium was used.

The strips coated with anti HRP II antibody were used to detect the presence of the HRP II antigen by immuno-chromatography.

The test was done using EDTA anticoagulated blood according to standard operating procedure (SOP) given by manufactures. Interpretation of the test result was done as below:

1. When only one pink- purple band appears in the control window marked as “C” the test considered to be negative.
2. When one control band and another one bands appear only at region of “Pf” the test considered to be positive for P.falciparum.
3. When one control band and one pink purple band appear only at region “Pan” the test considered to be positive for other species (non falciparum).
4. When one control band and another two bands appear at regions “Pf” and “Pan” the test considered to be positive for P. falciparum or mixed infection.
5. When no bands appear on device the test should be considered invalid [10].

3. Results:

A total of 406 samples were evaluated by thick and thin Leishman stained peripheral blood smear, QBC technique and antigen card test. The blood film result indicated that malarial parasite was detected in 112 (27.58%) cases by thick blood smear and in 81(19.95%) cases by thin peripheral blood smear.QBC technique detected 98(24.13%) cases of malaria and antigen card test detect 110 (27%) cases of malaria (Table 1). The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of antigen card test with respect to Leishman stain thick smear was 98.2%, 100%, 100% and 99.3%, respectively. The sensitivity of thin smear was low 72% but its specificity was 98%. The sensitivity and specificity of QBC were 87.5% and 99.0%, respectively with respect to Leishman stained thick smear (Table 2).

4. Discussion:

Rapid detection and effective treatment is a pre requisite in reducing morbidity and mortality due to malaria. Peripheral blood smear study is simple, least expensive, labor intensive, time consuming and therefore delay in diagnosis. Leishman's or Giemsa stained thick smears are considered to be the 'Gold standard' in diagnosis. However, the accurate species differentiation and quantitation of parasitemia is possible only when performed by skilled microscopist [11, 12]. Apart from clinical and microscopic examination, many rapid diagnostic tests have been developed. Most frequently, they deploy a dipstick or test strip bearing monoclonal antibodies detected against the target parasite antigen [2]. Newer technique like QBC and Antigen card test are rapid, simple and easy to interpret.

In the present study, while comparing the available different methods of rapid detection of malarial parasites with gold standard Leishman stained thick smear, the sensitivity of thin smear found to be lowest (72.3%) which may be because of the undefined ring stage of the parasite. While Parija et al [13] reported a very low sensitivity of (54.8%) leishman stained thin smear. In our study we got a high specificity and positive predictive value (98% and 93%) respectively (Table 2). Leishman stained thin blood smear detects malarial parasite only when there are 50 parasite/ ml of blood [5]. Even though this method can be used for species identification of malarial parasite.

When we compared QBC with Leishman stained thick smear the sensitivity of QBC was found to be low 87.5% but specificity, PPV and NPV was 99.0 %, 97.0 %, 95.4 % respectively. Although the sensitivity of QBC has been reported to be high as 92.6% by Mendiratta *et al* and 100 % by Bhandari et al [14, 3]. Whereas in our study we observed low sensitivity of QBC. One of the reasons for this could be that as the hospital is present in an endemic region for malaria the levels of parasitemia could have been low. In another study Benito et al [15] revealed that QBC has a low sensitivity and greater rapidity as compared to Leishman stained thick smear. Similarly in the present study we also observed same findings with lower sensitivity and higher specificity. QBC has the advantage that screening is much faster. However, it requires a fluorescence microscope which is expensive and the QBC mounts cannot be preserved, unlike the Leishman stained smears. One more disadvantage of QBC technique is that a permanent record of test cannot be kept [16].

In the present study we observed 98.2 % of sensitivity, 100% of specificity and 100% PPV in Parascreen antigen card test comparing with the thick smear. Similarly some authors also observed same findings [17, 2]. While few authors [13, 18] observed a lower sensitivity of 75% by using other kit (malarigen) based on similar principle, whereas the specificity (100%) and PPV (100%) were in accordance with our study.

However, in the present study parascreen antigen card test was negative in two cases which were positive cases in Leishman's stained thick smear; (Table1) these were probably false negatives because of immuno-chromatography (ICT) is unable to detect HRP II below 100 parasites/μL of blood. One should bear in mind that HRP II has been shown to persist in blood for 7-14 days and up to 28 days following antimalarial therapy; hence it is also important to be familiar with the history of antimalarial treatment of the individual patient to rule out false positives [19, 20].

In this study we also compared thin smear with antigen card test, 81 cases were positive by both these methods, while thin smear missed to rule out malaria in 29 cases which was positive for malaria by antigen card test (Table1). Which is comparatively similar to the study done by Iqbal et al [21] in their study microscopy missed 47 cases but these 47 cases were positive by optiMAL antigen test. However, the test was found to be user friendly and interpretation was more objective as compared to smear and QBC.

There were some limitations in the present study, sample size was small and it was a hospital based study, so can't represent whole population. There is need to perform such studies on larger and community based population.

Tables:

Table 1: Comparison of Leishman stained blood film and QBC with antigen card test

Results	Thick smear	Thin Smear	QBC	Antigen card test
Positive	112	81	98	110
Negative	294	325	308	296
Total	406	406	406	406

QBC= Quantitative Buffy Coat analysis

Table 2: Comparison of sensitivity and specificity of the various methods of malarial detection with Leishman stained thick smear

Particulars	Sensitivity	Specificity	PPV	NPV
Thin smear	72.3%	98.0 %	93.0 %	90.5%
QBC	87.5%	99.0 %	97.0 %	95.4%
Antigen test	98.2%	100 %	100 %	99.3%

PPV= positive predictive value, NPV = negative predictive value, QBC = Quantitative Buffy Coat.

Conclusion:

Since malaria is endemic in certain regions of India, we need to employ more sensitive tests, which are also rapid to detect low levels of parasitemia in population. Where QBC method is useful in laboratories only for screening large number of samples and also where appropriate laboratory facilities are available. But some peripheral health care units there are non available of skilled persons and good laboratory facilities for blood film examination. Therefore we recommend antigen card test which was simple, reliable, rapid and effective for the diagnosis of malaria. Even though the test can be a promising alternative to microscopy in remote and rural areas of our country.

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MALARIA JOURNAL

RESEARCH Open Access

Performance of a HRP-2/pLDH based rapid diagnostic test at the Bangladesh-India-Myanmar border areas for diagnosis of clinical malaria

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Abstract

Background: The rapid diagnostic test (RDT) has been adopted in contemporary malaria control and management programmes around the world as it represents a fast and apt alternative for malaria diagnosis in a resource-limited setting. This study assessed the performance of a HRP-2/pLDH based RDT (Parascreen® Pan/Pf) in a laboratory setting utilizing clinical samples obtained from the field.

Methods: Whole blood samples were obtained from febrile patients referred for malaria diagnosis by clinicians from two different Upazila Health Complexes (UHCs) located near the Bangladesh-India and Bangladesh-Myanmar border where malaria is endemic. RDT was performed on archived samples and sensitivity and specificity evaluated with expert microscopy (EM) and quantitative PCR (qPCR).

Results: A total of 327 clinical samples were made available for the study, of which 153 were *Plasmodium falciparum*-positive and 54 were *Plasmodium vivax*-positive. In comparison with EM, for *P. falciparum* malaria, the RDT had sensitivity: 96.0% (95% CI, 91.2-98.3) and specificity: 98.2% (95% CI, 94.6-99.5) and for *P. vivax*, sensitivity: 90.7% (95% CI, 78.9-96.5) and specificity: 98.9% (95% CI, 96.5-99.7). Comparison with qPCR showed, for *P. falciparum* malaria, sensitivity: 95.4% (95% CI, 90.5-98.0) and specificity: 98.8% (95% CI, 95.4-99.7) and for *P. vivax* malaria, sensitivity: 89.0% (95% CI,77.0-95.4) and specificity: 98.8% (95% CI, 96.5-99.7). Sensitivity varied according to different parasitaemia for falciparum and vivax malaria diagnosis.

Conclusion: *Parascreen® Pan/Pf Rapid test for malaria* showed acceptable sensitivity and specificity in border belt endemic areas of Bangladesh when compared with EM and qPCR.

Background

Malaria is often lethal with high potential expenditure for health if diagnosis is inaccurate [1]. Accurate diagnosis of malaria is of increasing importance as the prevalence of malaria is declining around the globe, making surveillance and screening more important for programme management [2,3] and to restrict the use of anti-malarial drugs to restrain the spread of drug resistance [4].

For decades, expert microscopy (EM) of peripheral thick and thin blood smears has been the standard diagnostic test for malaria, however, it is time consuming and requires substantial expertise [1,5]. Enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR)-based diagnostic tests have been introduced which ameliorate sensitivity and specificity of malaria diagnosis, but only in reference settings where well equipped laboratory facilities are available, making it difficult to implement in a field setting [6]. Other nascent molecular methods, such as loop-mediated isothermal amplification (LAMP) [7-9] and real-time quantitative nucleic acid sequence-based amplification (QT-NASBA) [10] are in use, but the efficacy of each is unproven.

After being introduced in the early 1990s, rapid diagnostic tests (RDTs) have become an attractive alternative to the above-mentioned methods in a resource-limited setting for malaria diagnostics. The antigen-based RDTs detect specific antigens produced by malaria parasites by reaction with bound antibodies on an absorbent nitrocellulose membrane. Among several types of RDTs the two-band tests and three-band tests are most

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widely used. Two-band tests either detect only one species (*Plasmodium falciparum)*, usually by detecting histidine-rich protein 2 (HRP2), or detect any of the four most common malaria parasites (*P. falciparum, Plasmodium vivax, Plasmodium malariae* and *Plasmodium ovale)*, typically by detecting pan-*Plasmodium*-specific lactate dehydrogenase (pLDH), while three-band tests detect both the *P. falciparum*-specific antigen HRP2 and the pLDH or any one species specific LDH (mostly *P. vivax*). The third band is the test control band [1,5,11].

Southeast Bangladesh, northeast India and southwest Myanmar are similar in geographical characteristics and endemic for malaria. *Plasmodium falciparum* is the most abundant parasite, followed by *P. vivax* in these countries [6,11,12]. The presence of *P. malariae* and *P. ovale* has also been reported in each country [13-16]. These three countries share their borders, making transborder malaria transmission plausible. The presence of all four parasites in these mostly remote and resource-limited areas illustrate the importance of a RDT that can detect all malaria parasites. Amongst the locally available RDTs, *Parascreen® Pan/Pf Rapid test for malaria* (Zephyr Biomedical Systems, India), hereafter noted as Parascreen, is a RDT that has the capability to detect all types of human malaria, as it detects *P. falciparum*-specific HRP-2 and pan-*Plasmodium*-specific LDH. It has been evaluated against microscopy and conventional PCR in field and laboratory settings [12,17-24]. Here, the assessment of Parascreen in a laboratory setting and its performance compared with EM and qPCR are described.

Methods

Study area and population

Whole blood samples were obtained from febrile patients with clinical symptoms referred for laboratory investigation between May 2009 to December 2010. The represented regions include Matiranga Upazila in Khagrachari district and Ramu Upazila in Cox's Bazar district, two different subdistricts of the southeastern part of Bangladesh from corresponding UHC. Matiranga borders Tripura state of India and Ramu borders Myanmar, where malaria is endemic [15,16] and is caused mainly by *P. falciparum* and *P. vivax*.

Sample collection

An expert medical technologist collected approximately 5 mL of blood from adult subjects and 3 mL from minor subjects by venipuncture. Thick and thin blood films were prepared in duplicate using two drops of blood for each sample. The remaining blood was preserved at −20°C in EDTA tube and transported to the Parasitology Laboratory, icddr,b in cool boxes maintaining the temperature below 4°C using ice bags.

Approval from Research Review Committee (RRC) and Ethical Review Committee (ERC) of icddr,b was obtained for this study. Permission for conducting the study was obtained from the National Malaria Control Programme (NMCP). All participants or legal guardians signed informed consent before participant enrolment and sample collection. Complete anonymity was maintained at each stage of the study.

Expert microscopy (EM)

Blood smears were stained with Giemsa and screened for parasites under the (100X) oil immersion lens at the field site by experienced microscopists in the corresponding UHC. The microscopy results were confirmed by a second independent, experienced microscopist who was blinded to prior results. Parasite density was determined by both microscopists counting the parasites and leucocytes [25] and the average was used for the study. When there was any disagreement in diagnosis by the two microscopists for any sample and mixed (*P. falciparum* and *P. vivax*) infection were excluded from the study.

Rapid diagnostic tests (RDTs)

Parascreen (Zephyr Biomedical Systems, India; Lot No 101159) is a three-band antigen detection RDT which comes in cassette format. It employs a recombinant antibody against pLDH to detect *Plasmodium*-specific LDH and anti-HRP2 antibody to detect *P. falciparum*-specific HRP2. All RDTs were performed on archived blood samples by trained and skilled laboratory personnel at the Parasitology Laboratory, icddr,b following the manufacturer's instructions. Briefly, one pink-purple line in the proximal area (control line) interprets negative for malaria; one pink-purple line in the middle area, along with the control line, interprets non-*P. falciparum* infection, exclusively *P. vivax* in this study; one pink-purple line, along with the previous two bands, interprets *P. falciparum* infection. If any of the two test lines or control line did not appear, the test was invalid and repeated.

DNA isolation

DNA was isolated using QIAamp DNA blood mini kit (Qiagen Sciences Inc, USA) following the manufacturer's instructions from 200 μL of archived whole blood.

qPCR

Quantitative PCR (qPCR) was performed on isolated DNA following the method described by Alam *et al.* [6] with Invitrogen® SYBR Green I supermix UDG (Life Technologies Corporation, USA). The sensitivity and specificity of qPCR for *P. falciparum* was 97.1 and 97.6%, respectively, while for *P. vivax* 95.2 and 98.1% [6]. Any mixed (*P. falciparum* and *P. vivax*) infection diagnosed by qPCR was not considered in this study.

Data analysis

All data were encoded in an Excel data sheet and the performance of RDT was calculated by means of the following indicators: sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and agreement (kappa) were calculated with their corresponding 95% confidence intervals (95% CI), using EM and qPCR as reference standards. Sensitivity was calculated as the proportion of positive RDT test results among malaria-positive samples identified by EM and qPCR, while specificity was calculated as the proportion of negative test results among the malaria-negative samples identified by the reference standards. PPV and NPV were obtained as the true positive results among all malaria-positive samples and the true negative results among all negative samples, respectively [26]. Agreement (k) analysis was conducted in IBM SPSS Statistics, version 17.0 (IBM Corporation, NY, USA) by creating a 2 × 2 contingency table.

Results

In total, 327 febrile patients were included in this study from two UHCs. The results of EM, qPCR and Parascreen are provided in Table 1. With EM, there were 207 (63.3%) positive malaria cases, of which 153 (73.9%) were *P. falciparum* infection and 54 (26.0%) were *P. vivax* infection. The parasite density for *P. falciparum* ranged between 16 and 261,480 parasites/μL (IQR: 7,500-50,100) with median value of 19,960 parasites/μL, while the parasite density for *P. vivax* ranged between 16 and 25,120 parasites/μL (IQR: 320–4,800) with median value of 1,140 parasites/μL. qPCR confirmed 208 (63.6%) positive malaria cases, of which 154 (74.0%) were *P. falciparum* and 54 (25.9%) were *P. vivax*. With Parascreen, there were 202 (61.7%) malaria positive cases, of which 150 (74.2%) were *P. falciparum* and 52 (25.7%) were *P. vivax* infection.

Table 2 represents the calculated indicators when Parascreen was compared with EM and qPCR. EM being the reference standard, Parascreen had the following results, for any kind of malaria detection, sensitivity: 97.1% (95% CI, 93.5-98.8) and specificity: 99.1% (95% CI, 96.8-99.9); for *P. falciparum* malaria detection, sensitivity: 96.0% (95% CI, 91.2-98.3) and specificity: 98.2% (95% CI, 94.6-99.5) and for *P. vivax* malaria detection, sensitivity: 90.7% (95% CI, 78.9-96.5) and specificity: 98.9% (95% CI, 96.5-99.7). When qPCR was used as the reference standard, Parascreen had the following results for any kind of malaria detection, sensitivity: 97.1% (95% CI, 93.5-98.8) and specificity: 100% (96.1-100.0); for *P. falciparum* malaria detection, sensitivity: 95.4% (95% CI, 90.5-98.0) and specificity: 98.8% (95% CI, 95.4-99.7) and for *P. vivax* malaria detection, sensitivity: 89.0% (95% CI, 77.0-95.4) and specificity: 98.8% (95% CI, 96.5-99.7).

Table 1 Parascreen® diagnosis results and comparison with diagnosis by EM and qPCR

Parasreen results		Microscopy			qPCR		
		Negative	Pf	Pv	Negative	Pf	Pv
Negative	125	119	3	3	119	3	3
Pf	150	1	147	2	0	148	2
Pv	52	0	3	49	0	3	49
Total	327	120	153	54	119	154	54

Parascreen showed higher sensitivity (93.3-100%) in detecting samples with parasite densities >500 parasites/μL for both *P. falciparum* and *P. vivax*, whereas for parasite densities ranging from 1–500 parasites/μL, the sensitivity was low (60.0%-83.3%) (Figure 1).

Discussion

Parascreen showed acceptable performance in this study with overall sensitivity and specificity of 97.1 and 99.1%, respectively, when compared to EM, and 97.1 and 100%, respectively, in comparison with qPCR. Parascreen can detect all types of non-falciparum malaria but in this study only *P. vivax* was considered as *P. malariae* and *P. ovale* cases were not present in the study samples [13,14]. Parascreen demonstrated varying sensitivity and specificity when compared with EM and qPCR depending on parasite species (*P. falciparum* and *P. vivax)* and parasitaemia of infections.

Several evaluation studies of Parascreen in different countries reported overall sensitivity ranging from 47.5 to 95.5% and specificity from 64.3 to 98.5% with varying performance for falciparum and non-falciparum malaria detection [12,17-21,23,24]. Parascreen has been evaluated by WHO RDT evaluation programme and for *P. falciparum* detection it showed almost 100% detection rates while for *P. vivax* it was approximately 30% [27]. Here, in this study, for *P. falciparum* detection, the sensitivity and specificity was also in concordance with the previous findings [12,20,21], while for *P. vivax* detection, improved sensitivity and specificity are reported. The improved sensitivity and specificity of *P. vivax* detection compared to previous findings may be due to the increased release of antigen through parasite lysis in the archived sample [1] or due to the improvement in the product. This finding is also corroborated by a meta analysis where mean sensitivity and specificity of 95.0 and 95.2%, respectively, for HRP-2 based assays and 93.2 and 98.5%, respectively, for pLDH based assays were calculated [28].

In a study in India, Parascreen showed 94.0% sensitivity and 72.0% specificity for *P. falciparum* and for *P. vivax* 77.2% sensitivity and 98.1% specificity were recorded when compared with EM and similar values observed when compared with PCR [12].

Table 2 Comparative indicators of Parascreen®, when using EM and qPCR as reference standard

Reference standard	Test	Results by Parascreen				
		Sensitivity [%(95% CI)]	Specificity [%(95% CI)]	PPV [%(95% CI)]	NPV [%(95% CI)]	Agreement (k)
EM	Overall	97.1 (93.5-98.8)	99.1 (94.7-99.9)	99.5 (96.8-99.9)	95.2 (89.4-98.0)	0.954
	Pf	96.0 (91.2-98.3)	98.2 (94.6-99.5)	98.0 (93.8-99.4)	96.6 (92.4-98.6)	0.945
	Pv	90.7 (78.9-96.5)	98.9 (96.5-99.7)	94.2 (83.0-98.4)	98.1 (95.5-99.3)	0.910
qPCR	Overall	97.1 (93.5-98.8)	100.0 (96.1-100.0)	100.0 (97.6-100.0)	95.2 (89.4-98.0)	0.961
	Pf	95.4 (90.5-98.0)	98.8 (95.4-99.7)	98.6 (94.7-99.7)	96.0 (94.7-99.7)	0.945
	Pv	89.0 (77.0-95.4)	98.8 (96.5-99.7)	94.2 (83.0-98.4)	97.8 (95.0-99.1)	0.899

In Myanmar, two RDTs with similar detection properties have been evaluated in field settings [11,26]. The SD 05FK60 RDT evaluated in the Rakhaine state of Myanmar showed 90.2% sensitivity and 98.5% specificity for *P. falciparum* and 79.4% sensitivity and 98.7% specificity for non-falciparum malaria [11]. The VIKIA Malaria Ag Pf/Pan™ test showed 98.0 and 100% sensitivity for *P. falciparum* and non-falciparum malaria, respectively, with specificity of 98.0 and 100%, respectively [26].

Onsite (Pf/Pan), a RDT with similar detection principle recently evaluated in Bangladesh, reported 94.2% sensitivity and 99.5% specificity for falciparum malaria detection and for vivax malaria detection it showed sensitivity and specificity of 97.3 and 98.7%, respectively [5] which showed slightly better sensitivity and specificity compared to Parascreen.

WHO recommends sensitivity ≥95% at ≥100 parasites/uL for RDTs [1]. In this study, for both falciparum and vivax malaria detection, sensitivity was less than the recommended values for low parasitaemia; however, considering fewer low parasitaemia samples, statistically valid conclusions have not been attained.

In this study, Parascreen was unable to detect three microscopically confirmed falciparum malaria samples with parasitaemia ranging from 112 to 2,600 parasites/uL. This might be caused by the degradation of HRP-2 target antigen as the study was carried out with archived samples. Intraspecies sequence variation [29], deletions or mutations of HRP-2 gene [30,31] among different *P. falciparum* isolates could also account for false negative tests. The extent of HRP-2 variations in Bangladesh is currently unknown, however variations or deletions in

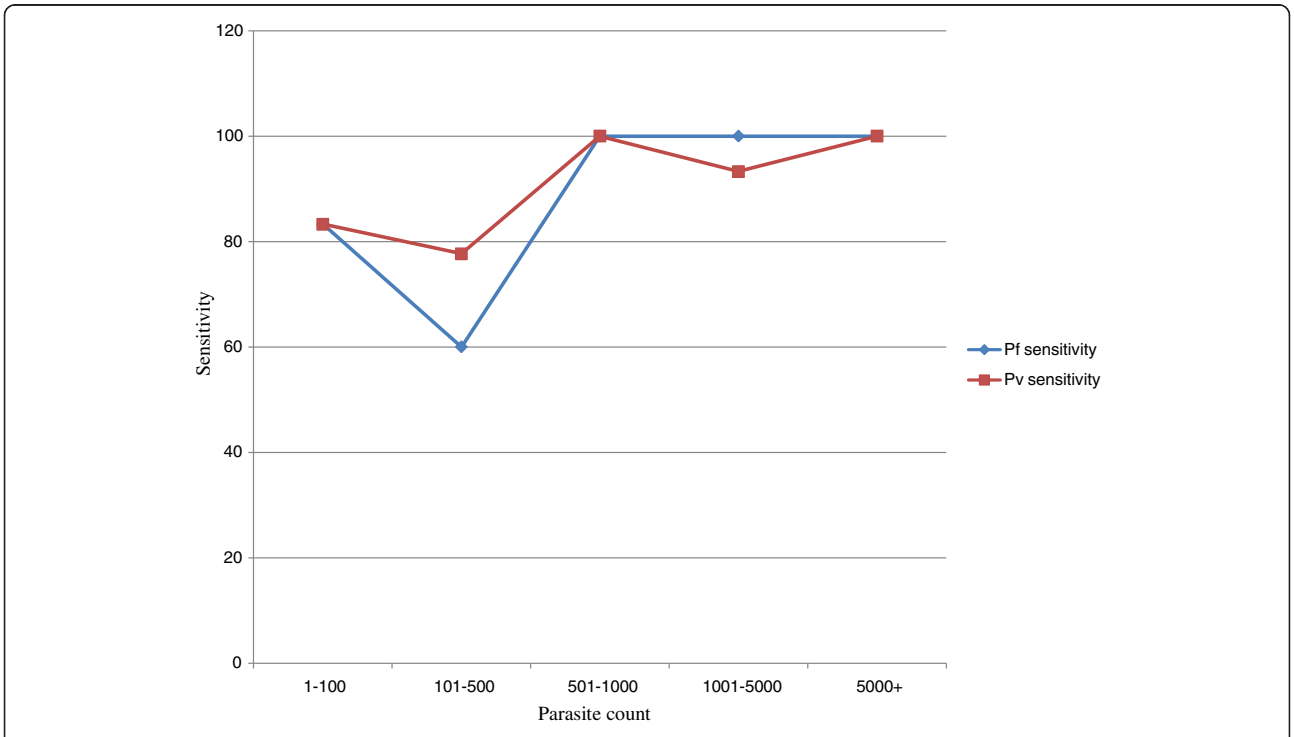

Figure 1 Varied sensitivity of Parascreen® (Pf/pan) according to different parasitaemia.

HRP-2 have been reported recently from India, [31] as well as some African countries [30,32]. In this study, three *P. falciparum* samples showed no HRP-2 test line but Pan specific test line, thus considered as *P. vivax*, as other types of malaria were absent in the study samples. The intraspecies variation, mutation or deletions in the HRP-2 gene can cause non-expression of HRP-2 [30] which may explain this. Parascreen identified three *P. vivax*-positive samples with parasitaemia ranging from 16 to 200 parasites/uL as negative. This might be due to low pLDH level, as pLDH level is directly proportional to parasitaemia [33]. In many studies, a reduced sensitivity for non-falciparum malaria detection, compared to falciparum detection, in combined HRP-2/pLDH RDTs has also been reported [5,6,11,26].

As all four malarial parasites co-exist in the Bangladesh-India-Myanmar border area, an important criterion for selection of an appropriate RDT is the capability to detect all types of malaria. It is advantageous to use Pf/Pan RDTs which can do so. The high predictive values for Parascreen indicate that it is able to detect true malaria cases as well as ruling out non-malaria cases. High sensitivity, specificity and predictive values for Parascreen present it as a viable alternative for malaria diagnostics in Bangladesh-India-Myanmar border areas where malaria is endemic.

The absence of *P. malariae* and *P. ovale* samples in this study restricts the findings to the detection performance of falcipaum and vivax malaria. The inclusion of *P. malariae* and *P. ovale* in the study samples is needed to assess non-falciparum malaria detection performance.

Conclusion

Parascreen showed acceptable performance for falciparum as well as vivax malaria diagnosis in standard experimental conditions. It can be employed in resource-limited settings to diagnose all types of human malaria.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RE and MSA conceptualized and designed the study, collected and identified samples, analyzed the data, drafted the manuscript and made final revisions. RE, ANM and MSA did sample analysis. WAK and RH made critical revision of the manuscript. RE and ANM performed laboratory tests. All the authors read the final version of the manuscript and approved.

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Acta Tropica

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Field evaluation of rapid diagnostic tests for malaria in Yaounde, Cameroon

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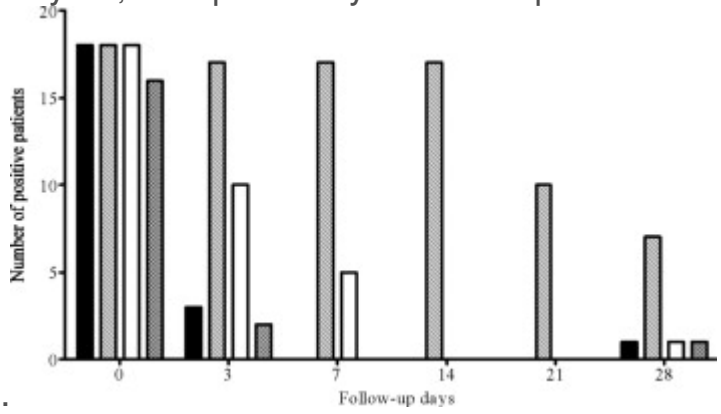
Abstract

Rapid diagnostic tests (RDTs) are affordable, alternative diagnostic tools. The present study aimed to evaluate RDTs available in Cameroon and compare their characteristics to follow the parasitological response of patients for 28 days. Malaria diagnosis was assessed in 179 febrile patients using conventional microscopy as the reference method. Parascreen detects both *Plasmodium falciparum*-specific histidine-rich protein 2 (Pf HRP-2) and Pan-specific plasmodial lactate dehydrogenase (pLDH) in all four human *Plasmodium* spp. Diaspot is based on the detection of Pf HRP-2. OptiMAL-IT (pLDH specific for *P. falciparum*and pLDH for all four human *Plasmodium* spp.) was assessed for comparison. The reliability of RDTs was evaluated by calculating the sensitivity, specificity, positive predictive value, negative predictive value, false-positive rate, false-negative rate, and likelihood ratio. The clinical outcome of 18 children treated with atovaquone–proguanil and followed for 28 days was evaluated

using microscopy and RDTs. Of 179 samples, 133 (74.3%) were pure *P. falciparum*-positive smears, 4 (2.2%) pure *P. malariae*-positive smears, and 42 (23.5%) negative smears. Parascreen and Diaspot had high sensitivity (>92%) and positive predictive values (>94%). The specificities for Parascreen and Diaspot were 81.0% and 90.5%, respectively. The false-positive rates and the false-negative rates were 19.0% and 4.5% for Parascreen and 9.5% and 8.3% for Diaspot, respectively. Most false-negatives occurred in samples with low parasitaemia (<500 asexual parasites/μL). The performance of RDTs was better at higher parasitaemia (>500 asexual parasites/μL). Four pure *P. malariae* were only detected by the pan-*Plasmodium* bands of Parascreen and OptiMAL-IT. In blood samples from patients treated and followed-up for 28 days, HRP2-based RDTs remained positive in most samples until Day 28. Despite negative smears, OptiMAL-IT remained positive in several patients until Day 7 but was negative in all patients from Day 14 onwards. RDTs can improve the management of febrile patients. The validity, ease of use, and cost of HRP2-based tests were comparable. However, one of the current weaknesses of the RDT-based strategy using the tests available in Cameroon is inadequate sensitivity for low parasitaemia. In some cases, RDT results may require correct interpretation based on clinical history, clinical examination, and microscopic diagnosis.

Graphical abstract

Number of patients who were positive with microscopy and different rapid diagnostic tests before (Day 0) and after treatment with atovaquone–proguanil during a 28-day follow-up period. Black bars, microscopy; hatched bars, Diaspot; white bars, OptiMAL-IT (Pf band); double hatched bars, Parascreen (Pan band). The Pf band of Parascreen was positive in all patients on Day 0, Day 3, and Day 7. Parascreen was not evaluated on Day 14, Day 21, and Day 28, except on Day 28 in one patient who had a recrudescent malaria.



Highlights

► Parascreen and Diaspot had high sensitivities and high positive predictive values. ► Most false-negatives were associated with low parasitaemia. ► Rapid diagnostic tests were not useful for follow-up after an effective treatment.

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Original Article

Parascreen as an alternative diagnostic tool for falciparum malaria

Jenny Ginting, Siska Mayasari, Munar Lubis, Syahril Pasaribu, Chairuddin P. Lubis

Abstract

Background Malaria is a parasitic disease with high morbidity and mortality. Rapid immunochromatographic are emerging to detect specific antigens of human plasmodia.

Objective To determine the sensitivity and specificity of Parascreen for the detection of *Plasmodium falciparum* in children.

Methods A diagnostic test study was performed in Mandailing Natal District, Penyabungan, North Sumatera. Subjects were public health center and hospital patients with symptoms of fever, pallor, headache, and diarrhea. Blood specimens were obtained for Parascreen testing. Microscopy of Giemsa-stained blood samples served as the gold standard.

Results One hundred and four subjects were studied. The sensitivity and specificity of Parascreen were 76% and 100%, respectively. Positive and negative predictive values of the test were 100% and 49%, respectively. Likelihood ratio was infinite for a positive test and 0.23% for a negative test.

Conclusion Parascreen is a useful and highly specific diagnostic tool for *P. falciparum* malaria **[Paediatr Indones 2008;48:220-3].**

Keywords: *malaria, Plasmodium falciparum, Parascreen, sensitivity, specificity*

Malaria remains a major health problem for children in tropical areas of the world, including Indonesia.[1,2] Every year, 200 million people are infected with malaria, resulting in two million deaths.[3,4] Most malarial deaths occur in infants and young children.[5,6] Malaria is caused by one or more of the four plasmodium species that infect humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium malariae*.[7,8] Malaria due to P. falciparum is the most common and most dangerous due to its ability to cause fatal cerebral malaria.[9-11]

Malaria presents a diagnostic challenge to laboratories in most countries.[12] Prompt and accurate diagnosis is the key to effective disease management; therefore, it is one of the main interventions of the global malaria control strategy.[13,14] Considered as the gold standard, microscopic examination of Giemsa-stained blood films is widely used because of its efficiency and low cost.[15-17] However, it is time consuming and requires proper equipment and trained personnel.[16] The World Health Organization has recognized the need to overcome problems concerning diagnostic microscopy and supports the development of non-microscopic alternatives.[11,16] Several diagnostic methods have been developed for detection of the *P. falciparum* malaria disease process.

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Immunological methods for this purpose have been found to be convenient and easy.[10] Parascreen is an immunochromatographic test (ICT) used for the rapid diagnosis of malaria which has been marketed for several years.[18] However, the performance of this test in the detection of P. *falciparum* malaria in Indonesian children has not been established. This study aims to determine the sensitivity and specificity of Parascreen for the detection of *P. falciparum* in children in Mandailing Natal District, Penyabungan, North Sumatera.

Methods

A diagnostic test study was conducted in Mandailing Natal District, Penyabungan, North Sumatera from October to November 2006. The study was approved by the Health Reseatch Ethics Committee of the Medical School, University of North Sumatera.

The required number of subjects based on the sample size formula for a diagnostic test was 104. We included patients who came to the public health center or hospital with symptoms of fever, pallor, headache, and diarrhea. Patients with history of receiving any antimalarial drug within one week prior to commencement of the study and those who refused examination were excluded.

The Parascreen test were done on all samples. Microscopy of Giemsa-stained thick and thin blood films were considered the gold standard. Parascreen testing as well as blood film preparation was performed directly from finger-pricked blood samples. Blood films were stained with 10% Giemsa solution and examined at a magnification of 1,000x by an expert microscopist. The microscopist was unaware of the patient's diagnosis or Parascreen test result. The initial thick and thin films were considered positive if parasites were seen in at least 100 high-power fields.

Parascreen Pan/Pf test (Zephyr Biomedical Systems, Verna, Goa, India) with 15 μl of finger-pricked capillary blood was performed according to the manufacturer's instructions by well-trained personnel. The results were read by designated physicians who were blinded to the microscopy results. The test was considered positive if the control line was visible in accordance with the specific histidine-rich protein-2 (HRP-2) and/or pan-malarial antigen line. A diagnosis of *P. falciparum* was made if the HRP-2 line was visible, with or without the pan-malarial antigen line.

Data was analyzed using SPSS 13.0 for Windows (SPSS Inc., Chicago, Illinois, USA). The performance of the Parascreen test for detection of P. *falciparum* was determined by calculating the sensitivity, specificity, positive and negative predictive values, and positive and negative likelihood ratios of this test. Test accuracy was defined as the proportion of subjects with a correct Parascreen result, calculated as the sum of true positives and true negatives divided by the total number of subjects.[19]

Results

One hundred and four subjects were recruited in this study. Fifty-five percent of the subjects were female. Most subjects (90%) were 6-12 years of age. The most common complaint was pallor (85%) and the most common physical finding was splenomegaly (7%). Subject characteristics are shown in **Table 1**.

Table 1. Subject characteristic

	n	%
Sex		
Female	57	55
Male	47	45
Age (years)		
6 - 12	94	90
> 12 - 15	8	8
> 15 - 18	2	2
Complaints		
Pallor	88	85
Fever	14	13
Headache	50	8
Diarrhea	7	7
Physical findings		
Jaundice	4	4
Hepatomegaly	5	5
Splenomegaly	7	7

Results of the Parascreen test and blood slide microscopy were used to construct a 2x2 table (**Table 2**). Based on microscopy results, the prevalence of P. *falciparum* malaria was 82%. The sensitivity, specificity, PPV, and NPV of the Parascreen test to detect P. falciparum were 76%, 100%, 100%, and 49%, respectively. The accuracy of the test was 81%. The likelihood ratio was infinite for a positive test and 0.23 for a negative test.

Table 2. Comparison between Parascreen and microscopy results

		Microscopy		n
		Positive	Negative	
Parascreen	Positive	65	0	65
	Negative	20	19	39
n		85	19	104

We calculated the sensitivity of the Parascreen test at different levels of P. falciparum parasitemia (**Table 3**). The test was not sensitive for parasitemia less than 100/μl. Sensitivity increased with increasing levels of parasitemia, and reached 100% at parasitemia above 400/μl.

Table 3. Sensitivity of the Parascreen test at different levels of P. falciparum parasitemia

Level of parasitemia (number of parasites per μL blood)	n	Number of positive Parascreen tests	Sensitivity
1 – 100	11	0	0
101 - 200	32	26	81%
201 – 400	24	21	87%
401 – 600	18	18	100%

Discussion

Of the 104 patients who met the case definition for clinical malaria, 55% were female. The age range was 6 to 18 years; most subjects were 6 to 12 years old. In Nias, North Sumatera, Marletta *et al*[20] found malaria to be most prevalent in the age group of 5-14 years. The difference in malaria morbidity rates across gender and age groups is caused by factors such as occupation, education, environment, population migration, and immunity.[9]

The diagnosis of malaria is based on anamnesis, physical examination, and laboratory findings. The gold standard for laboratory diagnosis of malaria is detection of parasites on microscopic examination of thick and thin blood smears. However, this method has several shortcomings, such as the need of a light microscope and a trained examiner. According to a recent survey of laboratories in West Nusa Tenggara, Indonesia, only 79% of the analysts evaluated were able to read the blood smear properly.[21]

In this study, false negative results were mostly found in subjects with low parasitemia levels (<100/μL), similar to the findings of Kakkilaya.[22] A study by Aslan *et al*[4] showed that the colour intensity of a rapid diagnostic test dipstick is induced by the parasitemia level.

In this study, we found that the Parascreen test had a sensitivity of 76% and specificity of 100%. In India, Singh[23] found that ICT malaria Pf/Pv, another rapid diagnostic test for malaria, had a sensitivity of 97% and specificity of 88%. Palmer[17] similarly evaluated the OptiMAL test, which had a sensitivity of 94% and specificity of 100%. In Sumba, Indonesia, Tjitra[16] found the sensitivity and specificity of ICT malaria Pf/Pv to be 95.5% and 89.8%, respectively. In the same district as the present study, Desrinawati[24] evaluated ICT malaria Pf/Pv and found a sensitivity of 76% and specificity of 69%. Jelinek[25] compared OptiMAL with ICT malaria Pf using PCR as the gold standard; this study found a sensitivity of 92% and specificity of 98% for ICT malaria Pf and a sensitivity of 89% and specificity of 99% for OptiMAL. In West Nusa Tenggara, Arum *et al*[21] found the sensitivity and specificity of ICT malaria Pf/Pv to be 100% and 97%, respectively.

In this study, we found an increase of sensitivity with increasing parasitemia levels, reaching 100% in parasitemia >400/μl. In Thailand, Coleman *et al*[26] reported that the sensitivity of ICT malaria Pf/Pv was 100% in parasitemia ≥500/μl, but only 23.3% in parasitemia <500/μl. Tjitra *et al*[16] obtained a sensitivity of 96% in parasitemia >500/μl, but only 29% in parasitemia <500/μl.

Sensitivity and specificity are constant indices of diagnostic test performance uninfluenced by disease prevalence and are used to derive likelihood ratios.[19] In this study, the Parascreen test had a reasonably good sensitivity and a high specificity, resulting in an infinite likelihood ratio for a positive test.

We conclude that, with a sensitivity of 76% and specificity of 100%, Parascreen can be used as an alternative diagnostic tool for P. *falciparum* malaria.

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Performance of Local Light Microscopy and the ParaScreen Pan/Pf Rapid Diagnostic Test to Detect Malaria in Health Centers in Northwest Ethiopia

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Abstract

Background: Diagnostic tests are recommended for suspected malaria cases before treatment, but comparative performance of microscopy and rapid diagnostic tests (RDTs) at rural health centers has rarely been studied compared to independent expert microscopy.

Methods: Participants (N = 1997) with presumptive malaria were recruited from ten health centers with a range of transmission intensities in Amhara Regional State, Northwest Ethiopia during October to December 2007. Microscopy and ParaScreen Pan/Pf® RDT were done immediately by health center technicians. Blood slides were re-examined later at a central laboratory by independent expert microscopists.

Results: Of 1,997 febrile patients, 475 (23.8%) were positive by expert microscopists, with 57.7% *P.falciparum*, 24.6% *P.vivax* and 17.7% mixed infections. Sensitivity of health center microscopists for any malaria species was >90% in five health centers (four of which had the highest prevalence), >70% in nine centers and 44% in one site with lowest prevalence. Specificity for health center microscopy was very good (>95%) in all centers. For ParaScreen RDT, sensitivity was ≥90% in three centers, ≥70% in six and <60% in four centers. Specificity was ≥90% in all centers except one where it was 85%.

Conclusions: Health center microscopists performed well in nine of the ten health centers; while for ParaScreen RDT they performed well in only six centers. Overall the accuracy of local microscopy exceeded that of RDT for all outcomes. This study supports the introduction of RDTs only if accompanied by appropriate training, frequent supervision and quality control at all levels. Deficiencies in RDT use at some health centers must be rectified before universal replacement of good routine microscopy with RDTs. Maintenance and strengthening of good quality microscopy remains a priority at health center level.

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Introduction

Accurate early case detection and prompt treatment with appropriate antimalarial drugs is the major strategy for effective case management in malaria patients [1]. Correct diagnosis is also vital for the malaria prevalence and incidence indicators used to evaluate the impact of malaria control interventions [2]. A parasite based diagnostic test (microscopy or rapid diagnostic test [RDT]) is now recommended, if available, instead of presumptive treatment for all persons with suspected malaria [3]. While this recommendation has been adopted in the latest version of the Ethiopia treatment guidelines [4], diagnostic test facilities are not always available and their quality has not been comprehensively assessed or compared under routine conditions.

We previously reported two studies on ParaScreen Pf/PAN RDT in Ethiopia, one from a large household survey in mainly asymptomatic persons [5] and one from ten health centers in Amhara region [6]. ParaScreen can distinguish between a *P.falicparum* (or mixed) infection, and a non *P.falciparum* infection. The sensitivity of ParaScreen compared to expert microscopy was relatively low in the household survey [5], but it performed better for persons with suspected malaria in the health facilities in Amhara region [6]. The health facility study directly compared two RDTs, ParaScreen and ParaCheck (detects *P.falciparum* only), done by the health center technicians with the results on the same individuals by expert microscopy. The ratio of *P.falciparum* to *P.vivax* was 64% to 46%. The findings indicated that overall, ParaScreen had adequate performance of

80% sensitivity for *P.falciparum* and 74% for *P.vivax*, with 97% and 99% specificity respectively. ParaCheck also performed well for *P.falciparum* but it is not designed to detect *P.vivax*, and has been replaced with multi-species RDTs supplied to all health posts (which do not have microscopy) in Ethiopia. The higher level Health Centers and Hospitals retain the use of microscopy for malaria diagnosis.

A recent study at three health centers in Oromia region observed slightly higher sensitivity but lower specificity for *P.falciparum* by ParaScreen (85.6% and 92.4% respectively) compared to expert microscopy than we previously observed in Amhara [7]. For *P.vivax* they observed 82.5% sensitivity and 96.2% specificity with ParaScreen. Overall regardless of other parameters used for comparing the performance of three RDTs, ParaScreen performed similarly to two other tests (CareStart and ICT Combo) for *P.falciparum* but CareStart had better specificity for *P.vivax*. The slide positivity rates among patients with suspected malaria by expert microscopy were very similar in the two studies (23.8% in Amhara [6] and 23.2% in Oromia regions [7].

Although in our previous study ParaScreen performance was acceptable overall in the Amhara health centers [6], variation was noted between health centers in the accuracy of both microscopy and RDT compared to the expert microscopists. This variation in performance at health center level is important because in Ethiopia, RDTs are routinely done at health posts (where microscopy is not available) by health extension workers, and immediate supportive supervision for these workers is expected to be provided by the cluster heath center staff at their respective catchment health posts. In addition during times of emergency, failure of microscopes and/or shortage of reagents, multispecies RDTs have to be used in the health centers, so detailed know-how on the performance of multispecies RDTs by the health center technicians is crucial. Therefore, we build on the previously reported results and conduct additional analysis with three aims:

1) To investigate the variation between health centers in the performance of the microscopists working in the health centers compared to expert microscopists;
2) To investigate further the variation between health centers in the accuracy of ParaScreen RDT performed on site, in comparison with results of expert microscopists.
3) To compare indirectly the performance of local microscopy and ParaScreen RDT for diagnosing malaria in NorthWest Ethiopia.

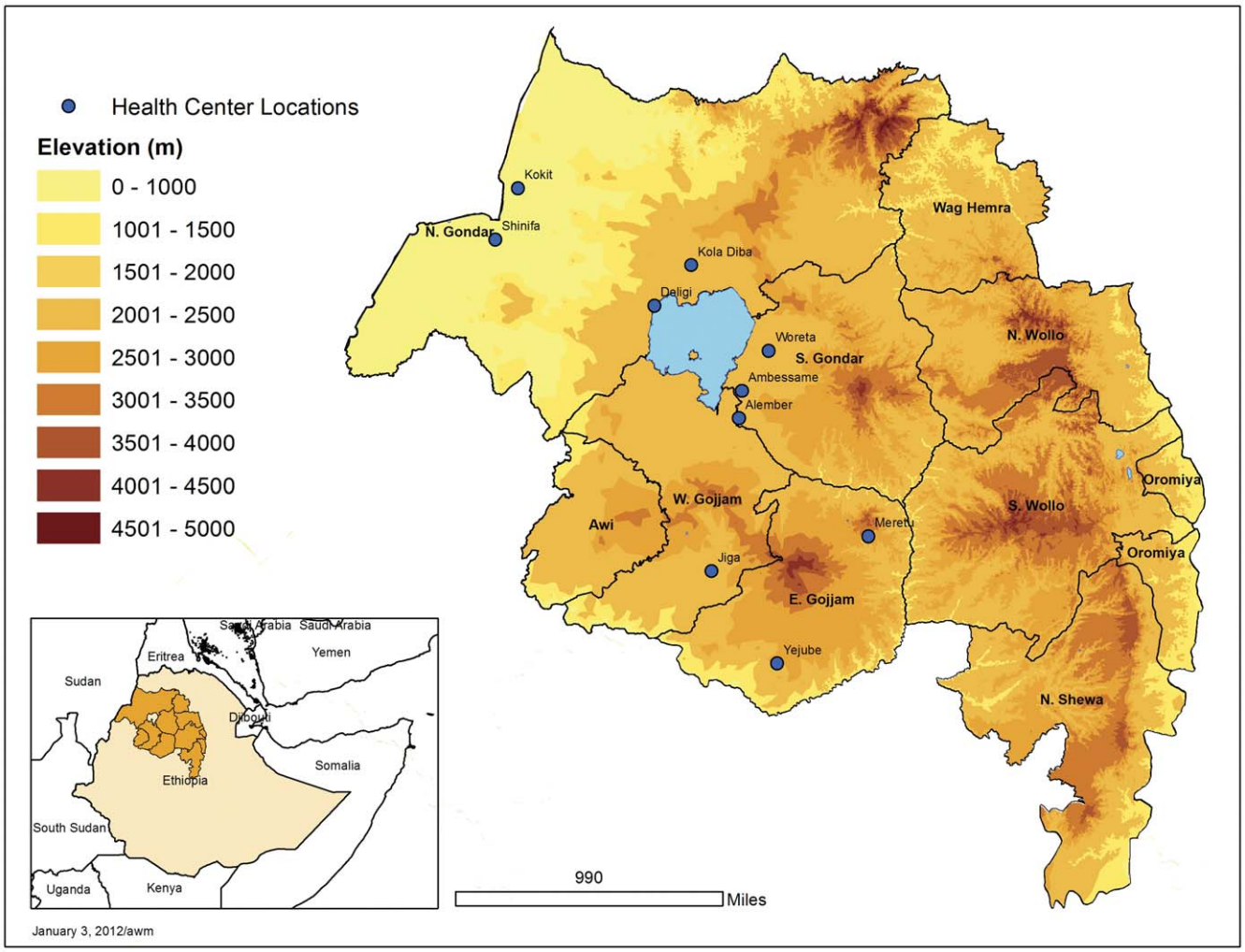

Figure 1. Location of health centers included in the study in Amhara National Regional State, Northwest Ethiopia.
doi:10.1371/journal.pone.0033014.g001

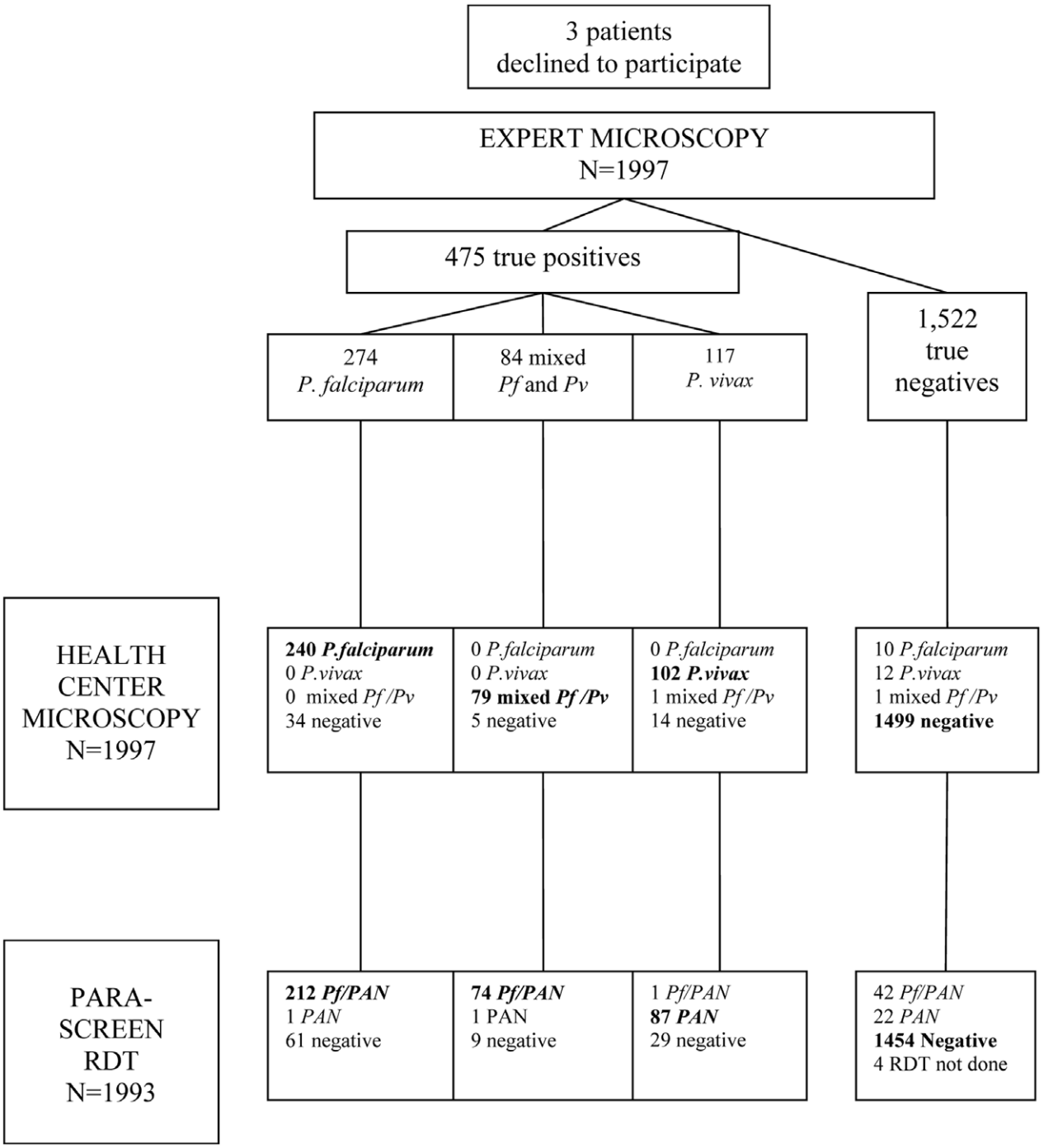

Figure 2. Flowchart of health center technician microscopy and ParaScreen RDT results compared to expert microscopy.
doi:10.1371/journal.pone.0033014.g002

Methods

Ethical Considerations

The study protocol received ethical approval from the Emory University Institutional Review Board (IRB 00006389) and the Amhara Regional Health Bureau (Reference No. R3H5.05/1/2760). Verbal informed consent was sought from each individual and from parents of children aged under 18 years; assent was sought from children 6 to 18 years in accordance with the tenets of the Declaration of Helsinki. All positive cases were treated at their respective health centers according to the treatment guidelines for malaria infection in Ethiopia. Personal identifiers were removed from the data set before the analyses were undertaken.

Study Settings and Population Selection

As previously described [6], the study was conducted in ten health centers (selected to cover a range of transmission intensities) in Northwest Ethiopia (Fig. 1) during the peak transmission period of malaria infection between 16th Oct and 30 Dec 2007. The

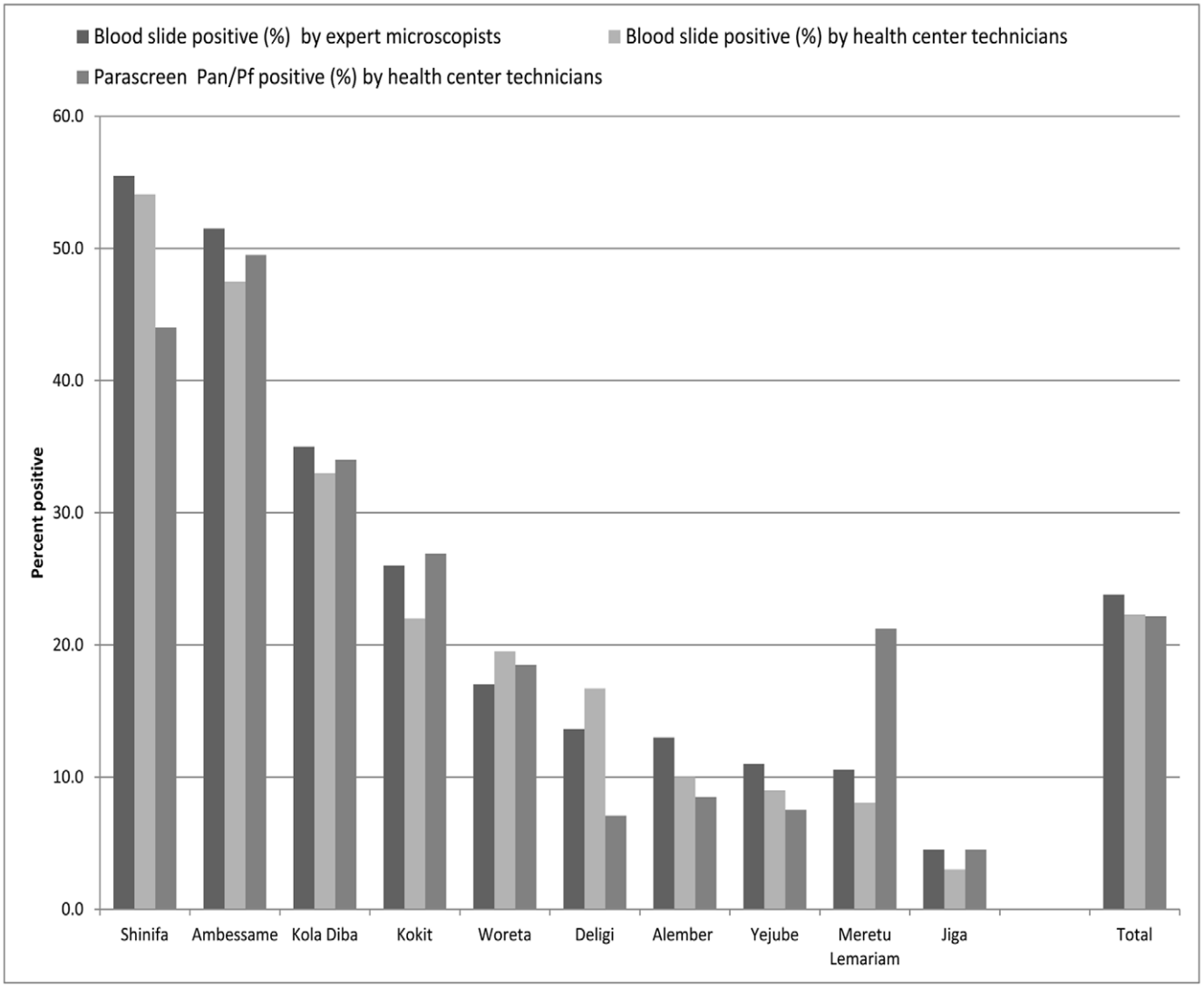

Figure 3. Test positive rate by health center.
doi:10.1371/journal.pone.0033014.g003

Table 1. Prevalence of malaria by expert microscopists, by health center and species.

Name of health center	Total No. examined	No. pos Pf or mixed	No. pos Pv	No. pos any species	% positive (any species)
Shinifa	200	95	16	111	55.5
Ambessame	200	67	36	103	51.5
Kola Diba	200	62	8	70	35.0
Kokit	200	47	5	52	26.0
Woreta	200	24	10	34	17.0
Deligi	198	16	11	27	13.6
Alember	200	20	6	26	13.0
Yejube	200	6	16	22	11.0
Meretu Lemariam	199	16	5	21	10.6
Jiga	200	5	2	9	4.5
TOTAL	**1997**	**358**	**115**	**475**	**23.8**

doi:10.1371/journal.pone.0033014.t001

coordinates of each health center were recorded using a Garmin ETrex GPS unit.

In each health center the first 200 self-presenting patients of any age and either sex who qualified as clinically presumptive malaria (i.e. an axillary temperature greater than or equal to 37.5°C or history of fever in the previous 48 hours) were recruited to the study after excluding individuals with other known causes of non malarial febrile illnesses or serious illness. After obtaining informed consent demographic data were recorded on a structured questionnaire and a finger-prick blood sample taken for blood film preparation and ParaScreen RDT processing.

Training

Among the ten technicians involved in this study, two held a university degree (BSc in medical laboratory technology) and the other eight held a diploma (or advanced diploma) in medical laboratory technology. Nine technicians had a minimum of five years' experience in malarious areas and the other had two and a half years' experience. All the technicians who participated during the training were from government health centers and had previous exposure and experience working with a monospecies malaria RDT (Paracheck Pf) that detects Pf only in their respective health centers.

The training, conducted for half a day at each health center, focused on technical operation of multispecies RDT (ParaScreen) based on the manufacturer's instruction, and procedure for standard blood smear preparation. This included how to handle RDTs, how to collect blood from finger prick for both RDT and smear preparation, how to use buffer for RDT, and RDT reading and interpretation. The procedures for blood films (thin and thick) preparation, staining and species identification were briefly addressed. During training before sample collection was started, simplified and detailed standard operating procedures (SOP) on both RDT and blood slide preparation and staining were prepared and distributed to all health centers that have participated in this study. Similarly, agreement was reached with registered health officers and clinical nurses about the selection criteria of febrile patients with suspected malaria that fulfill the requirement of the study. It was also agreed with the health officers and nurses that all patients involved in the study would be treated according to the malaria treatment algorithm and national guideline of the country.

The centers were visited four times during sample collection and processing, and there was frequent telephone communication whenever there was a need to clarify study related issues or during shortage of materials to be replaced.

Malaria Parasites Detection

Blood slide preparation. The finger-prick blood samples were collected by medical laboratory technicians and processed for thin and thick films according to standard WHO protocol [8], as

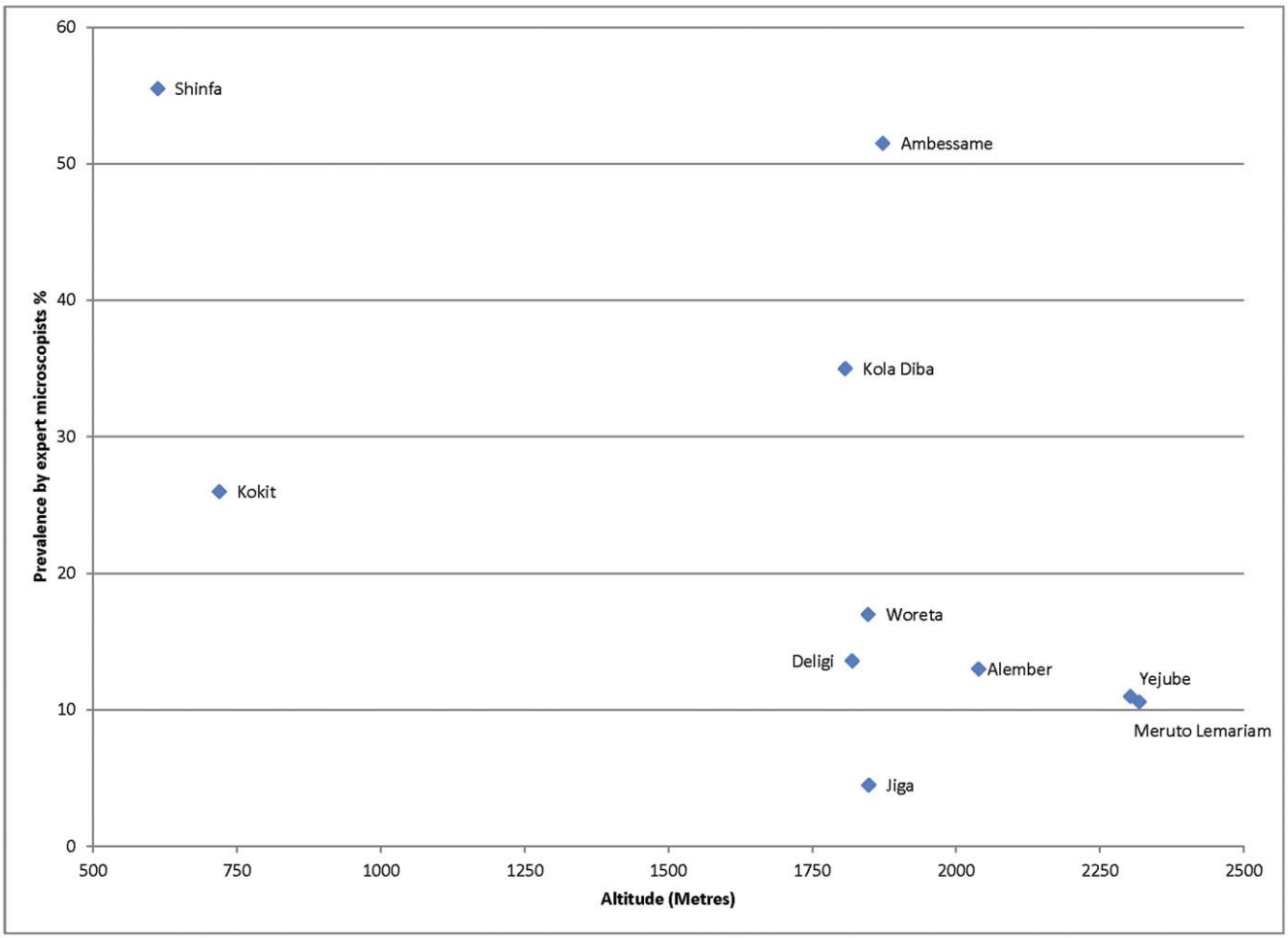

Figure 4. Malaria slide positivity rate in relation to altitude.
doi:10.1371/journal.pone.0033014.g004

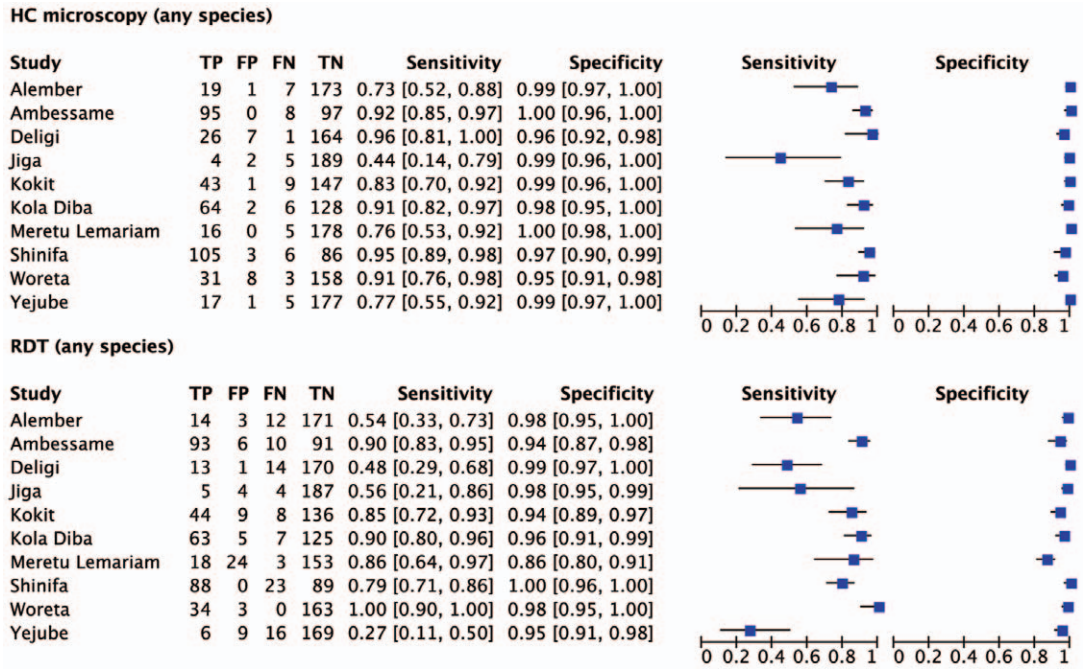

Figure 5. Sensitivity and specificity of local health centre microscopy and RDT compared to expert microscopy for the outcome 'positive for any malaria species', by health center. TP = true positive; FP = false positive; FN = false negative; TN = true negative.
doi:10.1371/journal.pone.0033014.g005

previously described [6]. Slides were also sent for expert microscopy at The Carter Center in Addis Ababa where they were examined in blinded fashion.

Rapid Diagnostic Tests. Patients were tested with ParaScreen Pan/Pf® (Zephyr Biomedical systems, Verna, Goa, India) device according to the manufacture's instruction.

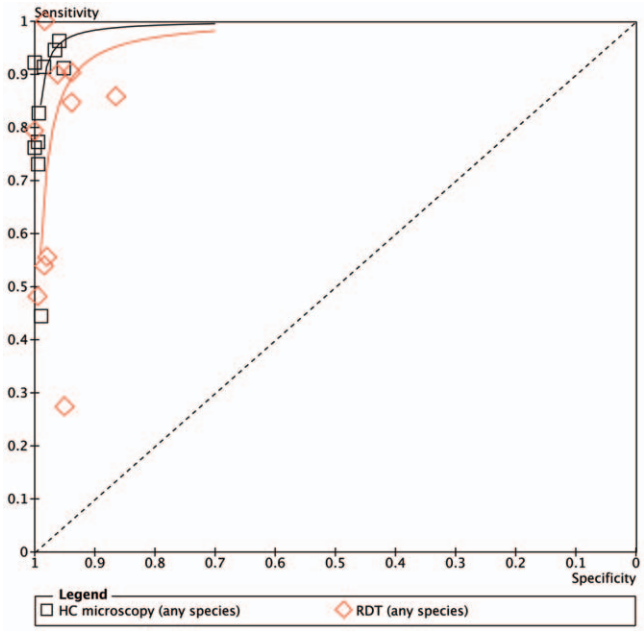

Figure 6. Summary receiver operator characteristic curve (SROC) for local health centre microscopy and RDT compared to expert microscopy for the outcome 'positive for any malaria species'.
doi:10.1371/journal.pone.0033014.g006

ParaScreen RDT had long expiry dates (6 months or more) and were stored according to the manufacturer's recommendations (4–30°C). Tests with no band at the control line were considered invalid. Band formation on the Pan-line only was considered to be evidence of non-falciparum malaria (presumably *P. vivax* infection) whilst bands at both Pan and Pf were considered *P.falciparum* or mixed infections.

Statistical analysis. Statistical analysis was conducted using SPSS version 15.0 (IBM http://www-01.ibm.com/software/analytics/spss/) and RevMAN 5.1 (Review Manager (RevMan) [Computer program]. Version 5.1. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2011). The performance of health center microscopy and of ParaScreen RDT was determined by calculating the sensitivity, specificity, positive predictive value and negative predictive value against reference laboratory microscopy as the gold standard. Sensitivity was calculated as the proportion of positive test results against true positives; specificity was calculated as a proportion of negative test results against true negatives. The positive predictive value was calculated as a proportion of true positive results among all positively reacting samples and the negative predictive was calculated as the proportion of true negative results among all negatively reacting samples. Proportions were compared using the chi-squared test. Summary receiver operator characteristic curves (SROC) were prepared in RevMAN for the two comparisons (local microscopy vs expert microscopy; RDT vs expert microscopy) and presented side by side for each of three outcomes (any malaria positive, *P.falciparum* or mixed, *P.vivax* or PAN only) by health center.

Results

Locations of the health centers are shown in Figure 1. Out of 2000 recruited patients, 1997 febrile cases were examined for malaria parasites by blood slide microscopy (198 to 200 per health

Table 2. Health center microscopy compared to expert microscopy: any malaria species.

Health center	Sensitivity % (95% CI)	Specificity % (95% CI)	Positive predictive value % (95% CI)	Negative predictive value % (95% CI)
Shinfa	94.6 (88.1–97.9)	96.6 (90.5–99.3)	97.2 (92.1–99.4)	93.5 (86.3–97.6)
Ambessame	92.2 (85.3–96.6)	100.0 (96.3–100)	100.0 (96.2–100)	92.4 (85.5–96.7)
Kola Diba	91.4 (82.3–96.8)	98.5 (94.6–99.8)	96.9 (89.5–99.6)	95.5 (90.5–98.3)
Kokit	82.7 (69.7–91.8)	99.3 (96.3–99.9)	97.7 (87.9–99.9)	94.2 (89.3–97.3)
Woreta	91.2 (76.3–98.1)	95.2 (90.7–97.9)	79.5 (63.5–90.7)	98.1 (94.7–99.6)
Deligi	96.3 (81.0–99.9)	95.9 (91.8–98.3)	78.8 (61.1–91.0)	99.4 (96.7–99.9)
Alember	73.1 (52.2–88.4)	99.4 (96.9–99.9)	95.0 (75.1–99.9)	96.1 (92.2–98.4)
Yejube	77.3 (54.6–92.2)	99.4 (96.9–99.9)	94.4 (72.7–99.9)	93.7 (93.7–99.1)
Meruto Lemariam	76.2 (52.8–91.8)	100.0 (97.9–100)	100.0 (79.4–100)	97.3 (93.4–99.1)
Jiga	44.4 (13.7–78.8)	98.9 (96.3–99.9)	66.7 (22.3–95.7)	97.4 (94.1–99.2)
TOTAL	**88.4 (85.2–91.2)**	**98.4 (97.6–98.9)**	**94.4 (91.8–96.3)**	**96.5 (95.4–97.3)**

doi:10.1371/journal.pone.0033014.t002

center). Out of these, 56.2% were males and the remaining were females. The age range was 8 months to 85 years with a mean of 20.7 years. Of the 1997 persons tested by slide, 1993 samples were also examined by ParaScreen RDT at the health centers. During supervisory visits to the health centers, it was observed in some health centers that the technicians were overloaded with different laboratory work due to high flow of outpatients seeking treatment and laboratory tests.

The results for all the health centers combined are shown in the flow chart in Figure 2. By expert microscopy (the gold standard), 23.8% of the 1997 patients tested were positive for malaria parasites, with a range from 4.5% to 55.5% by health center (Table 1 and Figure 3). Results for health center microscopy were overall 22.3% positive (N = 1997) with a range of 3.0 to 54.1%; and for ParaScreen RDT 22.2% positive (N = 1993) with a range of 4.5 to 49.5%. These differences between expert microscopists,

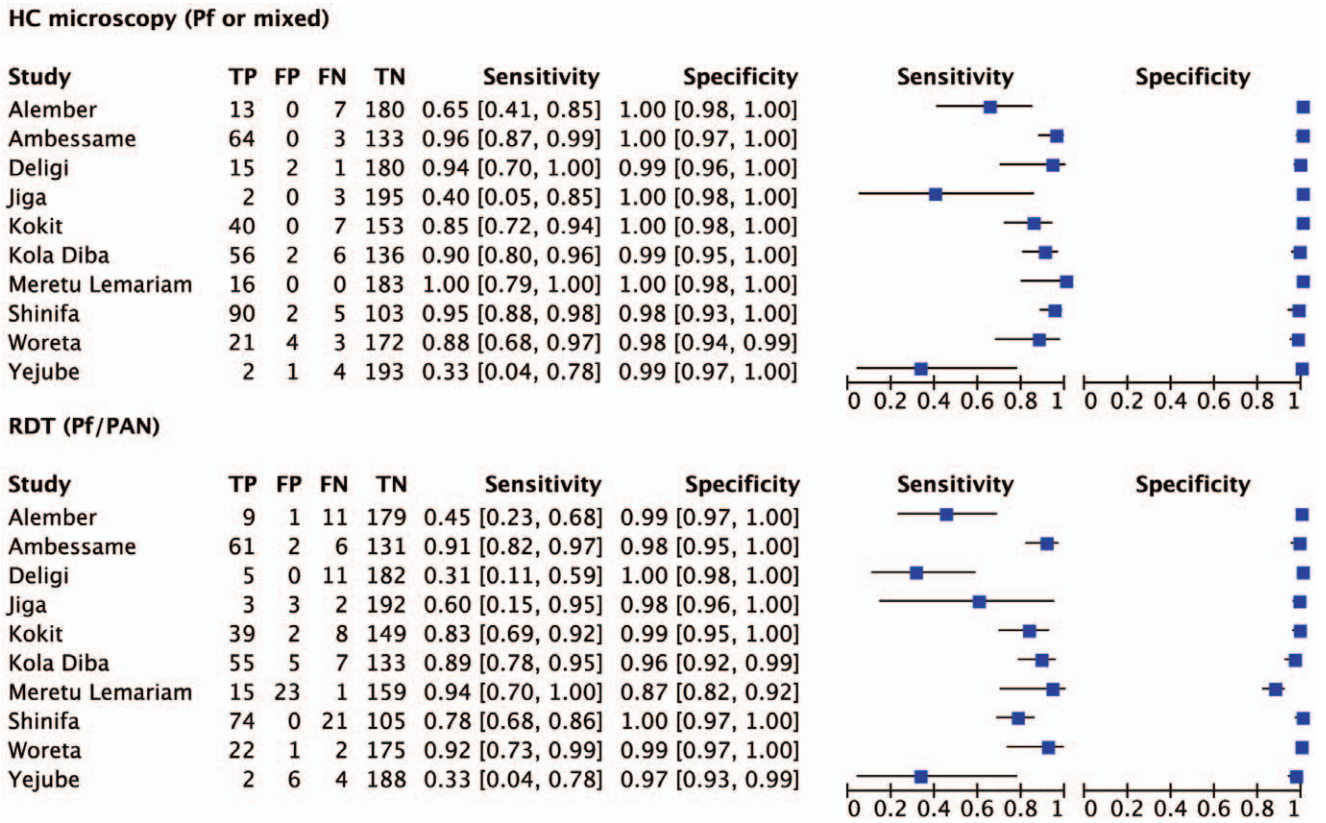

Figure 7. Sensitivity and specificity of local health centre microscopy and RDT compared to expert microscopy for the outcome 'positive for *P.falciparum* or mixed infection', by health center. TP = true positive; FP = false positive; FN = false negative; TN = true negative.
doi:10.1371/journal.pone.0033014.g007

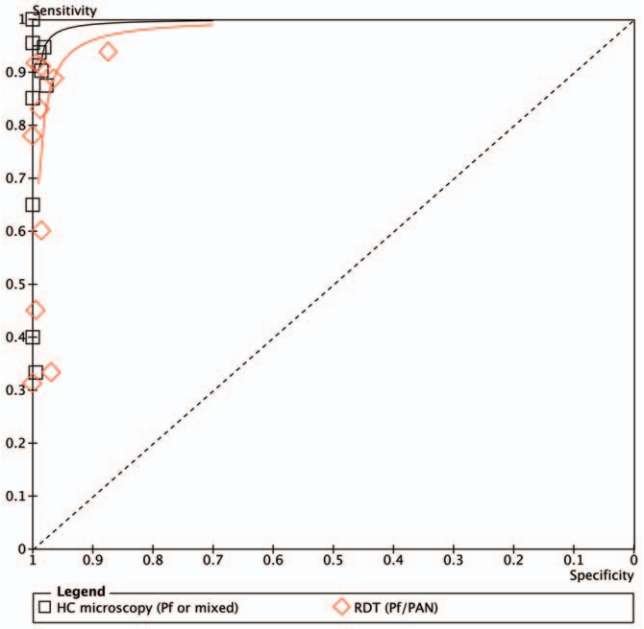

Figure 8. Summary receiver operator characteristic curve (SROC) for local health centre microscopy and RDT compared to expert microscopy for the outcome 'positive for *P.falciparum* or mixed infection'.
doi:10.1371/journal.pone.0033014.g008

health center microscopists and RDTs in overall percent positive are not statistically significant. However, they mask significant variation at the health center level.

Altitudinal variation in relation to malaria slide positivity is shown in Figure 4. In general there was a declining trend of positivity rate with altitude, with the lowest rates being observed at altitudes higher than 2000 meters above sea level, but there were two health centers between 1750 and 2000 meters above sea level with high slide positivity rates (Ambessame with 51.5% and Kola Diba with 35% slide positivity rate). The possible explanation for high malaria positive rate in these two health centers at high altitude could be that the majority of the patients were from the catchment villages of lower altitude of known malarious areas.

Health Center Microscopy Compared to Expert Microscopy

The majority of infections (57.7%) detected by expert microscopists were *P.falciparum* only, with 24.6% *P.vivax* and 17.7% mixed infections (Table 1 and Figure 2). The overall ratio of *P.falciparum* to *P.vivax* (1.78:1 for the experts) was comparable for the health center microscopists (1.69:1).

By individual health center, overall percent positive was not significantly different between health center and expert microscopists in any health center. However the general concordance in slide positive rate mentioned above and shown for the total sample in Figure 3 does not represent the complete picture, since there was not complete overlap in the positives or the species identified by the two sets of microscopists (Figure 2).

Figure 5 expresses the sensitivity and specificity for the outcome of malaria positive (any species) at each health center against expert microscopy, and Figure 6 shows the results in Summary Receiver Operator characteristic (SROC) format. The positive and negative predictive values are given in Table 2.

The overall sensitivity of microscopy for any malaria species by the health center microscopists was 88.4% (95% CI 85.2–91.2) and the specificity was 98.4% (95% CI 97.6–98.9). In the six health centers with highest prevalence (Shinifa, Ambessame, Kola Diba, Woreta, Deligi and Alember), sensitivity was greater than 90% in five of them and above 80% in Kokit (Figure 5). In three of the medium transmission areas (Meruto-Lemariam, Yejube and Alember), sensitivity of 70 to 80% was observed. Notably, Figure 5 shows very poor sensitivity by the health centre microscopist of 44.4% (95% CI 13.7–78.8) at Jiga health center, which had the lowest positivity rate of all the centers. Specificity was above 95% at all the centers.

Figures 7 and 8 show the equivalent results for the comparison of health center microscopy versus expert microscopy for *P.falciparum* or mixed infections. Positive and negative predictive values are given in Table 3. Sensitivity for *P.falciparum* was above 80% in 7 of the 10 centers, and specificity was 98% or higher in all (Figure 7). Two sites (Yejube and Jiga) had relatively low sensitivity for *P.falciparum* (<60%).

For *P.vivax* results are shown in Figs. 9 and 10. Sensitivity and specificity are shown graphically in Figure 9 while positive and negative predictive values are in Table 4. For *P.vivax* (Figure 9), the majority of the sites had sensitivity above 80% and specificity was

Table 3. Health center microscopy compared to expert microscopy: *P.falciparum* infection only.

Health center	Sensitivity % (95%CI)	Specificity % (95%CI)	Positive predictive value % (95%CI)	Negative predictive value % (95%CI)
Shinfa	93.3 (85.1–97.8)	98.4 (94.3–99.8)	97.2 (90.3–99.7)	96.1 (91.1–98.7)
Ambessame	93.6 (82.5–98.7)	100.0 (97.6–100.0)	100.0 (91.9–100)	98.1 (94.5–99.6)
Kola Diba	89.5 (78.5–96.0)	98.6 (95.0–99.8)	96.2 (87.0–99.5)	95.9 (99.3–98.5)
Kokit	89.5 (75.2–97.1)	100.0 (97.6–100)	100.0 (89.7–100)	97.6 (93.9–99.3)
Woreta	85.7 (57.2–98.2)	98.4 (95.4–99.7)	80.0 (51.9–95.7)	98.9 (96.1–99.9)
Deligi	100.0 (66.4–100)	98.9 (96.2–99.8)	81.8 (48.2–99.7)	100.0 (98.5–100.0)
Alember	30.0 (6.7–65.3)	100.0 (98.1–100)	100.0 (29.2–100)	96.5 (92.8–98.6)
Yejube	20.0 (0.5–71.6)	99.5 (97.2–99.9)	50.0 (1.3–98.7)	97.9 (94.9–99.5)
Meruto Lemariam	100.0 (79.4–100)	100.0 (98.0–100)	100.0 (79.4–100)	100.0 (98.0–100)
Jiga	0.0	100.0 (98.1–100)	0.0	98.5 (95.7–99.7)
TOTAL	**87.6 (83.1–91.3)**	**99.4 (98.9–99.7)**	**96.0 (92.8–98.1)**	**98.1 (97.3–98.7)**

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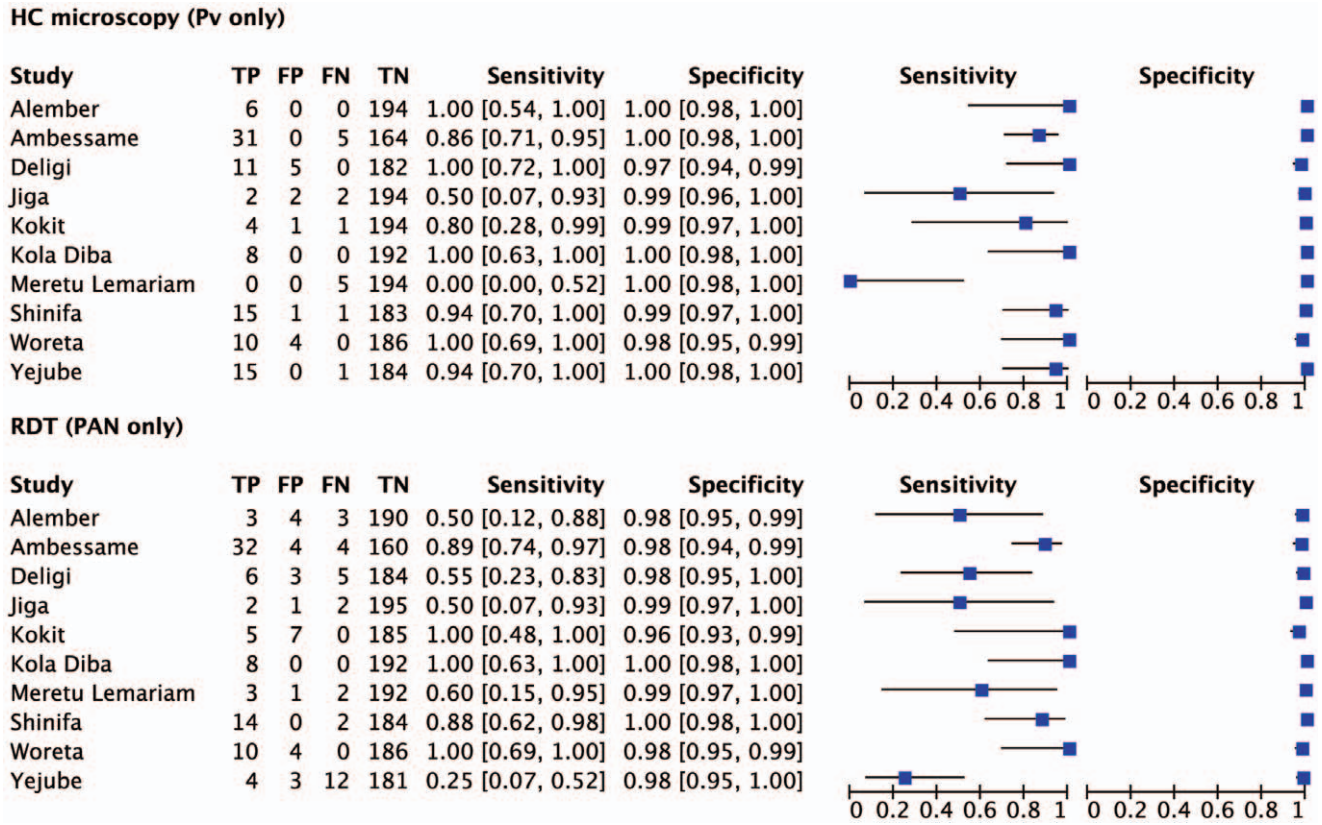

Figure 9. Sensitivity and specificity of local health centre microscopy and RDT compared to expert microscopy for the outcome '*P.vivax* or PAN only', by health center. TP = true positive; FP = false positive; FN = false negative; TN = true negative.
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very good; only one site (Meruto Lemariam) showed very poor sensitivity for *P.*vivax.

ParaScreen Rapid Diagnostic Test Compared to Expert Microscopy

For any malaria species (Figure 6), the overall sensitivity of RDTs was 79.4%. Only 3 of the health centers (Ambessame, Kola Diba and Woreta) had sensitivity over 90%), two (Kokit and Meruto Lemariam) were between 80 and 90%, one (Shinfa) was 79% and the other four were below 60% sensitivity. Specificity was very good overall with the exception of Meruto Lemarian with 86% specificity. The SROC curves for the outcomes of malaria positive (any species) are shown in Figure 7 while positive and negative predictive values are in Table 5.

The proportion of positives (any species) detected by RDT was significantly lower than the expert microscopists at two health

Table 4. Health center microscopy compared to expert microscopy: *P.vivax* only.

Health center	Sensitivity % (95%CI)	Specificity % (95%CI)	Positive predictive value % (95%CI)	Negative predictive value % (95%CI)
Shinfa	93.8 (69.8–99.8)	99.5 (97.0–99.9)	93.8 (69.8–99.8)	99.5 (97.0–99.9)
Ambessame	86.1 (70.5–95.3	100.0 (97.8–100)	100.0 (88.8–100)	97.0 (93.2–99.0)
Kola Diba	100.0 (63.1–100	100.0 (98.1–100)	100.0 (63.1–100)	100.0 (98.1–100)
Kokit	80.0 (28.4–99.5)	99.5 (97.2–99.9)	80.0 (28.4–99.5)	99.5 (97.2–99.9)
Woreta	100.0 (69.2–100)	97.9 (94.7–99.4)	71.4 (41.9–91.6)	100.0 (98.0–100)
Deligi	100.0 (75.1–100)	97.3 (93.9–99.1)	68.8 (41.3–88.9)	100.0 (97.9–100)
Alember	100.0 (54.1–100.0)	100.0 (98.1–100.0)	100.0 (54.1–100.0)	100.0 (98.1–100.0)
Yejube	93.8 (69.8–99.8)	100.0 (97.0–99.9)	100.0 (78.2–100)	99.5 (97.0–99.9)
Mertu-Lemariam	0.0	100.0 (98.1–100)	0.0	97.5 (94.2–99.2)
Jiga	50.0 (6.8–93.2)	98.9 (96.4–99.9)	50.0 (6.8–93.2)	98.9 (96.4–99.9)
TOTAL	**82.2 (79.7–92.6)**	**99.3 (98.8–99.6)**	**88.7 (81.5–93.8)**	**99.2 (98.7–99.6)**

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Table 5. Rapid Diagnostic Test (Pf/PAN or PAN) compared to expert microscopy: any malaria species.

Health center	Sensitivity % (95%CI)	Specificity % (95%CI)	Positive predictive value % (95%CI)	Negative predictive value % (95%CI)
Shinfa	79.3 (71.7–86.8)	100.0 (95.9–100.0)	100.0 (95.9–100.0)	79.5 (72.0–86.9)
Ambessame	90.3 (84.6–96.0)	93.8 (89.0–98.6)	93.9 (89.2–98.6)	90.1 (84.3–95.9)
Kola Diba	90.0 (83.0–97.0)	96.2 (98.2–99.5)	92.6 (86.4–98.6)	94.7 (90.9–98.5)
Kokit	84.6 (74.8–94.4)	93.9 (90.1–97.8)	83.0 (72.9–93.1)	94.6 (90.9–98.2)
Woreta	100.0 (89.7–100.0)	98.2 (94.8–99.6)	91.9 (78.1–98.3)	97.6 (93.9–99.3)
Deligi	48.1 (29.3–67.0)	99.4 (96.8–99.9)	92.9 (66.1–100.0)	92.4 (88.6–96.2)
Alember	53.8 (34.7–73.0)	98.3 (95.0–99.6)	82.4 (56.6–96.2)	93.4 (89.9–97.0)
Yejube	27.3 (8.7–45.9)	94.9 (91.7–98.2)	40.8 (15.2–64.8)	94.9 (91.7–98.2)
Meruto Lemariam	85.7 (70.7–100.7)	86.5 (81.5–91.5)	42.9 (27.9–57.8)	98.1 (95.9–100)
Jiga	55.6 (23.1–88.0)	97.9 (95.9–99.9)	55.6 (23.1–88.0)	97.9 (95.9–99.9)
TOTAL	**79.4 (75.5–82.9)**	**95.7 (94.6–96.7)**	**85.3 (81.6–88.5)**	**93.7 (92.4–94.9)**

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centers: Shinifa (44% RDT vs 55.5% expert microscopy, Chi-sq = 5.29, p = 0.021) and Deligi (7.1% RDT vs 13.6% expert microscopy, Chi-sq = 4.60, p = 0.032), while percent positive was higher by RDT at Meruto Lemariam (21.1% RDT vs. 13.2% expert microscopy, Chi-sq = 4.49, p = 0.034). The others were not significantly different.

For *P.falciparum* or mixed infection sensitivity and specificity are shown in Figure 7, SROC in Figure 8 and PPV and NPV given in Table 6. Four of the health centers (Deligi, Alember, Yejube and Jiga) with lower prevalence (see Table 1) performed poorly with RDTs (Figure 7). Meruto Lemariam was the exception among the health centers with low prevalence in achieving very good sensitivity for *P.falciparum*, although at the expense of specificity.

For *P.vivax* (PAN only by RDT), Figures 9 and 10 and Table 7 present the results. The same four low prevalence centers mentioned above (Deligi, Alember, Yejube and Jiga) performed very poorly (<60%) on RDT sensitivity, and Meruto Lemarian had only 60% sensitivity. The two centers with highest overall prevalence (Shinfa and Ambessame) had 88–89% sensitivity while the other 3 were over 90%.

ParaScreen Rapid Diagnostic Test Compared to Health Center Microscopy

Only indirect comparison is possible because the same technicians conducted both tests in each health center, which compromised the blinding. The relative accuracies of health centre microcopy and RDT for each of three outcomes (any species, Pf or mixed, Pv) are shown in the Summary ROC curves in Figures 6, 8 and 10 respectively. In each case the RDT predicted curve lies to the right and below (less accurate) that for HC microscopy.

The five sites with highest prevalence were relatively consistent in giving good or very good performance for both microscopy and RDT compared to expert microscopy. However overall, the performance of RDT was not as good as health center microscopy, and it was particularly poor in the five sites with lower prevalence. As expected, the RDTS performed in general less well for *P.vivax* than *P.falciparum*.

Discussion

Rapid diagnostic tests are being strongly promoted for wider use to ensure that all suspected malaria cases receive a diagnosis before treatment. Most RDT studies have tested whether RDTs are as

Table 6. Rapid Diagnostic Test (Pf/PAN) compared to expert microscopy: *P.falciparum* and mixed infections.

Health center	Sensitivity % (95%CI)	Specificity % (95%CI)	Positive predictive value % (95%CI)	Negative predictive value % (95%CI)
Shinfa	77.9 (69.9–86.2)	100.0 (96.5–100)	100.0 (95.1–100)	83.3 (76.8–89.8)
Ambessame	91.0 (84.2–97.9)	98.5 (94.7–99.8)	96.8 (89.0–99.6)	95.6 (92.2–99.0)
Kola Diba	88.7 (80.8–96.6)	96.4 (93.3–99.5)	91.7 (84.7–98.7)	95.0 (91.4–98.6)
Kokit	83.0 (72.2–93.7)	98.7 (95.4–99.8)	95.1 (83.5–99.4)	95.0 (91.6–98.4)
Woreta	91.7 (73.0–98.9	99.4 (96.9–99.8)	95.7 (78.1–99.9)	98.9 (95.9–99.8)
Deligi	31.3 (8.5–54.0)	100.0 (97.9–100)	100.0 (47.8–100)	94.3 (91.0–97.6)
Alember	45.0 (23.2–66.8)	99.4 (96.9–99.9)	90.0 (55.5–99.7)	94.2 (90.9–97.5)
Yejube	33.3 (4.4–71.1)	96.9 (94.5–99.3)	25.0 (5.0–55.0)	97.9 (95.9–99.9)
Meruto Lemariam	93.8 (69.8–99.8)	87.4 (82.6–92.2)	39.5 (23.9–55.0)	99.4 (96.6–99.9)
Jiga	60.0 (14.7–94.7)	98.5 (95.6–99.7)	50.0 (10.0–90.0)	98.9 (96.3–99.9)
TOTAL	**79.6 (75.1–83.7)**	**97.4 (96.5–98.1)**	**86.7 (82.8–90.4)**	**95.6 (94.5–96.6)**

doi:10.1371/journal.pone.0033014.t006

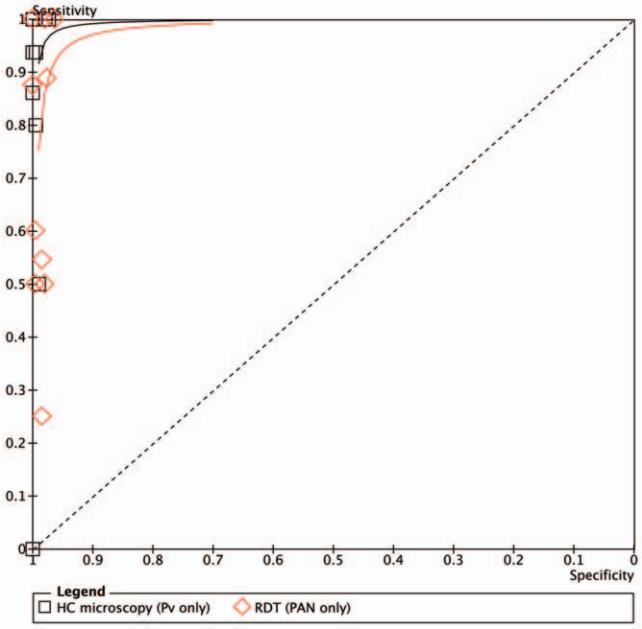

Figure 10. Summary receiver operator characteristic curve (SROC) for local health centre microscopy and RDT compared to expert microscopy for the outcome '*P.vivax* or PAN only'.
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accurate as expert microscopy, and these previous studies were mostly designed to assess the performance of the tests *per se*, rather than their accuracy in routine use. There have been few evaluations of the accuracy of RDTs compared to the *status quo* of routine health center microscopy, or of variation in performance of both routine microscopy and RDT between sites. The results of such studies point to differences in strict application of knowhow gained during training and previous work experience in malarious areas (for both methods) as well as storage or other possible factors that affect the correct use of RDTs. Even if RDTS are not as good as expert microscopy, in some cases they may be better than routine microscopy. In this study we address this issue indirectly by examining the performance of both routine microscopy and RDTs as performed in ten rural health centers, compared to the gold standard of expert microscopy.

Overall, microscopists in ten rural health centers in Amhara region, Northwest Ethiopia showed fair to very good performance compared to expert microscopy, with the exception of the health center with the lowest prevalence of 4.5% among suspected malaria cases. One other health center did badly with *P.vivax* slides only. Microscopists in health centers in these study sites of Northwest Ethiopia are performing to a standard higher than has been observed in some other malaria endemic areas [9]. However there are still some gaps and inconsistencies in microscope capacity, and lack of a standardized quality control system for diagnostics, as has been observed by others [10].

For RDTs, there was large variation between sites in the performance, with generally lower performance than for local microscopy, when each is compared to expert microscopy. Four of the ten sites (of the five with less malaria) performed very poorly on RDT sensitivity in general, and the other was very poor for *P.vivax*. Even one of the sites with high prevalence demonstrated only a fair level of sensitivity with RDTs. Sensitivity of the test (unlike positive predictive value) should not be affected by prevalence. A decrease in positive predictive value for RDT in one site with lower prevalence was also observed in Uganda [11].

During supervisory visits it was noted that although the technicians were observed to be proficient in performing the tests according to standard operating procedures, they were overloaded with the many other lab tests they are expected to perform in addition to malaria diagnosis. Under real world conditions, when pressed with large numbers of patients, they may use rapid staining methods and skimp on slide examination time or number of fields to be examined (especially if densities are low) just to satisfy the clients. Low prevalence in an area with few requests for malaria diagnosis gives the technician limited ability to maintain his or her skills in parasite identification by microscopy, or to practice reading and interpreting faint positive RDT tests. More quality control checks and frequent refresher trainings are needed in low incidence areas, or as malaria incidence declines due to extensive control efforts.

Overall our results demonstrate slightly lower sensitivity with RDTs than has been observed in Ethiopia and elsewhere [7,10]. The low sensitivity with ParaScreen in some sites means that cases are being missed while high false positive rates means that persons without malaria (and possibly with other infections) are getting

Table 7. Rapid Diagnostic Test (PAN only) compared to expert microscopy: *P.vivax* only.

Health center	Sensitivity % (95%CI)	Specificity % (95%CI)	Positive predictive value % (95%CI)	Negative predictive value % (95%CI)
Shinfa	87.5 (61.7–98.5)	100.0 (98.0–100.0)	100.0 (76.8–100)	98.9 (96.2–99.8)
Ambessame	88.9 (78.6–99.2)	97.6 (95.2–99.9)	88.9 (78.6–99.2)	97.6 (95.2–99.9)
Kola Diba	100.0 (63.1–100)	100.0 (98.1–100.0)	100.0 (63.1–100)	100.0 (98.1–100.0)
Kokit	100.0 (47.8–100)	96.4 (93.8–99.0)	41.7 (13.8–69.6)	100.0 (98.1–100)
Woreta	100.0 (69.2–100)	97.9 (95.9–99.9)	71.4 (47.8–95.1)	100.0 (98.0–100)
Deligi	54.5 (25.1–84.0)	98.4 (95.4–99.7)	66.7 (35.9–97.5)	97.4 (95.1–99.6)
Alember	50.0 (10.0–90.0)	97.9 (95.9–99.9	42.9 (6.2–79.5)	98.5 (95.5–99.7)
Yejube	25.0 (3.8–46.2)	98.4 (95.3–99.7)	57.1 (20.5–93.8)	93.8 (90.4–97.2)
Meruto Lemariam	60.0 (14.7–94.7)	99.5 (97.2–99.9)	75.0 (19.4–99.4)	99.0 (96.3–99.8)
Jiga	50.0 (10.0–90.0)	99.5 (97.2–99.9)	66.7 (9.4–99.2)	98.9 (96.4–99.8)
TOTAL	**74.4 (65.5–81.9)**	**98.6 (97.9–99.1)**	**76.3 (67.4–83.8)**	**98.4 (97.7–98.9)**

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treated for malaria in some sites. These findings suggest that there are deficiencies in strict application of training materials, lack of previous skill in performing multispecies RDTs, and/or possible problems in RDT handling conditions in some sites, in addition to large demands on technicians' time for other lab tests. Where no adequate and standard malaria microscopy exists (for example in health posts staffed by Health Extension Workers in moderate to high malarious areas), this study supports the introduction of multispecies RDTs for improvement of diagnosis of malaria, provided that they are accompanied by adequate training on procedure and limitations of the tests, as well as continual supervision and overall quality control mechanisms. However, microscopy in rural health centers remains the local 'gold standard' and should not be neglected for refresher training and supervision especially where problems are identified in particular centers as in this study.

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Author Contributions

Conceived and designed the experiments: TE PMG TG EBS FOR PME. Performed the experiments: TE TT BM AG ZT BA AWM AM. Analyzed the data: TE FA PMG AWM. Wrote the paper: TE PMG PME.

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RESEARCH Open Access

Reliability of rapid diagnostic tests in diagnosing pregnancy-associated malaria in north-eastern Tanzania

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Abstract

Background: Accurate diagnosis and prompt treatment of pregnancy-associated malaria (PAM) are key aspects in averting adverse pregnancy outcomes. Microscopy is the gold standard in malaria diagnosis, but it has limited detection and availability. When used appropriately, rapid diagnostic tests (RDTs) could be an ideal diagnostic complement to microscopy, due to their ease of use and adequate sensitivity in detecting even sub-microscopic infections. Polymerase chain reaction (PCR) is even more sensitive, but it is mainly used for research purposes. The accuracy and reliability of RDTs in diagnosing PAM was evaluated using microscopy and PCR.

Methods: A cohort of pregnant women in north-eastern Tanzania was followed throughout pregnancy for detection of plasmodial infection using venous and placental blood samples evaluated by histidine rich protein 2 (HRP-2) and parasite lactate dehydrogenase (pLDH) based RDTs (Parascreen™) or HRP-2 only (Paracheck Pf® and ParaHIT®f), microscopy and nested *Plasmodium* species diagnostic PCR.

Results: From a cohort of 924 pregnant women who completed the follow up, complete RDT and microscopy data was available for 5,555 blood samples and of these 442 samples were analysed by PCR. Of the 5,555 blood samples, 49 ((proportion and 95% confidence interval) 0.9% [0.7 -1.1]) samples were positive by microscopy and 91 (1.6% [1.3-2.0]) by RDT. Forty-six (50.5% [40.5 - 60.6]) and 45 (49.5% [39.4 – 59.5]) of the RDT positive samples were positive and negative by microscopy, respectively, whereas nineteen (42.2% [29.0 - 56.7]) of the microscopy negative, but RDT positive, samples were positive by PCR. Three (0.05% [0.02 - 0.2]) samples were positive by microscopy but negative by RDT. 351 of the 5,461 samples negative by both RDT and microscopy were tested by PCR and found negative. There was no statistically significant difference between the performances of the different RDTs.

Conclusions: Microscopy underestimated the real burden of malaria during pregnancy and RDTs performed better than microscopy in diagnosing PAM. In areas where intermittent preventive treatment during pregnancy may be abandoned due to low and decreasing malaria risk and instead replaced with active case management, screening with RDT is likely to identify most infections in pregnant women and out-performs microscopy as a diagnostic tool.

Keywords: Rapid diagnostic tests (RDTs), Reliability, Sensitivity, *Plasmodium falciparum*, Pregnancy-Associated Malaria (PAM), Microscopy, Polymerase chain reaction (PCR), Sub-microscopic infections, Pregnancy outcomes, Tanzania

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Background

Accurate diagnosis and prompt treatment of pregnancy-associated malaria (PAM) is essential to avert adverse pregnancy outcomes [1]. Detection of sub-microscopic infections is crucial in order to not only effect prompt treatment of asymptomatic cases, but also to identify and clear potential reservoirs of transmission [2,3] and to reduce malaria related morbidity and mortality. Presumptive treatment of malaria based on clinical diagnosis is relatively cheap but it is unreliable due to overlapping symptoms with non-malarial infections caused by viruses or bacteria [4] and could lead to over-diagnosis [5] as well. Wrong diagnoses may lead to presumptive medication and hence many patients may leave the health facility without the right treatment. Rational prescription of anti-malarials is not only important in saving on the cost of expensive drugs but it also prevents drug overuse that might result in the development of resistance [6]. Sub-microscopic infections during pregnancy might be associated with increased risk of adverse pregnancy outcomes including low birth weight babies and maternal anaemia [7,8]. Therefore, treatment of these infections may prevent potential risks of adverse pregnancy outcome [9].

PAM in the sub-Saharan Africa is caused by *Plasmodium falciparum*, and it is precipitated because VAR2CSA, member of the *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP-1) family expressed on the surface of infected erythrocytes (IEs) mediates sequestration of IEs in the intervillous spaces of the placenta by binding to chondroitin sulphate A (CSA) receptors [10,11]. The pathogenesis of PAM and its association with adverse pregnancy outcome [12,13], such as intrauterine growth retardation and low birth weight is not well understood but it is thought to be caused by impairment in nutrient transport to the foetus [14], with possible effects on growth regulating hormones [15] and trophoblast invasion [16]. As a result of placental sequestration it is often difficult to detect IEs in the peripheral blood using microscopy [17]. Furthermore, malarial infections are usually asymptomatic among adults in malaria endemic regions, decreasing the chances of clinical detection by using clinical algorithms. Primi- and secundigravidae as opposed to multigravidae are most affected as they lack sufficient previous exposure to allow the development of protective immunity [18,19].

Microscopic examination of blood smears has been the gold standard for malaria diagnosis but it is compromised by poor infrastructure and the need for individuals with expertise in microscopy who are not necessarily available in many health facilities in malaria endemic regions [20]. Furthermore, microscopy is not sensitive enough [5,9,21-24], it requires good quality reagents, well maintained microscopes, and is time consuming [25]. Studies conducted in many malaria endemic regions show better sensitivity of RDTs as compared to microscopy [26-29] in malaria diagnosis and it is suggested that RDTs could be used as a supplementary diagnostic tool to aid evidence-based decision making in malaria treatment. The use of RDTs requires neither extensive training [30] nor substantial investment in infrastructure as compared to microscopy. However, there are a number of challenges that need to be addressed for optimal and effective utilization of RDTs in malaria diagnosis in order to provide reliable and credible diagnoses [31]. There should among others be; frequent quality controls and assurances, optimal storage conditions as well as updates on newly available or improved RDTs. If proper instructions are not given to staff especially in the rural communities on how to properly handle these RDTs, their expected usefulness as an alternative diagnostic tool for malaria diagnosis would be highly compromised.

The performance of Parascreen™ has previously been assessed under field conditions [32] involving children with clinical suspicion of malaria in a rural area of Kenya and gave results that were in agreement with other malarial diagnostic tests. Likewise, Paracheck Pf® and ParaHIT®f have also been assessed in community studies within the study area [33,34]. However, during these cross-sectional and longitudinal community studies [33] it has been shown that Paracheck Pf® and ParaHIT®f which are HRP-2 based RDTs were not very sensitive in diagnosing parasite densities of less than 200 asexual stages/μl in asymptomatic children. The sensitivity of different RDTs can also be improved by increasing the concentration of detection antibodies. This is an important component in the manufacturing process, which is usually coupled by frequent evaluations of test performances. Nevertheless their use, despite the low sensitivity at very low parasitaemia has significantly reduced over-prescription of anti-malarials among individuals without malarial infection and they performed well in diagnosing those with symptoms of malaria [5].

Many studies that assess the sensitivity and specificity of RDTs of malaria mainly utilize microscopy as the gold standard [35-37]. However, RDTs detecting histidine rich protein 2 (HRP-2) have the problem of detecting HRP-2 antigen circulating in the blood more than two weeks after IEs have been cleared from the blood stream, resulting in high false positive rates [38,39]. In the study presented here, RDTs that detect both HRP-2 and pLDH as well as HRP-2 only antigens were used in order to detect *P. falciparum* infections, and to also identify non-*falciparum* species in the area [40].

Other sensitive alternative tests superior to microscopy and RDTs such as PCR and real-time quantitative nucleic acid sequence-based amplification (real time QT-NASBA) are also available [28,41], but they are mainly being utilized for epidemiological studies rather than facilitating treatment.

Failure to detect asymptomatic and sub-microscopic infections may leave a large part of the population with untreated infections that may lead to persistent maternal anaemia [42,43] and adverse consequences for the foetus. Simple and easy to use malarial diagnostic tools with adequate sensitivity such as RDTs are therefore required [44] for effective management of PAM. As part of a study entitled strategies to prevent pregnancy-associated malaria (STOPPAM), a sub-study with the aim of assessing the reliability of RDTs in diagnosing PAM was conducted.

Methods

Study design

A prospective cohort study on pregnant women was conducted from September 2008 to October 2010. Enrolled women had gestational age of ≤ 24 weeks, were residents within an accessible area of Korogwe District in order to facilitate follow up, had given written informed consent to participate and were willing to deliver at Korogwe District Hospital (KDH). After inclusion, the cohort was followed up through three pre-scheduled antenatal clinic visits (every 2–6 weeks depending on gestational age) until delivery at KDH and satellite outreach dispensaries within Korogwe District, and they were also seen outside the pre-scheduled visits (at any time) whenever necessary. Venous blood samples were collected during each visit and placental blood at delivery for detection of malaria parasites and evaluation of haematological and other biological parameters. Ultrasound sonography was performed at inclusion to ascertain the gestational age and at each antenatal visit to assess intrauterine foetal growth.

Study site

This study was carried out in Korogwe District, north-eastern Tanzania. The district is inhabited by approximately 261,004 individuals, with a growth rate of 1.4% per annum according to the 2002 Tanzanian human population census report [45]. The district can be topographically stratified into lowland and highland zones, and malaria transmission is perennial with the highest transmission in the lowlands and after long rains [46,47] and low transmission in the highlands at the onset of short rains. *P. falciparum* is the dominant malaria species transmitted by *Anopheles gambiae* s.s and *Anopheles funestus* [48] with entomological inoculation rates of 91 infective bites/person per year [46]. The district has been under constant surveillance of malaria since 2003. Of late, there has been a progressive decline in malaria parasite slide positivity rates in the area [49,50] that transformed the area from hyper-endemic to meso/hypo endemic. Obstetric care is provided at KDH, and at other Health Centres and dispensaries within Korogwe District. More details about the study area can be found in Mmbando *et al.* [49]. According to the 2004–2005 demographic and health survey (DHS) report, the coverage of intermittent preventive treatment during pregnancy with sulphadoxine-pyrimethamine (IPTp-SP) in Tanga Region where Korogwe District is situated was 61.9% [51] as opposed to 90% during the STOPPAM study indicating the importance of sensitization and its impact on utilization of health services.

Ethical approval, sensitization meetings and informed consent

The study protocol was approved by the Tanzania Medical Research Coordinating Committee with reference number NIMR/HQ/R.8a/Vol. IX/688. Sensitization meetings about the study goals and expectations were held in all catchment villages. All procedures were conducted in consistent with good clinical and laboratory practices. All participants gave written informed consent.

Study samples

A cohort of 924 pregnant women was followed up from enrolment until delivery, and a total of 5,905 samples were collected. Of those, 5,167 venous and 388/650 placental blood samples, for which there were complete RDT and microscopy datasets, were randomly selected for analysis. The calculation of the sample size for the primary study "STOPPAM" was based on the placental hospital study that was previously conducted at KDH and reported a placental parasite prevalence ranging from 10 – 18%. The study screened 1,171 and excluded 176 pregnant women to have the final sample size of 995 pregnant women meeting the inclusion criteria for enrolment. Seventy one pregnant women were lost to follow up due to various reasons (Figure 1).

Blood drawing

Five to ten ml of venous blood was drawn at inclusion, at scheduled and unscheduled antenatal clinic visits, and just before/after delivery. Ten ml of unperfused placental blood was also collected within 15 minutes of delivery for women who gave birth at the labour ward of KDH. The venous and placental blood for malaria thick and thin blood smear preparations as well as filter paper blood spots for DNA extraction were collected in ethylenediamine-tetraacetic acid (EDTA) minicollect tubes, whereas for RDTs whole blood was directly added to the test device.

Malaria rapid diagnostic tests

Parascreen™ (Zephyr Biomedicals Goa, India) an RDT that detects histidine rich protein 2 (HRP-2) antigen from *P. falciparum* and parasite lactate dehydrogenase (pLDH) from the *Plasmodium* species was used for

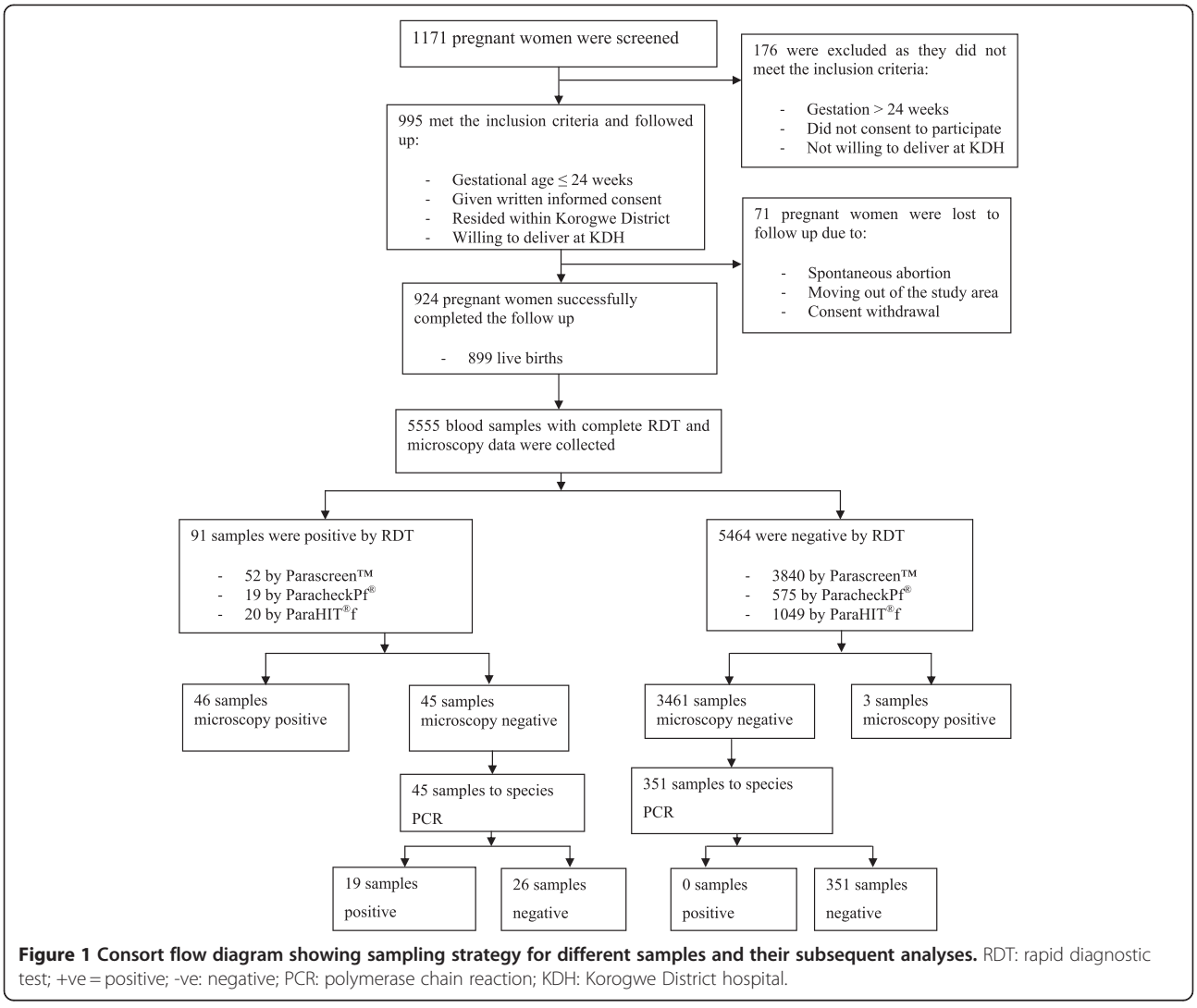

Figure 1 Consort flow diagram showing sampling strategy for different samples and their subsequent analyses. RDT: rapid diagnostic test; +ve = positive; -ve: negative; PCR: polymerase chain reaction; KDH: Korogwe District hospital.

the majority of samples in this study. A minority of samples were tested by Paracheck Pf® (Orchid Biomedical Systems –Mumbai, India) or ParaHIT®f (Span diagnostics Ltd – Surat, India), the commonly available RDTs in the study area. Paracheck Pf® and ParaHIT®f only detect HRP-2 antigen from *P. falciparum*. All tests were performed following the manufacturer's instructions. The laboratory and clinical personnel were trained on how to perform and interpret the RDTs results. As of the 2011 WHO round 3 data on malarial RDTs performances, all the RDTs used in this study have been shown to perform well in all three rounds of testing for performances.

Microscopic examination of thick and thin blood smears

Thick and thin blood smears from whole EDTA venous and placental blood samples were prepared on glass slide, air dried, stained for 30 minutes with 5% Giemsa stain, then washed gently in tap water, air dried and finally examined with a 100x objective lens under oil immersion. Before a slide was declared negative 100 microscopical thick film fields were scanned. Asexual parasite density was recorded as number of asexual stage parasites per 200 leucocytes, and converted to parasite count per microlitre, by using the actual count of leucocytes as estimated by Sysmex KX-21 N haematological analyser (Kobe, Japan). If the parasite count was less than 10, it was recorded per 500 leucocytes. All slides were read twice by two independent experienced microscopists and results from the two readings with a difference of less than 50% were considered definitive. Smears with discordant results were re-examined by a third experienced microscopist (blinded to the first two readings) and results from two readers that were in agreement were considered final. All laboratory technologists reading the blood slides participated in the proficiency microscopy examination and were certified by the National Institute for Communicable Diseases (NICD), South Africa.

Plasmodium species diagnostic PCR

In order to circumvent the problem of false negatives due to the inability of microscopy in detecting sub-microscopic infections or false positives due to the detection of circulating HRP-2 antigen by RDT even after parasite clearance, all the RDT positive but microscopy negative samples as well as a proportion of both the RDT and microscopy negative samples were analysed using nested *Plasmodium* species diagnostic PCR assay. Fifty microlitre of EDTA blood was added on a premade template of Whatman number 3 filter paper and allowed to dry at room temperature and stored in silica gel to preserve the DNA integrity. Briefly, half sector of the filter spot was excised and incubated with 0.5% saponin (SIGMA™) in 1x PBS and incubated overnight to remove the haemoglobin. The DNA was extracted by Chelex 100 resin method as explained by Wooden *et al.* [52] with some modifications. The DNA supernatant was carefully transferred to a new 96 wells PCR plate without touching the Chelex 100 resin and stored at −20°C until use. The parasite DNA was amplified by outer and nested species diagnostic PCR according to Snounou *et al.* [53] and the PCR products were analysed in 1.5% ethidium bromide stained UltraPure™ agarose gel (Invitrogen) with a Gene ruler™ 50 bp DNA ladder (Lonza, Belgium). The gels were visualized under UV trans-illuminator from BIO-RAD.

Management of malaria

All women with confirmed malarial infection based on RDTs and/or microscopy were treated with anti-malarial drugs. Uncomplicated and asymptomatic infections were treated by administration of quinine in the first trimester and artemether-lumefantrine during the second or third trimester.

Data management and analyses

All data were documented on case record forms and double entered into Microsoft Access database, cleaned, validated and transferred into R. version 2.12.0 statistical package for analyses. Statistical significance level was considered at $\alpha = 0.05$. Baseline characteristics (demographic, clinical and parasitological) were analysed using descriptive statistics. Sensitivity was defined as the proportion of true malarial cases (positive blood smears and/or PCRs) that were correctly identified by positive RDTs whereas specificity was the proportion of true negative malarial cases (negative blood smears/negative PCRs) that were correctly identified by negative RDTs. Positive predictive value was the proportion of true malarial cases (positive blood smears and/or PCR) among the individuals with the positive RDTs. Negative predictive value was the proportion of true negative malarial cases (negative blood smears/PCRs) among the total number of negative RDT tests. Accuracy was defined as the proportion of all tests that gave correct results (True Positive + True Negative)/ number of all tests.

Results

In total, 1,171 pregnant women were screened, 995 (85%) met the inclusion criteria and were enrolled and followed up. Seventy-one women were lost to follow up or excluded before delivery due to spontaneous abortion or moving out of the study area. Of the enrolled cohort, 924/995 (93%) successfully completed follow up, from whom 5,555 blood samples with complete RDT and microscopy data were selected for further analyses (Figure 1). Of the enrolled cohort, 471 women were primi/secundigravid and 524 were multigravid with mean ages of 22.6 ± 4.2 and 30.7 ± 5.3 years for primi/secundigravid and multigravid women, respectively. There were 899 successful live births among the 924 pregnant women who successfully completed the follow up. *P. falciparum* was the only malarial parasite detected by microscopy. In women with a positive slide reading, the median asexual parasite density/μl was 2,090 [range; 40–390,748] and 4,163 [range; 40–45, 760] for primi-/secundigravid and multigravid, respectively, (Table 1). 3,892 samples were tested with Parascreen™, an RDT which detects both *P. falciparum* and non-*falciparum* parasites. Thirteen samples (0.3% [95% CI: 0.2 – 0.6] were RDT positive for non-*falciparum* species, and of these 11 were also RDT-positive for *P. falciparum*. As stated above non-*falciparum* parasites were not detected by slide reading and the remaining part of the article only compares detection of *P. falciparum* by the different methods employed since it is the one mainly involved in PAM pathogenesis.

Comparison of rapid diagnostic test with microscopy results

Overall 91/5,555 (1.6% [1.3-2.0]) samples were positive for malarial parasite antigen based on RDTs whereas 49/5,555 (0.9% [95% CI; 0.7 –1.1]) were positive by microscopy (Table 2). Of the 91 RDT positive samples, 46 (50.5% [95% CI: 40.5 - 60.6] were microscopy positive whilst 45 (49.5% [95% CI; 39.4 – 59.5]) were microscopy

Table 1 Age, mean haemoglobin and parasite density of positive slides among the pregnant women included in the study

Parameter	Gravidity	
	Primi/Secundigravid (n = 471)	Multigravid (n = 524)
Age in years (Mean ± SD)	22.6 ± 4.2	30.7 ± 5.3
Mean haemoglobin level (g/dL) [range]	10.45 [3.1 – 22.8]	10.53 [3.8 – 22.4]
Median asexual parasite density/μl [range]	2090 [40–390748]	4163 [40–45760]

SD: standard deviation; dL: deciliter; μl: microlitre.

negative. To test whether the 45 microscopy negative but RDT positive samples were genuinely negative or false positive, a species diagnostic PCR assay was performed. Three (0.05% [0.02 - 0.2]) samples were positive by microscopy but negative by RDT. These samples were not tested by PCR assay, but the slide readings were confirmed by two independent expert slide readers as positive with median parasite density of 224.75 [range; 86 – 486].

PCR confirmation of the RDT positive but microscopy negative cases

The 45 RDT positive but microscopy negative samples were checked by nested PCR to ascertain whether they were sub-microscopic infections. Interestingly, 19/45 (42.2% [95% CI; 29.0 - 56.7] were positive by PCR (Table 3).

Nested species diagnostic PCR correction of RDT and microscopy negative samples

A proportion 351/650 (54%) of the available placental blood samples that were malarial negative by both RDT and microscopy were checked by nested PCR targeting *P. falciparum* as it is the most prevalent species in the study area and the one responsible for PAM pathology. Only 650 placental blood samples could be collected due to clotted placental blood (e.g. retained placenta or the need for the project nurses to stay in the labour room to resuscitate the newborn, delaying the transportation of the placenta to the laboratory for processing) and 190 women gave birth outside the KDH setting making it impossible to collect placental blood. Due to limited resources and time, only a small proportion of all the negative samples could be checked by PCR. The 351 placental instead of venous blood samples were randomly selected as microscopy could have missed some parasites in the peripheral blood due to parasite sequestration in the placenta and also due to the presence of debris and other contaminants in the placental blood that might have resulted to poor quality blood smears not easily readable. Interestingly, all these samples were negative by PCR indicating that both microscopy and RDTs performed equally well in diagnosing true negative cases.

Table 3 PCR analysis results of samples that were RDT positive but microscopy negative

RDT type	No. microscopy negative samples	PCR results	
		Positive	Negative
Parascreen™	30	12 (40.0%)	18 (60.0%)
ParacheckPf®	9	6 (66.7%)	3 (33.3%)
ParaHIT®f	6	1 (16.7%)	5 (83.3%)
Total	**45**	**19 (42.2%)**	**26 (57.8%)**

PCR: polymerase chain reaction; RDT: rapid diagnostic test.

Performance of different RDTs after PCR correction

The performance of the different RDTs and microscopy was compared in the 442 samples in which PCR was performed and using the PCR results as the golden standard (Table 4). There was no statistically significant difference between the performances of the different RDTs. However, the study was not designed to directly compare the different RDTs and these RDTs were not employed on the same samples. All the PCR and slide positive samples were *P. falciparum.* Probably the use of a modified Snounou PCR using *P. ovale. wallikeri* and *P. ovale. curtisi* primers might have increased the number of positive PCRs and hence the overall performance of the RDTs.

Discussion

The performance of RDTs in diagnosing PAM was evaluated against microscopy and nested *Plasmodium* species diagnostic PCR in a cohort of pregnant women in north-eastern Tanzania. The use of RDTs might act as an appropriate complementary diagnostic tool for malaria instead of only relying on presumptive treatment based on clinical grounds in areas with limited expert microscopy and laboratory infrastructure. Prescription of any drug during pregnancy is a challenging task due to potential risks of harming the foetus [54], overprescription and subsequent risk of drug resistance development [55]. Simple, cheap, reliable, accurate, easy to use, sensitive and specific diagnostic tests that can identify genuine malarial cases are the only means of allowing accurate malaria detection and rational treatment. With the escalating anti-malarial drug resistance which

Table 2 Performance of different brands of rapid diagnostic tests in diagnosing PAM

RDT type	No. of Samples	Results				Parasite density (asexual stages/μl)	
		Rapid Diagnostic Test		Microscopy		Median	Range
		Positive	Negative	Positive	Negative		
Parascreen™	3892	52 (1.3%)	3840 (98.7%)	28 (0.7%)	3864 (99.3%)	2565	39.5–101208
Paracheck®Pf	594	19 (3.2%)	575 (96.8%)	8 (1.3%)	586 (98.7%)	2004.5	581.5-23587.5
ParaHIT®f	1069	20 (1.9%)	1049 (98.1%)	13 (1.8%)	1056 (98.8%)	1023.63	242.5–390748
Total	**5555**	**91 (1.6%)**	**5464 (98.4%)**	**49 (0.9%)**	**5506 (99.1%)**	**2090**	**40–390748**

RDT: rapid diagnostic test; μl: microlitre.

Table 4 Comparison of RDT and microscopy performance using PCR as golden standard in the 442 samples where PCR results were available

Diagnostic category	No. of samples	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
		% [95% CI]	% [95% CI]	% [95% CI]	% [95% CI]
Parascreen™	301	100.[89.9–100.0]	93.3 [89.6 -95.7]	65.4 [51.8-76.9]	100[98.5- 100.0]
Paracheck Pf®	64	100 [8 0.6-100]	93.8 [83.2 – 97.9]	84.2 [62.4-94.5]	100 [92.1-100.0]
ParaHIT®f	77	100 [79.6–100.0]	91.9 [82.5– 96.5]	75.0 [53.1 – 88.8]	91.9 [84.9-95.8]
Microscopy	442	70.8 [58.0 –81.1]	93.1[89.9-95.4]	63.9 [51.2-74.6]	94.5 [92.0–96.8]

PPV: positive predictive value; NPV: negative predictive value; 95% CI: 95% confidence interval.

necessitates the deployment of expensive artemisinin-based combination therapy, there is a need to prescribe anti-malarial drugs only to patients with true malarial illness [56]. The present study demonstrates that RDTs can act as a diagnostic tool to manage malaria during pregnancy in resource poor settings with limited access to expert microscopy as they are easy to use and perform better than microscopy. Based on the PCR results, the different types of RDTs used in this study were able to capture over 40% of sub-microscopic infections missed by microscopy.

According to the current Ministry of Health and Social Welfare's policy in Tanzania on malaria diagnosis, the use of RDTs is not yet implemented as a routine practice for pregnant women at antenatal clinics. Therefore, there is a need to provide evidence-based data on the best diagnostic supplement/alternative for malaria diagnosis during pregnancy in areas with limited laboratory infrastructure. This will assist the National Malaria Control Programme in Tanzania and beyond when considering RDTs as a possible routine diagnostic tool in malaria diagnosis during pregnancy. According to the recent WHO report [57] on the performance of different RDTs, it has been shown that they are easy to use, are heat stable and have the ability to detect parasitaemia as low as 200 asexual stages/μl. This makes RDTs an ideal diagnostic supplement to malaria diagnosis in resource constrained settings.

In many of the malaria endemic regions including Tanzania, healthcare delivery in peripheral settings is compromised by the lack of well equipped laboratories and personnel with sufficient expertise in malaria microscopy [58]. Microscopy could be as sensitive as RDTs or even more sensitive when done well. However, adequate infrastructure, maintenance of good quality microscopy and proper training on expert microscopy are not always present in many malaria endemic settings. Mismanagement of sub-microscopic infections could result in low but persistent parasitaemia that may culminate in adverse pregnancy outcomes [42,43]. Underdiagnosis and/or wrong diagnosis of true malarial infections may lead to infections going untreated or being wrongly treated as non-malarial illnesses, with subsequent adverse pregnancy outcomes and/or acting as potential reservoirs of transmission. However, in all malaria endemic settings, children with febrile illnesses are treated by anti-malarials and/or other antimicrobials following the World Health Organization guidelines' on integrated management of childhood illnesses.

Singer *et al.* [59] have shown that microscopy underestimates the real malarial burden during pregnancy. Nonetheless in their study, contrary to this study, PCR detected more positive cases as compared to RDTs, whilst assessing only placental blood samples. The present study might differ from that of Singer and colleagues in malaria transmission intensities and also in the current study only a small proportion of RDT and microscopy negative samples were checked by PCR due to limited resources and time. However, when taken as a proportion of placental blood the 351 samples checked by PCR accounts for 54% of the available placental blood samples with complete data. The message portrayed here is that microscopy was shown to have underestimated the true malarial prevalence after PCR correction.

The persistence of HRP-2 circulation in the blood more than two weeks even after successful clearance of IEs in the bloodstream is one of the concerns on the usefulness of HRP-2 based RDTs in malaria diagnosis, as has been reported by many studies [35,36,38,39,60]. However, in most of these studies microscopy was used as gold standard without PCR correction and this might have categorized sub-microscopic infections as false positives due to the limited sensitivity of microscopy. In the present study, all the RDT positive but microscopy negative samples were checked by PCR and the analyses showed that a large proportion of the RDT positive but microscopy negative samples were in fact sub-microscopic infections. Treatment of these few false positive women with anti-malarials might provide some prophylactic effect against subsequent infections outweighing the risk of not treating genuine sub-microscopic infections missed by microscopy that could have a profound effect on the pregnancy outcome. The relatively poor performance of microscopy compared to RDT cannot be explained by suboptimal conditions for microscopy as the present study was conducted in

parallel with a large clinical trial [61]. Therefore, laboratory conditions were excellent and all the laboratory technologists had ample experience in malaria diagnosis and were undergoing proficiency microscopy tests on a regular basis.

The performance of RDTs in malaria diagnosis in the present study is in agreement with studies by Tjitra, Batwala and Tham *et al.* [26,27,62] showing that RDTs were performing better than microscopy in malaria diagnosis under field conditions. Likewise, Bell *et al.* [38] conducted a study in the Philippines in an area of low endemicity and reported that sub-microscopic infections missed by microscopy but captured by RDTs were actually true infections after PCR correction. On the other hand, the current study is not in agreement with a study by Schachterle *et al.* [63] that showed that RDTs had high rates of false positives and negatives in a region of hypoendemicity. However, the results of that study were purely based on microscopy data without PCR correction.

RDTs missed some few infections, which were positive in repeated microscopic investigations. This could be due to assay degradation as a result of humid conditions or batch variability of the RDTs [64], delay in HRP-2 surge after increased parasite density [38] or due to deletion of *hrp-2* genes in some parasites [60]. Some studies have also reported reduced sensitivity of RDTs as a result of low parasitaemia [33,65] and this could explain the reason for the few cases with low parasite densities missed by RDTs [49,50].

This study indicates that RDTs outperform expert microscopy in detecting asymptomatic *P. falciparum* in pregnant women. Given the difficulties in establishing reliable microscopy based diagnostic services, RDTs are good alternative for the detection and in the management of *P. falciparum* infections in pregnant women. RDTs can both be used to detect infections not cleared by IPTp or to detect infections where the malaria endemicity is too low to warrant IPTp.

Competing interests
All authors declare no any conflict of interest.

Authors' contributions
DTRM, CS, JL, TT, AS, ML, PD, AJFL and MA designed the study. DTRM, JL, CS, MO, SB, CP, DJ, PM, DA and MA conducted the study and participated in the laboratory analyses. DTRM drafted the manuscript. All authors reviewed the manuscript and provided critical inputs. All authors read and approved the final manuscript.

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Comparison of Parascreen Pan/Pf, Paracheck Pf and light microscopy for detection of malaria among febrile patients, Northwest Ethiopia

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Abstract

Two malaria rapid diagnostic tests (RDT), Parascreen Pan/Pf® and Paracheck Pf®, were tested in rural health centres in Ethiopia against independent expert microscopy (the gold standard). Participants (n =1997) presented with presumptive malaria to ten health centers in Amhara Regional State during the 2007 peak malaria season (October to December). By microscopy, 475 (23.8%) suspected malaria cases were positive, of which 57.7% were P. falciparum; 24.6% P. vivax and 17.7% mixed infections. Parascreen and Paracheck were positive for 442 (22.1%) and 277 (13.9%) febrile patients, respectively. For Parascreen, P. falciparum sensitivity was 79.6%, specificity 97.4%, positive predictive value (PPV) 86.9%, and negative predictive value (NPV) 95.6%. For Parascreen, P. vivax sensitivity was 74.4%, specificity 98.6%, PPV 76.3% and NPV 98.4%. For Paracheck, P. falciparum sensitivity was 73.7%, specificity 99.2%, PPV 95.3%, NPV 94.5%. Sensitivity was significantly higher for both tests ($P < 0.05$) when parasite density was >100/μl of blood; in these cases Parascreen was 90.7% and 91.5% sensitive for P. falciparum and P. vivax, respectively, while Paracheck was 87.9% sensitive for P. falciparum. Parascreen thus performed adequately for both P. falciparum and P. vivax compared to expert microscopy and is more useful than Paracheck where microscopy is unavailable.

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Field and laboratory comparative evaluation of rapid malaria diagnostic tests versus traditional and molecular techniques in India

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Abstract

Background: Malaria presents a diagnostic challenge in most tropical countries. Microscopy remains the gold standard for diagnosing malaria infections in clinical practice and research. However, microscopy is labour intensive, requires significant skills and time, which causes therapeutic delays. The objective of obtaining result quickly from the examination of blood samples from patients with suspected malaria is now made possible with the introduction of rapid malaria diagnostic tests (RDTs). Several RDTs are available, which are fast, reliable and simple to use and can detect *Plasmodium falciparum* and non-falciparum infections or both. A study was conducted in tribal areas of central India to measure the overall performance of several RDTs for diagnosis of *P. falciparum* and non-falciparum infections in comparison with traditional and molecular techniques. Such data will be used to guide procurement decisions of policy makers and programme managers.

Methods: Five commercially available RDTs were tested simultaneously in field in parallel with peripheral blood smears in outbreak-affected areas. The evaluation is designed to provide comparative data on the performance of each RDT. In addition, molecular method i.e. polymerase chain reaction (PCR) was also carried out to compare all three methods.

Results: A total of 372 patients with a clinical suspicion of malaria from Bajag Primary Health Centre (PHC) of district Dindori and Satanwada PHC of district Shivpuri attending the field clinics of Regional Medical Research Centre were included in the study. The analysis revealed that the First Response Malaria Antigen pLDH/HRP2 combo test was 94.7% sensitive (95% CI 89.5-97.7) and 69.9% specific (95% CI 63.6-75.6) for *P. falciparum*. However, for non-falciparum infections (*Plasmodium vivax*) the test was 84.2% sensitive (95% CI 72.1-92.5) and 96.5% specific (95% CI 93.8-98.2). The Parascreen represented a good alternative. All other RDTs were relatively less sensitive for both *P. falciparum* and non-falciparum infections.

Conclusions: The results in this study show comparative performance between microscopy, various RDTs and PCR. Despite some inherent limitation in the five RDTs tested, First Response clearly has an advantage over other RDTs. The results suggest that RDTs could play and will play an important role in malaria diagnosis.

Background

Malaria is a disease of global importance that results in 300 - 660 million cases annually and an estimated 2.2 billion people are at risk of infection [1]. Of the 2.5 million reported cases in the South East Asia, India alone contributes about 70% of the total malaria cases [2]. Currently, 80.5% of the 109 billion population of India lives in malaria risk areas [3]. Malaria presents a diagnostic challenge in most resource poor areas where malaria is endemic. In such areas malaria diagnosis is often made only on the basis of clinical symptoms although this is alarmingly inaccurate [4]. The role of the laboratory diagnosis of malaria is primarily to support clinical care [5]. The traditional method for the detection of the malaria parasite is the examination of thick and thin blood smears by microscopy. The shortcomings of microscopy for

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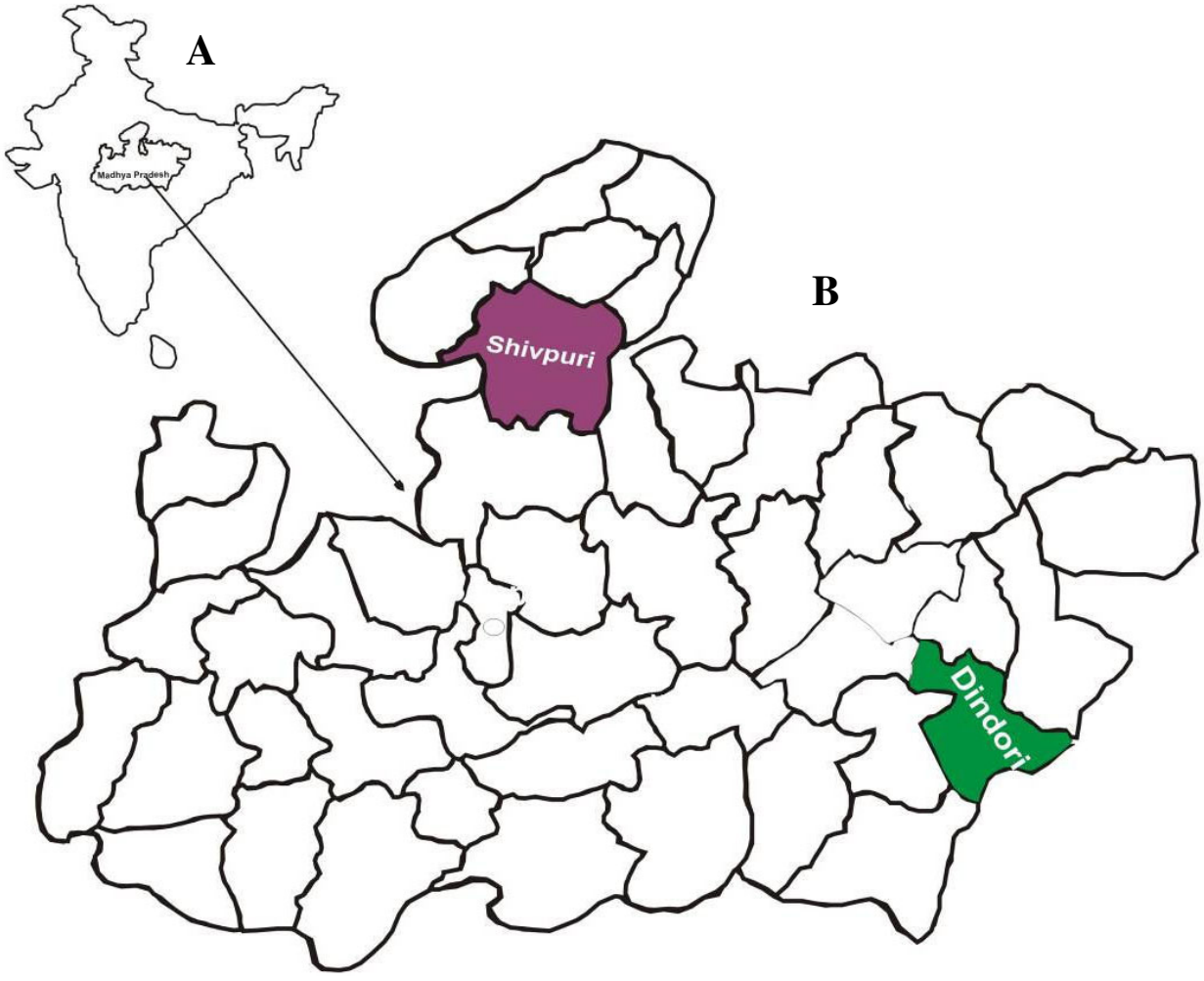

Figure 1 (A) Map of India showing Madhya Pradesh, (B) Shivpuri and Dindori district.

malaria diagnosis are well known [6,7]. These diagnostic limitations affect medical care provided, as malaria is a potentially fatal disease, usually curable if diagnosed quickly [8]. The World Health Organization has recognized the urgent need for simple and cost effective diagnostic tests for malaria to overcome the deficiencies of both light microscopy and clinical diagnosis [9]. The need for a simple, sensitive diagnostic test has led to the development of rapid diagnostic tests (RDTs) among other alternative techniques. Initially the use of RDTs met stiff resistance by the malaria community because of its cost. However, a number of reports from policy makers have acknowledged that RDTs may have their place because expert microscopy in malaria-endemic countries is hard to establish and cost of RDTs has been greatly reduced [10]. Further, the recently introduced artemisinin based combination therapy (ACT) is given to patients only when the diagnosis has been confirmed parasitologically. However, providing parasitological results at all levels of health care presents a serious challenge. Expanding the use of blood slide microscopy is a possible solution but the cost and logistic challenges in remote area restricts the use of microscopy to hospitals and well-equipped laboratories. It takes great skill and years of experience to learn to accurately read a malaria slide. The use of RDTs for *Plasmodium falciparum* malaria is being implemented by National Vector Borne Disease Control Programme (NVBDCP) to improve diagnostic efficiency in peripheral health care settings in central India. Further, although *P. falciparum* causes the most severe disease, recent reports of significant morbidity and drug resistance in *Plasmodium vivax* infections are generating new interest in *P. vivax* [11,12]. The first generation RDT tests were specific for *P. falciparum* [13-15], but the development of new rapid tests by including a Pan-malaria test line allowed detection of non-falciparum infections also [16,17]. Subsequently increasing numbers of similar products have been developed [7,18,19].

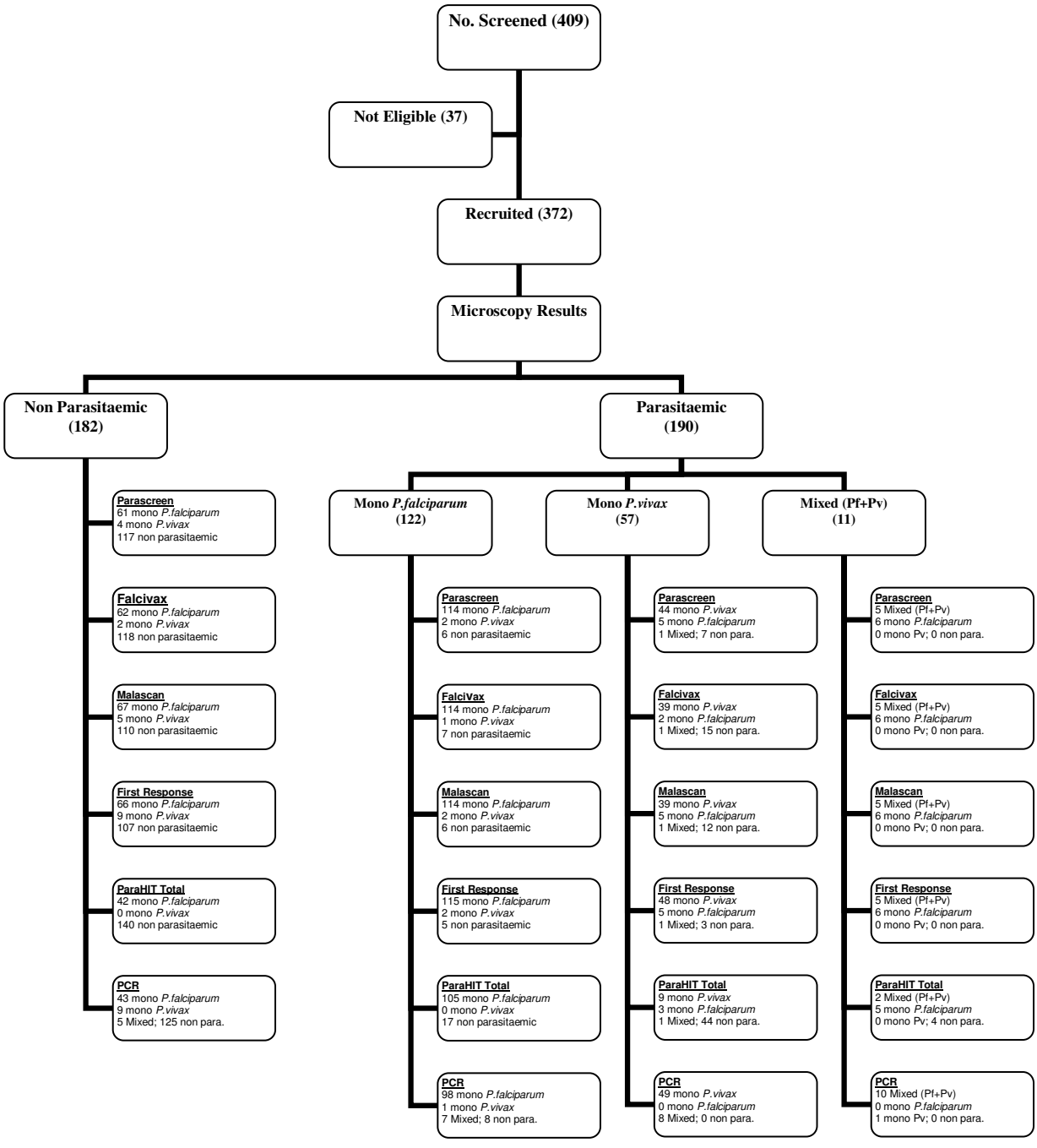

Figure 2 Total field clinic attendance and patients recruited for malaria screening by Rapid Diagnostic Tests, Microscopy and PCR.

We undertook a study on evaluation to assess the performance of commercially available malaria RDTs in comparison with microscopy and polymerase chain reaction (PCR) in an area where both *P. falciparum* and *P. vivax* are co-endemic. This would allow direct product comparisons that would assist the policy makers and programme managers in taking procurement decisions and would ultimately encourage improvement in the quality of manufacturing. Five RDTs evaluated for this purpose were selected on the basis of two main criteria i.e. tests detecting both *P. falciparum* and non-falciparum infections and commercial availability.

Methods

Study area

The study was conducted in 10 villages of Bajag Primary Health Centre of district Dindori (22°57' N latitude and 81°41' E longitude) between August to December, 2009.

Table 1: Characteristics of evaluated rapid malaria tests

		Prascreen Deviice (Pan/Pf)	Malascan Device (Pf/Pan)	Falcivax (Pv/Pf)	Frist Response Malaria pLDH/HRP2 combo	Para HIT Total
Plasmodium species targeted (F= P.*falciparum*V = *P.vivax*P = PAN)		P,F	F,P	F,V	F,P	F,P
Target Antigen		HRP2/pLDH	HRP2/Aldolase	HRP2/Vivax specific pLDH	HRP2/pLDH	HRP2/Aldolase/pLDH
Format		Cassette	Cassette	Cassette	Cassette	Dipstick
Sequence and type of bound antibody	**C**	√	√	√	√	√
	T_1	pLDH	Aldolase	pLDH	pLDH	**Aldolase/**pLDH
	T_2	HRP2	HRP2	HRP2	HRP2	HRP2
Required Volume (μ) of whole blood		5	5	5	5	8
Buffer Volume		4 Drops	4 Drops	4 Drops	2 Drops	4 Drops
Intermediate step		-	-	-	-	Buffer into tube blood on stick, stick on tube
Time to results (mins)		15	15	15	20	15
Maximum Reading time (mins)		30	30	30	-	30

Table 2: Comparative performance of Rapid Diagnostic Test kits (Parascreen, Falcivax, Malascan, First Response and ParaHIT Total) with traditional light microscopy and Polymerase Chain Reaction (PCR) for diagnosis of Malaria

	Parascreen	Falcivax	Malascan	First Response	ParaHIT Total
Light Microscopy as Gold Standard (Reference)					
Sensitivity	93.2	88.4	90.5	95.8	65.8
(95% CI)	(88.6-96.3)	(83.0-92.6)	(85.4-94.3)	(91.9-98.2)	(58.6-72.5)
Specificity	64.3	64.8	60.4	58.8	76.9
(95% CI)	(56.9-71.2)	(57.4-71.8)	(52.9-67.6)	(51.3-66.0)	(70.1-82.8)
Positive Likelihood Ratio	2.6	2.5	2.3	2.3	2.9
(95% CI)	(2.1-3.2)	(2.1-3.1)	(1.9-2.8)	(1.9-2.8)	(2.2-3.8)
Negative Likelihood Ratio	0.1	0.2	0.2	0.1	0.44
(95% CI)	(0.1-0.2)	(0.1-0.3)	(0.1-0.3)	(0.04-0.14)	(0.36-0.55)
Positive Predictive Value	73.1	72.4	70.5	70.8	74.9
(95% CI)	(67.1-78.6)	(67.2-78.1)	(64.3-76.1)	(64.8-76.3)	(67.6-81.2)
Negative Predictive Value	90.0	84.3	85.9	93.0	68.3
(95% CI)	(83.5-94.6)	(77.2-89.9)	(78.7-91.4)	(86.8-96.9)	(61.4-74.6)
Percentage Agreement (Accuracy)	79.0	76.9	75.8	77.7	71.2
Kappa	0.58	0.53	0.51	0.55	0.43
PCR as Gold Standard (Reference)					
Sensitivity	86.6	83.1	85.7	89.2	61.0
(95% CI)	(81.5-90.7)	(77.7-87.7)	(80.5-90.0)	(84.4-92.9)	(54.4-67.4)
Specificity	73.7	75.2	70.7	67.7	85.0
(95% CI)	(65.3-80.9)	(67.0-82.3)	(62.2-78.2)	(59.0-75.5)	(77.7-90.6)
Positive Likelihood Ratio	3.3	3.4	2.9	2.8	4.1
(95% CI)	(2.5-4.4)	(2.5-4.5)	(2.2-3.8)	(2.2-3.5)	(2.7-6.2)
Negative Likelihood Ratio	0.2	0.2	0.2	0.2	0.46
(95% CI)	(0.1-0.3)	(0.2-0.3)	(0.1-0.3)	(0.11-0.24)	(0.38-0.55)
Positive Predictive Value	85.1	85.3	83.5	82.7	87.6
(95% CI)	(79.9-89.4)	(80.0-89.7)	(78.2-88.0)	(77.5-87.2)	(81.5-92.2)
Negative Predictive Value	76.0	71.9	74.0	78.3	55.7
(95% CI)	(67.7-83.1)	(63.7-79.2)	(65.5-81.4)	(69.6-85.4)	(48.5-62.6)
Percentage Agreement (Accuracy)	81.9	80.2	80.2	81.3	69.8
Kappa	0.61	0.58	0.57	0.59	0.41

Dindori is a highly malarious district in Madhya Pradesh (Figure 1) contributing 12% of malaria in the state while its population is only 1% of state [20]. The villages of Bajag PHC are very remote, forested and inaccessible for 4-6 months during rainy season. The average annual rainfall is 1,400 mm. The inhabitants of these villages are ethnic group of Baiga primitive tribe. They are very poorly clothed and have immense faith in sorcery and witchcraft. There is no public transport system and health facilities are non-existent. *Plasmodium falciparum* is the predominant infection. The RDT evaluation was also carried out in 10 villages of Satanwada Primary Health Centre, District Shivpuri (25°4' N latitude and 77°44' E longitude). The inhabitants of the villages are ethnic group of Saharia primitive tribe who live in small one room hutments which are very overcrowded and unhygienic. *Plasmodium vivax* is the predominant infection in this area unlike Bajag PHC. The study area is hot, dry and the average annual rainfall is 875 mm.

Table 3: Comparative performance of Rapid Diagnostic Test kits (Parascreen, Falcivax, Malascan, First Response and ParaHIT Total) with traditional light microscopy and Polymerase Chain Reaction (PCR) for diagnosis of *P.falciparum*

	Parascreen	Falcivax	Malascan	First Response	ParaHIT Total
Light Microscopy as Gold Standard (Reference)					
Sensitivity	94.0	94.0	94.0	94.7	84.2
(95% CI)	(88.52-97.4)	(88.5-97.4)	(88.5-97.4)	(89.5-97.7)	(76.9-90.0)
Specificity	72.0	72.8	69.5	69.9	80.8
(95% CI)	(65.8-77.6)	(66.7-78.3)	(63.2-75.2)	(63.6-75.6)	(75.2-85.6)
Positive Likelihood Ratio	3.4	3.46	3.08	3.14	4.38
(95% CI)	(2.7-4.1)	(2.80-4.27)	(2.53-3.74)	(2.58-3.83)	(3.34-5.73)
Negative Likelihood Ratio	0.08	0.08	0.09	0.08	0.20
(95% CI)	(0.04-0.16)	(0.04-0.16)	(0.04-0.17)	(0.04-0.16)	(0.13-0.29)
Positive Predictive Value	65.1	65.8	63.1	63.6	70.9
(95% CI)	(57.9-71.8)	(58.6-72.5)	(56.0-69.9)	(56.5-70.3)	(63.1-77.8)
Negative Predictive Value	95.6	95.6	95.4	96.0	90.2
(95% CI)	(91.4-98.1)	(91.5-98.1)	(91.1-98.0)	(91.9-98.4)	(85.4-93.8)
Percentage Agreement (Accuracy)	79.8	80.4	78.2	78.8	82.0
Kappa	0.60	0.61	0.57	0.58	0.62
PCR as Gold Standard (Reference)					
Sensitivity	83.6	84.8	84.8	83.6	73.7
(95% CI)	(77.2-88.8)	(78.5-89.8)	(78.5-89.8)	(77.2-88.8)	(66.4-80.1)
Specificity	78.2	80.3	76.2	75.6	86.5
(95% CI)	(71.7-83.8)	(74.0-85.7)	(69.5-82.0)	(69.0-81.5)	(80.9-91.0)
Positive Likelihood Ratio	3.84	4.31	3.56	3.43	5.47
(95% CI)	(2.92-5.06)	(3.22-5.77)	(2.74-4.61)	(2.65-4.44)	(3.78-7.91)
Negative Likelihood Ratio	0.21	0.19	0.20	0.22	0.30
(95% CI)	(0.15-0.30)	(0.13-0.27)	(0.14-0.29)	(0.15-0.31)	(0.24-0.39)
Positive Predictive Value	77.3	79.2	75.9	75.3	82.9
(95% CI)	(70.62-83.1)	(72.6-84.9)	(69.2-89.8)	(68.5-81.2)	(76.0-88.5)
Negative Predictive Value	84.4	85.6	85.0	83.9	78.8
(95% CI)	(78.2-89.3)	(79.7-90.4)	(78.8-89.9)	(77.6-89.0)	(72.6-84.1)
Percentage Agreement (Accuracy)	80.8	82.4	80.2	79.4	80.5
Kappa	0.62	0.65	0.61	0.59	0.61

Study design

All patients irrespective of their age and sex except pregnant women presenting at the field clinic with a clinical suspicion of malaria were included in the study after taking written informed consent. This study was approved by the ethics committee of the Regional Medical Research Centre (ICMR).

Demographic and clinical information was recorded from each patient and all five RDTs were tested simultaneously in field in parallel with peripheral blood smear for microscopic examination in the laboratory. Before the initiation of the study, a one-day workshop was organized to provide training in blood collection from finger prick, test procedure for each RDT and interpretation of the test results as per manufacturer's instructions. The results of each RDT were recorded between 15-30 minutes as per the manufacturer's instructions. 2-3 drops of finger prick blood samples were also collected in heparinised tube/filter paper for PCR to be conducted in the laboratory.

Table 4: Comparative performance of Rapid Diagnostic Test kits (Parascreen, Falcivax, Malascan, First Response and ParaHIT Total) with traditional light microscopy and Polymerase Chain Reaction (PCR) for diagnosis of *P.vivax*

	Parascreen	Falcivax	Malascan	First Response	ParaHIT Total
Light Microscopy as Gold Standard (Reference)					
Sensitivity	77.2	68.4	68.4	84.2	15.8
(95% CI)	(64.2-87.3)	(54.8-80.1)	(54.8-80.1)	(72.1-92.5)	(7.5-27.9)
Specificity	98.1	99.0	97.8	96.5	100.0
(95% CI)	(95.9-99.3)	(97.2-99.8)	(95.5-99.1)	(93.8-98.2)	(98.8-100.0)
Positive Likelihood Ratio	40.5	71.8	30.8	24.1	
(95% CI)	(18.1-90.6)	(23.0-224.6)	(14.5-65.4)	(13.4-43.6)	
Negative Likelihood Ratio	0.23	0.32	0.32	0.16	0.84
(95% CI)	(0.14-0.37)	(0.22-0.47)	(0.22-0.47)	(0.09-0.30)	(0.75-0.94)
Positive Predictive Value	88.0	92.9	84.8	81.4	100.0
(95% CI)	(75.7-95.5)	(80.5-98.5)	(71.1-93.7)	(69.1-90.3)	(66.4-100.0)
Negative Predictive Value	96.0	94.5	94.5	97.1	86.8
(95% CI)	(93.2-97.8)	(91.5-96.7)	(91.4-96.7)	(94.6-98.7)	(82.9-90.1)
Percentage Agreement (Accuracy)	94.9	94.4	93.3	94.6	87.1
Kappa	0.80	0.76	0.72	0.80	0.24
PCR as Gold Standard (Reference)					
Sensitivity	68.3	61.7	63.3	76.7	13.3
(95% CI)	(55.0-79.7)	(48.2-73.9)	(49.9-75.4)	(64.0-86.6)	(5.9-24.6)
Specificity	97.0	98.4	97.4	95.7	99.7
(95% CI)	(94.5-98.6)	(96.2-99.5)	(94.9-98.9)	(92.8-97.7)	(98.2-100.0)
Positive Likelihood Ratio	23.1	37.5	24.1	17.9	40.5
(95% CI)	(11.9-44.9)	(15.4-91.5)	(11.8-49.0)	(10.4-31.1)	(5.2-318.1)
Negative Likelihood Ratio	0.33	0.39	0.38	0.24	0.87
(95% CI)	(0.22-0.47)	(0.28-0.54)	(0.27-0.53)	(0.15-0.39)	(0.79-0.96)
Positive Predictive Value	82.0	88.1	82.6	78.0	88.9
(95% CI)	(68.6-91.4)	(74.4-96.0)	(68.6.-92.2)	(65.3-87.7)	(51.8-99.7)
Negative Predictive Value	93.9	92.9	93.1	95.4	85.4
(95% CI)	(90.7-96.3)	(89.5-95.4)	(89.7-95.6)	(92.4-97.5)	(81.2-88.9)
Percentage Agreement (Accuracy)	92.3	92.3	91.8	92.6	85.4
Kappa	0.70	0.68	0.67	0.73	0.20

The five RDTs used in this study are - Parascreen Device (rapid test for malaria Pan/Pf), Falcivax Device (rapid test for malaria Pv/Pf), Malascan Device (rapid test for malaria Pf/Pan) (all from Zephyer Biomedicals Goa), ParaHIT Total (rapid test for Pf & Pan Malaria species) (SPAN Diagnostics Ltd, Surat) and First Response Malaria Antigen Combo Card test (pLDH/HRP2) (Premier medical corporation Mumbai). These RDTs were provided by their manufacturers for the evaluation. The detailed characteristics of each test are shown in Table 1.

All RDTs were tested by two Research Assistants to minimize variability. The blood films were examined by an experienced microscopist in the laboratory without reference to the results of RDTs and clinical history of patient. The results of both microscopy and RDTs were matched by an independent expert. A slide was considered positive if atleast one asexual form of parasite was detected in 100 microscopic fields in thick blood film. Blood parasite density was determined from the thick films by counting the number of parasites against 200

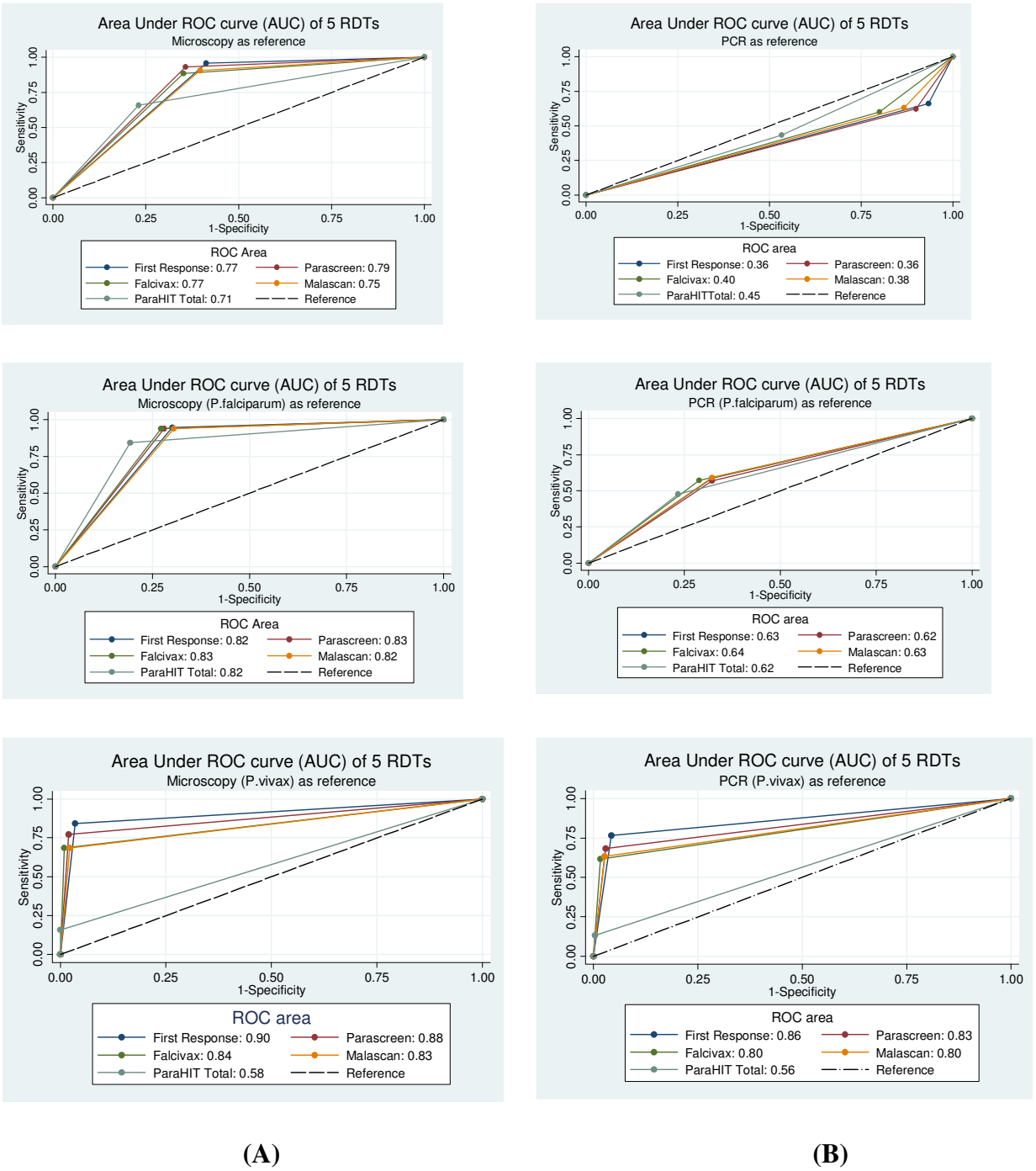

Figure 3 Area under Receiver Operating Characteristic (ROC) curve (AUC) of 5 RDTs Vs light microscopy (A) and PCR (B) as reference test.

white blood cells (WBC) and assuming that each subject had 8000 white blood cells/μl of blood. All negative slides that test positive on the RDT/PCR or all positive slides that test negative on the RDT/PCR were re-examined by another expert technician blinded to the results of microscopy, RDT/PCR and clinical status of the patients. The PCR was performed blind on coded samples by an independent Research Assistant unaware of clinical status of patients, result of RDTs and microscopic examination. Every person positive for falciparum malaria by RDT or by microscopy was treated with a combination of artesunate and sulphadoxine-pyremethamine (ACT) or

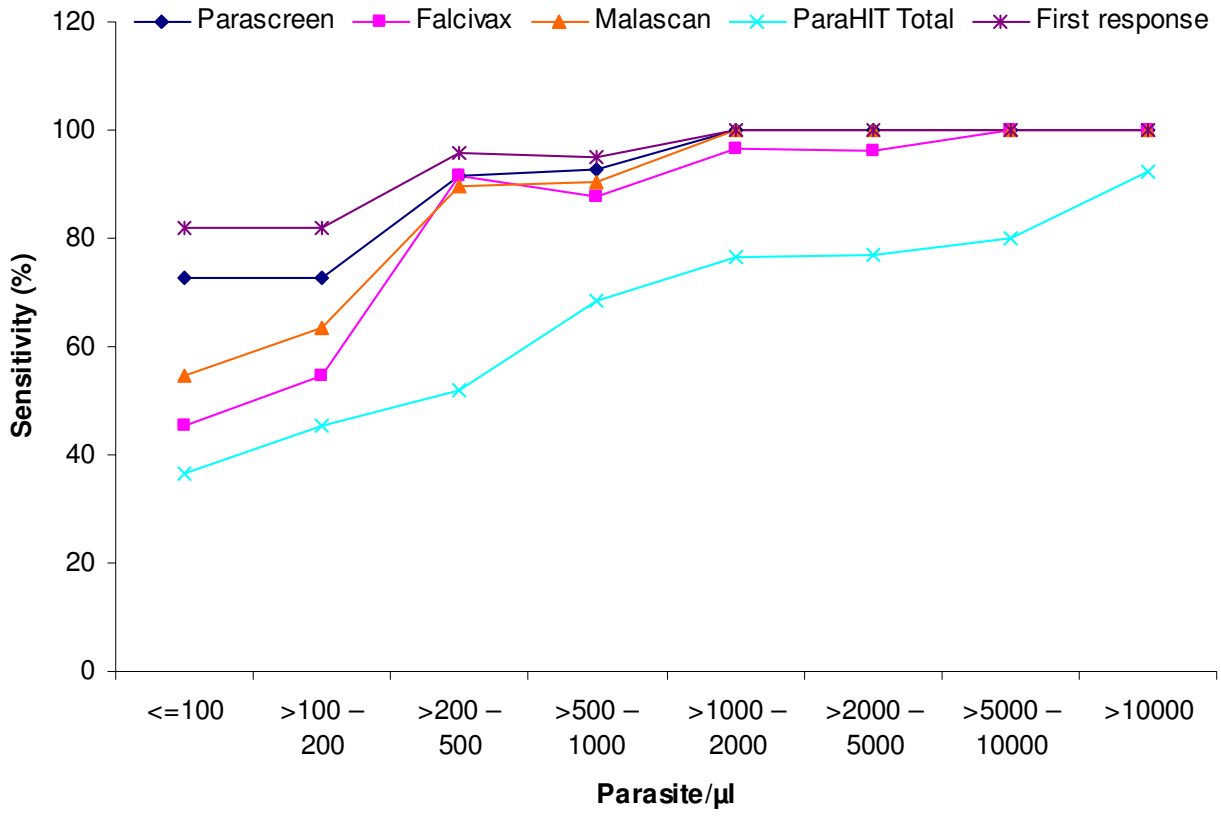

Figure 4 Showing sensitivity of five RDTs according to parasitaemia

with chloroquine (CQ) if RDT/microscopy showed non-falciparum infection.

For testing temperature stability of the tests, RDTs were stored at 25°C on receipt in the study sites, then allocated to separate groups for storage at 35°C & 45°C for 90 days, at 60°C for 48 hours, and at -10°C for 60 minutes before testing [21]. At the start of the study, the incubators were stabilized at the required temperature for three days before the RDTs to be tested were placed inside. RDTs were removed from storage to reach room temperature for 2 hours before testing and comparisons were made with control RDTs kept at 25°C until use and with microscopy. The malaria RDTs used in this study were from single lots of commercially available products.

Polymerase chain reaction

The DNA was isolated from the blood by using the commercially available DNA extraction kit (Bio Basic Inc) as per manufacturer protocol and also from archive blood spots by Tris-EDTA (TE) buffer method. PCR for the identification of malaria parasite was performed following the standard methods [22].

Data analysis

Results of the RDT and microscopy examination were recorded on separate forms. After double key data entry, the database was rechecked for all inconsistent entries and errors were corrected. Data were then analysed using STATA 8.2 (StataCorp, College Station Texas, USA). The figures for sensitivity, specificity, predictive values, accuracy, the area under the receiver operator characteristic curve (AUC) and the likelihood ratios were calculated using the 'diagt' command in Stata [23]. All estimated parameters are detailed with a 95% Confidence Interval (CI) unless stated otherwise.

Results

During the study period, 409 patients (age 1 to 69 years) attended the two sites 236 patients (57.7%) were screened at Dindori and 173 (42.2%) were screened at Shivpuri (mean age 15.45 ± 14.15). 37 patients (9%) were excluded as not fulfilling the study enrolment criteria due to recent anti-malarial intake. 372 patients were eligible and all these patients were recruited (mean age 15.03 ± 14.07). All recruited patients were tested by microscopy, RDT and PCR (Figure 2).

A total of 190 (51.1%) were found infected by microscopy, 57 (15.3%) with *P. vivax*, 122 with *P. falciparum* (32.8%) and 11 (3%) with both *P. vivax* and *P. falciparum*. The overall sensitivity and specificity of First Response for malaria was 95.8 and 58.8%, Parascreen 93.2 and

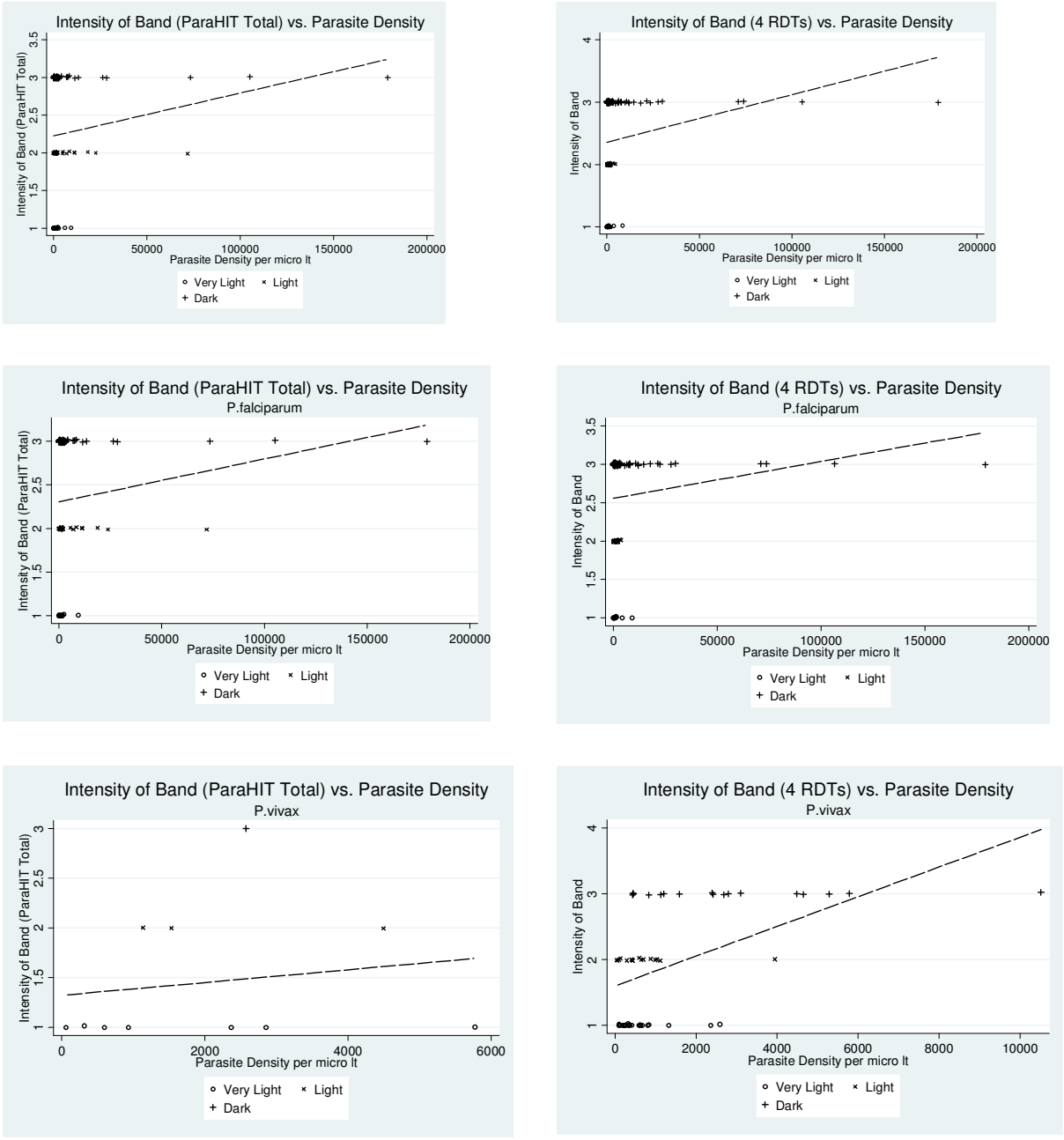

Figure 5 Scatter plot showing association between intensity of band (A: ParaHIT Total; B: All other 4 RDTs) and Parasite density/µl.

64.3%, Malascan 90.5 and 60.4%, Falcivax 88.4 and 64.8% and by ParaHIT Total 65.8 and 76.9% respectively (Table 2). The highest sensitivity was recorded by First Response and was considered here as reference test for comparing the sensitivity and specificity of other RDTs. Parascreen was found to be 41% less sensitive than First Response (OR 0.59, 95%CI 0.24 - 1.48), though, this difference was not significant statistically ($P > 0.05$). Falcivax was 67% less sensitive (OR 0.33, 95%CI 0.14 - 0.78) and Malascan was 58% less sensitive (OR 0.42, 95%CI 0.18 - 1.00) than First Response and these differences were significant statistically ($P < 0.05$). ParaHIT Total showed lowest sensitivity (OR 0.08, 95% CI 0.04 - 0.19) and highly significant statistically ($P < 0.0001$). Thus, Parascreen was the first

alternative to First Response. Similarly, further analysis showed that the specificity of Parascreen, Falcivax and Malascan were 1.07 to 1.26 times more when compared to First Response which is not significant (P > 0.05). However, specificity of ParaHIT Total was more than 2 times when compared to the First Response (OR 2.34, 95% CI 1.47 - 3.71), which was highly significant statistically.

The species wise analysis revealed that the sensitivity for *P. falciparum* was highest (94.7%) by First Response (Table 3), while lowest for ParaHIT Total, (84.2%). The specificity for *P. falciparum* was highest (80.8%) by ParaHIT Total while lowest 69.5% by Malascan. The positive predictive value (PPV) for *P. falciparum* was highest by ParaHIT Total (70.9%) while lowest by Malascan (63.1%). The negative predictive value (NPV) was highest (96.0%) by First Response while lowest (90.2%) by ParaHIT Total. The false positive rate for *P. falciparum* was highest (30.5%) by Malascan (73/239) while lowest (19.2%) by ParaHIT Total (46/239). Like wise the false negative rate for *P. falciparum* was highest (15.8%) by ParaHIT Total (21/133) and lowest (5.3%) by First Response (7/133). When PCR was used as a reference standard the corresponding values for sensitivity, specificity, PPV, NPV and accuracy for each RDT are shown in Table 3.

For non-falciparum infections i.e. *P. vivax* the sensitivity of the test when compared with microscopy was 84.2% by First Response, while only 15.8% by ParaHIT Total (Table 4). Specificity of the test was 100% by ParaHIT Total and 96.5% by First Response. Similarly, PPV was highest (100%) for ParaHIT Total while lowest (81.4%) for First Response. On the contrary, NPV was highest for First Response (97.1%) while lowest (86.8%) by ParaHIT Total. False positive rate for *P. vivax* was highest (3.5%) by First Response (11/315) and none by ParaHIT Total (0/315). On the contrary, false negative rate for *P. vivax* was lowest (15.8%) by First Response (9/57) and highest (84.2%) by ParaHIT Total (48/57). The values of sensitivity, specificity, PPV, NPV and accuracy using PCR as reference standard are shown in Table 4. Area under Receiver Operating Characteristic (ROC) curve (AUC) of five RDTs *vs* microscopy was computed for diagnosing malaria, *P. falciparum* and *P. vivax* (Figure 3) The AUC of different RDTs were significantly different for malaria (χ^2 = 18.21, P < 0.001), and for *P. vivax* (χ^2 = 108.29, P < 0.0001) but not significant for *P. falciparum* (χ^2 = 8.47, P > 0.05).

The analysis of RDTs sensitivity according to parasitaemia revealed that the First Response was most sensitive for diagnosis of P. falciparum (95.0%) and P. vivax (88.0%) malaria as compared to other 4 RDTs especially for levels of parasitaemia above 200 parasite/μl (Figure 4). It should be noted that in this study because of the requirement for fever in patients from a high transmission area, there were no cases in which the parasite density was ≤ 40 parasites/μl.

Analysis of intensity of band and parasite density revealed that there was a weaker correlation in band intensity and parasite density for ParaHIT Total r = 0.13 (P > 0.05). Although all other four RDTs was also showing a weak positive correlation in band intensity and parasite density but it was statistically significant r = 0.17 (P < 0.025). Species wise further analysis revealed that in *P. vivax* all four RDTs i.e. Parascreen, Falcivax, Malascan and First Response showed very strong positive correlation in band intensity and parasite density (r = 0.50, P < 0.0001) while no statistically significant correlation was seen in *P. falciparum* (Figure 5).

Further, exposure of all RDTs to high temperature i.e. 35°C, 45° & 60°C and low temperature (-10°C) did not cause any loss of sensitivity for both *P. falciparum* and *P. vivax* except ParaHIT Total when compared with microscopy and PCR. However, there was some reduction in test line-intensity at high temperature.

Discussion

This comparative evaluation was carried out in outbreak-affected areas. From a malaria transmission perspective in both the areas, the RDTs can play a key role in rapid diagnosis and prompt treatment of malaria where resistance to CQ also necessitates the use of more expensive ACT. As RDT can be conducted immediately in the field clinic while the patient is present, the most important point for the villagers is the knowledge that they are infected with malaria parasite. On the contrary, the delay in the results of microscopic diagnosis is a serious obstacle for the operation of a malaria control programme in remote areas. Despite some inherent limitations, out of five tests evaluated, the First response was highly sensitive for the diagnosis of *P. falciparum* and non-falciparum especially for levels of parasitaemia above 200 parasite/μl. On the other hand its specificity was much lower than its sensitivity. Having a relatively low specificity which leads to an over-diagnosis and to an over treatment of non-malaria cases was, however, considered as less serious in such outbreak affected areas than having a low sensitivity which may lead to a potentially fatal condition being missed [24]. However, in a field setting such as ours, a negative RDT corresponds in the vast majority of cases to a non-infected individual. The high NPV allow us to confidently diagnose negative test patients as non-malaria patients [25]. Thus the risk of missing an infected individual is very small by most RDTs used in this evaluation. In Ethiopia high NPV was also recorded using Parascreen RDT in a population-based study [26] and in a health facility based study [27]. However, the sensitivity of all RDTs except First Response for non-*P. falciparum* infec-

tions is low (16-77%) as reported earlier from India using First Response and Falcivax RDTs [19,28].

The performance of RDTs can be adversely affected at the temperature to which they are exposed when transported [21]. Temperatures of 35°C to 45°C are common in malaria-endemic regions and higher temperatures may be encountered during transportation. Further inadvertent freezing has also been recorded during routine shipment [29]. All types of RDTs (except ParaHIT Total) perform satisfactorily at all temperatures although we do not know whether the performance of RDTs will be equally good at low parasitaemia as recorded by some investigators [21].

A diagnostic test which is to be used in a peripheral health facility, particularly in resource poor areas, has to be simple and fast to perform by less qualified staff. Among the five RDTs tested, First Response require 20 minutes while all other RDTs require 30 minutes before classifying the RDT as negative test. Further, the First Response need only 2 drop of buffer while all other RDTs need 4 drops. Thus the First Response clearly has an advantage over other RDTs.

Conclusions

The two potential alternatives to microscopy are, PCR and RDTs. Primers exist for the reliable identification of the human malarias by PCR assays [22,30]. However, this is largely a research tool unsuited for routine use in the field or clinical laboratory. Given the logistic and financial difficulties of the PCR in field settings, only microscopy and RDTs are viable options at present and PCR remain a future alternative to these tests when inexpensive hand held diagnostic point of care (POC) instrumentation to detect malaria is available [31]. The practical and fast nature of RDTs make them the only currently viable supplement to or replacement of microscopy based diagnosis. Thus, RDTs could play and will play an important role in malaria diagnosis in the future. However, there are reservations about how well these RDTs perform as many commercially available RDTs lack the consistency, quality control and performance capabilities as claimed by the manufacturers making their use ineffective or potentially dangerous [32]. Further, can RDTs be operated by villagers, school-teachers or forest workers, in forested inaccessible areas so that they can penetrate into areas where microscope and health facilities are non existent? This is an important question which can only be answered when more experimental next generation RDTs are available.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NS*: Study design, Data analysis and manuscript preparation. MMS: Clinical and field work. MKS: Field work and RDTs analysis. RKM: Field work and RDTs analysis. SS: Laboratory experiments. PKB: Laboratory experiments and PCR analysis. MPS: Data analysis, interpretation and manuscript preparation. AS: Data analysis and interpretation. AG: Study design, test interpretation and manuscript preparation. All authors read and approved the final manuscript.

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COST EFFECTIVENESS AND RESOURCE ALLOCATION

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Cost-effectiveness of three malaria treatment strategies in rural Tigray, Ethiopia where both Plasmodium falciparum and Plasmodium vivax co-dominate

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Abstract

Background: Malaria transmission in Ethiopia is unstable and the disease is a major public health problem. Both, *p.falciparum* (60%) and *p.vivax* (40%) co-dominantly exist. The national guideline recommends three different diagnosis and treatment strategies at health post level: i) the use of a *p.falciparum/vivax* specific RDT as diagnosis tool and to treat with artemether-lumefantrine (AL), chloroquine (CQ) or referral if the patient was diagnosed with *p.falciparum*, *p.vivax* or no malaria, respectively (parascreen pan/pf based strategy); ii) the use of a *p.falciparum* specific RDT and AL for *p.falciparum* cases and CQ for the rest (paracheck pf based strategy); and iii) the use of AL for all cases diagnosed presumptively as malaria (presumptive based strategy). This study aimed to assess the cost-effectiveness of the recommended three diagnosis and treatment strategies in the Tigray region of Ethiopia.

Methods: The study was conducted under a routine health service delivery following the national malaria diagnosis and treatment guideline. Every suspected malaria case, who presented to a health extension worker either at a village or health post, was included. Costing, from the provider's perspective, only included diagnosis and antimalarial drugs. Effectiveness was measured by the number of correctly treated cases (CTC) and average and incremental cost-effectiveness calculated. One-way and two-way sensitivity analyses were conducted for selected parameters.

Results: In total 2,422 subjects and 35 health posts were enrolled in the study. The average cost-effectiveness ratio showed that the parascreen pan/pf based strategy was more cost-effective (US$1.69/CTC) than both the paracheck pf (US$4.66/CTC) and the presumptive (US$11.08/CTC) based strategies. The incremental cost for the parascreen pan/pf based strategy was US$0.59/CTC to manage 65% more cases. The sensitivity analysis also confirmed parascreen pan/pf based strategy as the most cost-effective.

Conclusion: This study showed that the parascreen pan/pf based strategy should be the preferred option to be used at health post level in rural Tigray. This finding is relevant nationwide as the entire country's malaria epidemiology is similar to the study area.

Background

Malaria continues to be a global challenge with half of the world's population at risk of the disease. In 2006 about 250 million episodes of malaria occurred globally with nearly a million deaths, mostly of children under 5 years of age. More than 85% of this disease burden was concentrated in countries in Sub-Saharan Africa (SSA). Ethiopia was one of the five main contributors to the overall African malaria burden [1,2].

In Ethiopia, despite the long history of malaria control since the 1950s, the disease is still a major public health problem [3]. Though some improvements, both in mortality and morbidity, have been recently achieved, malaria has been consistently reported as one of the three leading causes of morbidity and mortality over the

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past years [4]. Malaria in Ethiopia is seasonal, predominantly unstable and focal, depending largely on rainfall and altitude. Two transmission seasons are known: major (September to December) and minor (April to May). The unstable nature of malaria makes the population non-immune and prone to focal and cyclical epidemics. Unlike most SSA countries where *p.falciparum* almost accounts for all malaria infection, in Ethiopia, both *p.falciparum and p.vivax* are co-dominant, the former accounting for approximately 60% of all cases. In the low transmission season *p.vivax* increases its proportion due to its relapsing nature and the seasonal drop in *p.falciparum* infection [3,5,6].

In fighting against this deadly disease, early diagnosis and prompt treatment is one of the most basic and effective global strategies [7,8]. The effectiveness of this strategy is highly dependent on the national policy of providing effective diagnosis and first-line antimalarial drugs, and in the delivery system.

In 2004, Ethiopia made two important policy changes which favoured this strategy. Firstly, it launched a community-based health care system, the Health Extension Programme (HEP), to achieve significant essential health care coverage. HEP is the grass-root level of the primary health care (PHC) through the provision of two health extension workers (HEWs) in a health post (HP) at tabia (sub-district) level to serve approximately 5,000 inhabitants. HEWs are high school graduated women with one year of training on the components of the HEP programmes. HEP is a package of sixteen basic health components. All components of the programme comprise health promotion and prevention activities except the malaria intervention which, in addition, incorporates diagnosis and treatment [9]. HEP has been successfully implemented throughout the country including Tigray. Currently, there are more than 1,220 health extension workers in Tigray and the coverage has increased from 30% in 2006/7 to above 70% in 2007/8 [10].

Secondly, the country has made two changes on its national malaria diagnosis and treatment guideline. Malaria confirmatory diagnosis using rapid diagnostic tests (RDTs) replaced presumptive diagnosis, while maintaining the latter approach where the former is unavailable [8]. A presumptive malaria case is a patient who exhibits fever or history of fever within the past 48 hrs in the absence of clear symptoms indicating alternative causes of fever. RDTs are tests based on the detection of antigens released from the malaria parasites in lysed blood [11]. The second change included a shift in the treatment of *p.falciparum* from monotherapy sulphadoxine-pyrimethamine (SP) to artemisinin-based combination therapy (ACT), namely artemether-lumefantrine (AL), while keeping chloroquine (CQ) for treating *p.vivax*. The guideline recommends three different diagnosis and treatment strategies: i) if malaria is diagnosed with a falciparum-specific and pan-specific device, treat *p.falciparum* cases with AL, *p.vivax* with CQ and refer negatives to a higher level; ii) if malaria is diagnosed with only a *p.falciparum*-specific device, treat positive (*p.falciparum*) cases with AL and all the remaining with CQ; and iii) if malaria is diagnosed presumptively, treat all suspected cases with AL [8]. *P.falciparum* positive patients for whom AL is contra-indicated have to be treated with quinine and patients with one or more signs and symptoms of severity should be referred immediately to the nearest higher facility.

In the study year, 2007, on top of the presumptive diagnosis, two types of RDTs were in use at the health-post level in the study area: parascreen pan/pf (Zephyr Biomedical, Goa, India) and paracheck pf (Orchid Biomedical Systems, Goa, India); the former is able to identify both *p.falciparum* and *p.vivax* while the latter targets only *p.falciparum*. While paracheck pf was the commonly used RDT at health post level since 2004, parascreen pan/pf had been recently introduced.

Several studies on RDT cost-effectiveness (CE) have been conducted in the past years. Most of these studies were focused in areas of high malaria transmission and *p.falciparum*. Almost all were comparing potentially similar types of RDTs either with microscope and/or presumptive diagnosis [12-18]. However, none of them are similar to the Ethiopia malaria epidemiological context and to the current national diagnosis and treatment strategies.

Therefore, this operational research was designed to assess the cost-effectiveness of the recommended three diagnosis and treatment strategies in the Tigray region of Ethiopia. This will provide evidence to assist decision makers on which strategy is the most appropriate in the region

Methods

Study area

Tigray regional state is located in northern Ethiopia and is divided into 47 woredas (districts). The region has approximately 4.3 million inhabitants most of whom (81.2%) live in rural areas [19]. The majority of the population works in agriculture. Famine and drought are regular occurrences in the region. As in the rest of Ethiopia, malaria transmission in Tigray is very seasonal and occurs mainly at altitudes up to 2,200 meters above sea level (masl). Around 65% of the population is at risk of malaria and the disease was the number one cause of outpatient cases, admissions and deaths. In 2006, it accounted for 28% of all the patients treated in the regions' health facilities [20]. Previous efforts to control the problem have included insecticide residual spraying and environmental management. Since 2005,

distribution of long-lasting insecticidal nets is gradually covering all malarious villages.

The health system in Tigray is essentially the same as in the rest of Ethiopia, i.e., a four-tier system with Primary Health Care Units (PHCUs) at the grass-roots level. There are five zone-level hospitals, six district hospitals and one referral hospital in Mekelle, the capital.

Sampling procedure

In order to capture epidemiological variations, the study was stratified into the three commonly known malaria strata in the country: stratum-I (<1000 masl), stratum-II (1000-1500 masl) and stratum-III (1501-2000 masl). A district in a given stratum with a high number of villages was selected to represent its respective stratum. Four districts were selected: Kafta-Humera, Tahtay-Adiyabo, and Mereb-leke plus Raya-Azebo from strata I, II, and III respectively. Two districts were included in the strata III for being the largest strata. The districts populations ranged from 91,379 in Tahtay-Adiyabo to 136,039 in Raya-Azebo. In all the study districts, malaria has constituted a leading cause of the disease burden over years. For instance, in 2007/8 it accounted for 21%-28% of outpatient visits in the districts [20].

The study was conducted under a routine HEP service following the national malaria diagnosis and treatment guideline during the main transmission months of 2007. Half of the health posts (7-8) in each district were randomly selected.

Patient enrolment and management

All diagnosis and treatment procedures were done by the HEWs under routine conditions following the national guideline. HEWs (enumerators) were trained with a major focus on how to interpret the result of the newly introduced parascreen pan/pf device, blood film preparation and data collection. No additional training was given on paracheck pf as it had been used for years.

Every suspected malaria case, who presented to a HEW either at a village or health post, was included. Following the national malaria guideline, patients were excluded if they: i) exhibited signs and symptoms of severe malaria or any other severe disease, ii) had taken antimalarial drugs (AL or quinine) within the previous two weeks, and iii) were infants under three-months-old or were pregnant mothers during their first trimester for whom AL is contraindicated.

Previous years have shown a slide positivity rate (SPR) of approximately 30% in the high-transmission season [20]. For this anticipated SPR, with a confidence level of 95%, an absolute precision of five percentage points (25% to 35%) and a design effect of two), the required sample size was 646 patients for each stratum.

Patient history, including demographic data, signs and symptoms related to current illness (chief complaint) and medication, was collected. A finger-pricked blood sample from each subject was taken for the two types of RDTs, according to the RDT manufacturer's instructions (leaflet enclosed within the kit) and a blood film (thick and thin) for the microscope examination following the World Health Organization (WHO) guideline [21]. Patients were treated for malaria if one of the RDTs was positive.

The reference expert microscopy

Performance of the three alternative diagnostic and treatment strategies were calculated vis-à-vis the light microscopy. Blood films were stained with 3% Giemsa stain and examined by two independent (first and second) microscopists using ×1000 oil immersion following the WHO guideline [21]. The independent readings were compared for concordance of presence or absence of asexual/sexual forms of *plasmodium* and its species. A third senior microscopist examined discordant slides and his/her findings taken as true diagnostic outcome. A negative was declared after 200 microscopic fields read without finding a parasite. The first and the second microscopists were unaware of the RDT results and the third reader was blind to the results of both the RDTs and the preceding microscopists.

Data analysis

The cost-effectiveness (CE) of the three different diagnosis and treatment strategies was compared. The strategies included: i) the use of parascreen pan/pf as diagnosis tool and to treat with AL, CQ or referral if the patient was diagnosed with p.falciparum, p.vivax or no malaria respectively (parascreen pan/pf based strategy); ii) the use of paracheck pf and AL for p.falciparum cases and CQ for the rest (paracheck pf based strategy); and iii) the use of AL for all cases diagnosed presumptively as malaria (presumptive based strategy). All data were entered in to Microsoft Excel version 8. Effectiveness was calculated using Epi Info™ version 3.5 [22] and the cost and cost-effectiveness were calculated using Microsoft Excel 8.

Costing

Costing was undertaken from the provider's perspective (government) at the health post level and restricted only to the first visit of a patient. At this facility level, the entire malaria diagnosis and treatment service is free of charge.

Costing considered only diagnosis and antimalarial drugs because the fixed costs (infrastructure, supervision, training and HEWs salaries) were assumed not to differ among the comparative strategies. The cost of these items is also shared with other health programmes. RDT provision, compared to presumptive diagnosis, comprises other operational and management costs at

different levels in addition to the cost of the test kit; however, this cost was reasonably assumed as similar for both RDT-based strategies and traded-off with the expenditure reduction on drug management and transport as RDT application decreased the amount of AL needed. RDT costing was at the manufacturer's price and was calculated as the total number of presumptive patients multiplied by the unit price of each type of RDT kit (including lancets, swabs, pipette, buffer solution and desiccant).

AL costing was calculated at the manufacturer's cost (but not CQ) as it has been provided at no profit, as per the special pricing agreement between WHO and the manufacturer [23]. Antimalarial drug cost was calculated following the malaria diagnosis and treatment guideline at the peripheral level. Being age dependent, the number of cases in each treatment regimen was multiplied by the cost of the respective treatment course of either AL or CQ. Unit costs were obtained from the Tigray Health Bureau (THB) pharmacy unit for the year 2007. The following items were not including in costing: RDT reading time, RDT wastage and RDT training cost.

Effectiveness indicator and cost-effectiveness measure

RDTs, highly specific and less sensitive compared to presumptive diagnosis, are mainly introduced since presumptive treatment is non-specific while it is 100% sensitive. Therefore, there is a need to balance the risk between improving specificity (excluding non-malaria cases) and reducing sensitivity (missing malaria cases) while replacing presumptive with RDTs. Taking this into account, we selected the number of correctly treated cases (CTC) as the measure of effectiveness on the basis of the malaria diagnosis and treatment strategies. This indicator accommodates both concerns: detecting the malaria cases (sensitivity) and excluding the non-malaria cases (specificity) supporting the public health goal of properly managing all causes of illness. In low malaria prevalence areas such as Tigray [24], all malaria infections, even with low-level parasitaemia, are associated with clinical illness in all age groups. In such malaria epidemiology, there is no evidence if missing malaria cases is more or less dangerous than missing non-malaria cases or the vice-versa. Therefore, it was assumed that the weight of correctly or mistakenly treating cases of any disease including malaria was equal. A non-malaria case identified by the parascreen pan/pf was referred to a higher health facility level - this meant that this patient was correctly treated. The number of correctly treated cases was then calculated as the number of true positives plus the number of true negatives cases.

Cost-effectiveness was estimated as average cost-effectiveness ratio (ACER) and incremental cost-effectiveness ratio (ICER). ACER was calculated as a cost of diagnosis and treatment of a given strategy divided by the number of CTCs. To find out if an extra cost in a strategy produced an extra effect (health benefit), ICER analysis was conducted where the strategies were ranked by increasing cost and then the additional cost in one strategy was divided by the additional CTCs [25].

Sensitivity analysis

Sensitivity analysis for selected parameters for which the cost-effectiveness is more sensitive was conducted. Changes in some variables may have skewed some findings; to allow for this, a one-way sensitivity analysis was carried out on changes in AL cost and SPR. A reduction in cost for AL was incorporated into the analysis since the price of AL has been constantly decreasing throughout the last few years (even though drug resistance may necessitate the purchase of more expensive antimalarial drugs in the future). We did not consider changes on RDT price, as it seems unlikely to drop in the near future for at least two possible reasons: firstly, there is a huge gap between the demand and supply - for instance, in 2006, only 16 million RDTs were distributed while 80 million courses of ACTs were used [1]. Secondly, despite the potential high demand, the prices have been kept constant in the last years.

Change in SPR as a function of seasonal variation is inevitable. We considered a minor transmission season (the point estimate was of the major season), whilst assuming the diagnostic performance remained constant. A two-way sensitivity analysis was also carried out at a reduced AL cost during a low transmission season.

Ethical clearance

Ethical clearance was obtained from Tigray Health Bureau, Mekelle, Ethiopia. District Health Offices were informed of the study and its purposes. The purpose of the study was explained to the participants. Verbal consent was obtained from (patient/patient's guardian) as the majority of the rural population is illiterate. No patient refused to participate. Confidentiality of patient identity was maintained for every enrolled patient by assigning a unique identification number that was labelled on the RDT devices, blood film slides, data collection forms and database.

Results

Characteristics of the subjects

In total 2,422 subjects from all three strata and 35 health posts were enrolled in the study. Overall, 26.63% (n = 645), 28.0% (n = 677) and 45.42% (n = 1100) of the subjects were from strata I, II and III, respectively. In total, 37.2% (n = 901) were female, 13.96% (n = 338) were children aged under five years, 18.66% (n = 452) aged between 5-14 years and the remaining 67.38% were 15 years or above. The age of the study subjects ranged from three months to 85 years with a mean of 24.18

(median of 21 years). Eighteen percent of them sought treatment within one day since the onset of illness. Most of the patients (86.21%) appeared with fever and the remaining with a history of fever.

Microscope result

The microscope examination of thick blood smear showed a crude (all species and all stages) SPR of 27.29% (n = 661) with 68.53% (n = 453) of the positive samples being *p.falciparum (+/-*gametocytes, gametocyte alone and mixed) and 31.47% (n = 208) *p.vivax (+/-gametocytes*) (Table 1). The stratified SPR was 46.51%, 26.88% and 16.27% with a *p. falciparum* proportion of 68%, 69.78% and 69.77% for stratum I, II and III, respectively. There were 27 cases of gametocytes, out of which 26 were in the presence of asexual stage. There was only one mixed infection of *p.falciparum* and *p.vivax*. From the operational point of view, all these 28 cases were considered as *p.falciparum*. There was one case of *p.vivax* in the presence of gametocytes which was considered as *p.vivax*.

Cost analysis

The unit cost was US$ 0.59 (US$ = 9.00 Ethiopian birr for 2007) for the paracheck pf kit, US$1.05 for the parascreen pan/pf kit and US$ 0.03 for a pair of gloves. A treatment course of AL cost US$ 0.60, 1.20, 1.80 and 2.40 according to the treatment regimen (age) group. Each CQ tablet cost US$0.006.

The cost analysis indicated that the presumptive-based strategy (BS) was higher by 27.69% and 46.1% than the cost of the parascreen-BS and paracheck-BS, respectively. In the RDT-BS, the tests' cost accounted for the majority of the expenditure, 55.52% in paracheck-BS and 72.08% in parascreen-BS. AL constituted 41%, 27.65% and 100% of the total cost of paracheck-BS, parascreen-BS and presumptive-BS, respectively. Cost of chloroquine was insignificant which was 3.48% in paracheck-BS and less than 1% in parascreen-BS.

Table 1 Summary result of the comparison between the expert microscopy and the RDTs, Tigray, Ethiopia, 2007

Expert Microscope	Paracheck pf		Parascreen pan/pf		Total (microscope)
	Positive	Negative	Positive	Negative	
P.falciparum					
Positive	402	51	377	76	453
Negative	114	1855	97	1872	1969
Total	516	1906	474	1948	2422
P.vivax					
Positive	-	-	155	53	208
Negative	-	-	53	2161	2214
Total	-	-	208	2214	2422

P.falciparum positive is: asexual +/- sexual, asexual +/- p.vivax; P.vivax positive is: asexual +/- sexual; Paracheck pf negative is meant no-p.falciparum; Parascreen pan/pf p.vivax positive meant non p.falciparum malaria.

Effectiveness indicator and cost-effectiveness

Out of the 661 malaria and 1761 non-malaria cases, parascreen-BS correctly treated 88.48% cases (377 *p.falciparum*, 155 *p.vivax* and 1611 negatives) (Table 2). It failed to identify 11.52% patients, out of which 5.33% were malaria patients (76 *p.falciparum* and 53 *p.vivax*) who would have been left untreated (false negatives) and 6.19% (97 false *p.falciparum* and 53 false *p.vivax*) would have been incorrectly given antimalarial drugs (Table 1). Paracheck-BS correctly treated 23.95% cases (402 *p.falciparum* and 178 *p.vivax*) and mislabelled 76.05% (n = 1842). Out of these, 3.34% were malaria (51 *p.falciparum* classified as *p.vivax* and 30 *p.vivax* as *p.falciparum*) and 72.70% (n = 1761) were non-malaria (114 cases classified as *p.falciparum* out of which 11 were *p.vivax* and 1647 as *p.vivax* when they were not). The presumptive-BS captured all the *p.falciparum*, (18.7%, n = 453) but mistreated 1969 cases (81.30%) as *p.falciparum*, out of which 8.59% (208) were *p.vivax* and 72.71% were non-malaria (Table 2).

The CE analysis showed that the parascreen-BS was the most cost-effective with ACER US$ 1.69/CTC followed by US$ 4.66/CTC for the paracheck-BS and US $11.08/CTC for the presumptive-BS (Table 3). ICER analysis was conducted to find out whether this additional cost was worth paying to get the added effect. Presumptive-BS was highly dominated (less effect for more money) by parascreen-BS. Therefore, the ICER calculation was limited to parascreen-BS over paracheck-BS. At the base case, the additional cost on parascreen-BS over paracheck-BS would be able to treat an

Table 2 Effectiveness and cost ($US) of the three different diagnostic strategies, Tigray, Ethiopia, 2007

Description	Different treatment strategies		
	Presumptive n (%)	Paracheck-BS n (%)	Parascreen-BS n (%)
Correctly treated *p. falciparum* cases	453	402	377
Correctly treated p.*vivax* cases	0	178	155
Correctly treated non-malaria cases	0	0	1611
Total correctly treated cases	453 (18.70)	580 (23.95)	2143 (88.48)
Test Cost	0	1501.64 (55.52)	2615.76 (72.08)
AL cost	5017.2	1108.80 (41.00)	1003.20 (27.65)
CQ cost	0	94.05(3.48)	9.80(0.27)
Total cost	5017.20	2704.49	3628.76

Table 3 Average and incremental cost-effectiveness ratios among the three diagnosis strategies, Tigray, Ethiopia, 2007

Diagnostic based strategy	Cost	Correctly treated cases	ACER	Incremental cost	Incremental effect	ICER	Remark
Paracheck	2704.5	580	4.66	-	-	-	
Parascreen	3628.8	2143	1.69	924.27	1563	0.59	
Presumptive	5017.2	453	11.08	1388.44	-1690	-0.82	dominated

additional 64.5% (n = 1563) of patients correctly with an incremental cost of US$0.59/patient.

Sensitivity analysis

Taking into account the AL cost in the International Drug Price Indicator (2008 version) that showed a reduction of 32.8% (lowest dose), 33.25% (for the middle doses) and to 36.9% (adult dose) [26], a sensitivity analysis revealed a high reduction in the cost of the presumptive-BS by 37.14%, in paracheck-BS by 14.93% and in parascreen-BS by 10.05% (Table 4). The base case ACER was improved by 36.20% (from US$11.08 $US7.05) in presumptive-BS, by 14.81% (from US$ 4.66 to $US 3.97) in paracheck-BS and by 10.05% (from US$ 1.69 to $US 1.52) in parascreen-BS. Despite the significant drop in ACER, presumptive-BS was still dominated by parascreen-BS. The ICER of parascreen-BS over paracheck-BS was increased from $US0.59 to $US0.62 for each additional 1563 correctly treated cases.

The sensitivity analysis at 15% SPR during the minor transmission season with 35% *p.falciparum* to 65% *p.vivax*, with no change in the diagnostic performance of the strategies to the base case, showed a reduction in the proportion of correctly treated cases in the presumptive and paracheck-BS. The proportion of CTC was, however, increased in the parascreen-BS strategy (Table 4). The base case ACER decreased in parascreen-BS (from $US 1.69 to $US 1.29/CTC) and increased in the paracheck (from $US 4.66 to $US 6.11/CTC) and presumptive-BS (from $US 11.08 to $US 39.51/CTC) per correctly treated case. This illustrated that the cost-effectiveness increased by 23.67% in the parascreen-BS, decreased in the paracheck-BS by 31.12% and deteriorated significantly in the presumptive-BS by 258%. Since presumptive-BS was dominated, the IECR was recalculated as parascreen-BS over paracheck-BS. The base case of $US 0.59 dropped to $US 0.51/additional correctly treated case (Table 4).

A two-way sensitivity analysis (Table 5) at reduced cost of AL during the minor transmission season showed an increase in the ACER from $US 4.66 to $US 5.75 and from $US 11.08 to $US 25.14 in the paracheck-BS and presumptive-BS, respectively, while it dropped from $US 1.69 to $US 1.25 in the parascreen-BS. The two-way sensitivity analysis showed that presumptive-BS continued to be dominated by parascreen-BS.

Discussion

This is, to our knowledge, the first empirical study in Ethiopia evaluating the economic implications of the malaria diagnostic and treatment strategies currently implemented in the country. It is also a unique study in that it compared two RDTs targeting different plasmodium-specific antigens (*p.falciparum* and *p.vivax* vs. only *p.falciparum*) from an operational point of view.

This study has supported two central facts regarding the malaria transmission pattern in the region: firstly, our result of SPR (27.3%) and species composition of

Table 4 Sensitivity analysis at reduced AL cost and low-transmission for three malaria diagnostic strategies, Tigray, 2007

	At reduced AL price			Low transmission season (15% SPR)		
Comparison	Strategy			Strategies		
	Paracheck-BS	Parascreen-BS	Presumptive-BS	Paracheck-BS	Parascreen-BS	Presumptive-BS
Base case cost	2704.49	3629.76	5017.20	2704.49	3629.76	5017.2
Total new cost	2300.80	3264.20	3192.70	1926.05	2908.56	5017.20
Cost change within strategy	403.69	364.56	1886.50	778.44	720.20	0
Cost change in (%)	(14.93)	(10.05)	(37.14)	(28.78)	(19.85)	0
Correctly treated cases (n, %)	580 (23.95)	2143 (88.48)	453 (18.7)	315 (13.01)	2253 (93.02)	127 (5.24)
ACER at reduced AL and SPR	3.97	1.52	7.05	6.11	1.29	39.51
ACER Change from base case, %	(14.81)	(10.06)	(36.2)	(+31.1)	(23.67)	(+258)
Cost difference b/n strategy	0.00	963.40	-71.50	0.00	982.51	2108.64
Effect difference b/n strategy	0.00	1563.00	-1690	0.00	1938	-2124
ICER	-	0.62	dominated	-	0.51	dominated

+ ACER indicates higher value than the base case.

Table 5 A two-way sensitivity cost-effectiveness analysis at reduced cost of AL during low-transmission season, Tigray, 2007

Diagnostic strategies	Cost	Correctly treated cases	ACER	Incremental cost	Incremental effect	ICER
Paracheck-BS	1812.61	315	5.75	0	0	0
Parascreen-BS	2806.27	2253	1.25	993.66	1938	0.51
Presumptive-BS	3193.00	127	25.14	386.73	-2126	-0.18 (dominated)

p.falciparum to *p.vivax* (68.5% to 31.5%) is highly consistent with the commonly quoted statistics in serial reports of the THB [20]. Secondly, it has confirmed that malaria in the region varies from place to place due to differing altitude. The SPR was 46.51% for the lower stratum, 26.88% for the middle, and 16% for the highest stratum whilst showing a similar proportion of *p.falciparum* to *p.vivax* (range 68%-69.78%). As many other studies have indicated [17,27,28], this research has also revealed that the shift from presumptive-BS to RDT-BS is clearly of significant benefit in the era of ACT. In our context where malaria transmission is low, the likelihood of a fever episode being due to malaria, even during the peak transmission season, is on average 30%. Approximately one-third of this corresponds to *p. vivax*, increasing to two-thirds during the minor transmission season. The prevalence and proportion of the species bitterly challenges the presumptive-BS strategy as it leads to mistreat numerous *p.vivax* and false non-malaria cases. The need of using RDT-BS is therefore not debatable. Instead, the discussion should be tailored toward which type of RDT is the more cost-effective to ensure the maximum number of patients receive appropriate treatment. Accordingly, parascreen-BS was found to be the more cost-effective. The ICER showed that, if we invest in parascreen-BS instead of paracheck-BS, we can properly manage 65% (1563) additional cases for as little as $ 0.59/patient. If we spend on presumptive-BS instead of parascreen-BS, the cost rises to US$ 0.82/ patient (highly dominated). In fact, the cost-effectiveness of RDT-BS over presumptive-BS was partially increased at the expense of some missed malaria cases, since the RDTs are less sensitive than the presumptive-BS. We are also aware that if the effectiveness measure would have been only malaria cases, the paracheck-BS would have been the more cost-effective. However, the health benefit with the parascreen-BS is higher as more non-malaria cases get appropriate treatment and the saving is greater by avoiding over prescription. Over-treatment of malaria results in considerable morbidity and mortality by delaying the correct treatment of non-malaria illness and by contributing to the development and spread of antimalarial resistance strains.

The sensitivity analysis showed that the cost-effectiveness of the strategies varied depending on the season and AL cost. With the AL price drop, all alternatives improved their cost-effectiveness; however, in the low-transmission season, both the paracheck-BS and the presumptive-BS suffered while the parascreen-BS still improved. This shows that parascreen-BS is even more cost effective with reduced AL cost and during low-transmission season, which is the longest period of the year (December-August). Though no sensitivity analysis was made with regard to the different malaria strata, the higher the elevation, the lower the SPR makes parascreen pan/pf still more cost effective. Studies conducted in semi-immune populations have shown a higher cost effectiveness of RDTs in children <5 years compared to other age groups [14,29]. In our case, where all age groups share practically equal risk of malaria, this sensitivity analysis was not relevant.

Though the literature on the cost-effectiveness of RDTs has been growing in the last years [12-18,28], no comparable study designs to ours were found. The focus of all the studies has been on one type of plasmodium-specific RDT, either *p.falciparum* specific [13,14,17] or in combination with *p.vivax* [12,16]. Our study compared both types of plasmodium-specific RDTs at the same time.

Methodological considerations

There are some considerations to take into account which can potentially affect the findings of this research. Firstly, our study was limited to the health-provider perspective at the rural health post level. If a full societal perspective had been used to capture the distributional impact of the intervention, the epidemiological and economical advantages of the best RDT-BS might have been even higher. One limitation was that the study design did not allow us to capture whether HEWs complied with the guideline in their therapeutic decision-making. The HEW prescription report might not show the actual practice. Experience from the field and recent studies have shown that health workers are prescribing antimalarial drugs regardless of negative test results [17,30-35].

Cost calculation did not include the RDT reading time, RDT wastage and RDT training cost. The former is difficult to measure because the reading time might include attending several patients. RDTs could be wasted for different reasons such as poor transport,

storage conditions and due to inappropriate use. To estimate these wastage's costs would have been extremely difficult. The few hours training on RDT, which it is a long-term investment, made also difficult to allocate the cost to the patients. In our study, weights to malaria and non-malaria cases were assumed to be equal since our study population is non-immune. In some studies conducted in semi-immune populations, more weight has been given to non-malaria patients because malaria severity is less in adults [13,28].

Conclusions

The study has shown that the most cost-effective strategy was the one which used parascreen pan/pf in the treatment of malaria. The finding is relevant not only for Tigray region but also for the whole country, since malaria epidemiology follows a similar pattern nationally. Since 2008, the only available strategies at the health post level in the country have been the paracheck pf-BS and presumptive-BS. Our finding, pointing the superiority of the parascreen pan/pf based strategy, call decision-makers to reconsider this policy.

These results will be, however, pertinent only if an adequate supply of RDT and first-line antimalarial drugs at the health-post level are ensured and if HEWs comply with test results. Furthermore, and importantly, proper management of RDTs and adequate training and continuous supervision of HEWs should also be maintained. Finally, a study that captures the final health outcome of malaria diagnosis and treatment strategies and assesses HEWs' compliance with test results should be top research priorities in the region

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Authors' contributions

HL developed the study design, collected and analysed data and drafted the manuscript. MSS, CL, GB contributed to the study design and critically read and improved the manuscript. All authors read and approved the final manuscript.

Conflicts of interests

The authors declare that they have no competing interests.

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Point-of-care tests for malaria

Horizon Scan Report 0040
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Clinical Question:

In the primary care setting, what is the accuracy and utility of malaria point-of-care (POC) tests in the detection of parasitaemia caused by *Plasmodium* species, compared to standard laboratory practice using Microscopy and/or Polymerase Chain Reaction (PCR)?

Background, Current Practice and Advantages over Existing Technology:

Background:

Malaria is an important infectious disease, caused by the protozoan *Plasmodium* and transmitted by inoculation with an infected Anopheles mosquito. A variety of *Plasmodium* species cause malaria, typically producing cyclical systemic symptoms including fever, headache, vomiting and lethargy. Infection with *Plasmodium falciparum* can result in severe disease, and can lead to neurological sequelae including cerebral malaria and at worst death.

The World Health Organisation (WHO) World Malaria Report of 2009 estimates 243 million cases of malaria worldwide in 2008, the majority of which (85%) occurred in Africa, followed by South-East Asia (10%) and then the Eastern Mediterranean (4%).(**1**)

Whilst the largest burden of disease rests in Africa, the burden of malaria is increasing in non-endemic, industrialized areas due to imported disease in returning travellers who have no immunity (**2**). Many travellers do not comply with use of appropriate chemoprophylaxis and insect protection measures (**3**). For the reasons outlined above, malaria is an important differential diagnosis in febrile patients who have travelled to malaria endemic regions.

Current Practice and Advantages over Existing Technology:

a) **Primary care assessment of patients with suspected malaria**
   Existing Technology: Patient is clinically reviewed by General Practitioner (GP) and if malaria is suspected, liaison takes place with Infectious Diseases Registrar/medical registrar, with subsequent assessment of the patient in an Infectious Diseases Unit or appropriate Medical Assessment Unit. It is unlikely that blood samples would be sent from General Practice, due to the time delay that this would incur. However, were this to take place, blood samples would be sent from General Practice to the local hospital laboratory for analysis of thick and thin blood films for *Plasmodium* forms. Results would typically be sent back to the GP within

24 hours. Depending on the significance of the result, this may or may not need to be relayed to the Infectious Diseases Registrar and hospital admission planned.

Benefits of malaria POC testing: Rapid (within minutes) positive or negative malaria result, expediting referral to the Infectious Diseases team if positive, and investigation of other causes of febrile illness if negative without referral to the Infectious Diseases team. This technology could therefore allow assessment to move from a secondary care setting to primary care. This may lower testing thresholds.

b) Secondary care assessment of patients with suspected malaria

Existing Technology: Patients with suspected malaria in secondary care are frequently managed on Infectious Diseases wards and have an EDTA blood sample taken and analysed in the hospital laboratory. Here, the specimen is analysed under a microscope for *Plasmodium* forms. A diagnosis and/or level of parasitaemia is then estimated and appropriate treatment commenced if necessary. Other tests, such as PCR, may also be employed as a reference test.

Benefits of malaria POC testing: Rapid (within minutes) result of malaria infection, allowing prompt initiation of appropriate treatment. POC tests can be used in conjunction with microscopy, the latter helping to identify the specific *Plasmodium* species so as to direct treatment.

Details of Technology:

Malaria POC tests are generally portable, hand-held devices, the majority of which employ lateral-flow immunochromatography to detect *Plasmodium* antigens in a finger-prick sample of blood. A positive or negative result can be generated in as little as 10 minutes, allowing rapid diagnosis or exclusion of malaria. Their rapidity and also simplicity of use, not requiring specialist knowledge or equipment, are seen as their principle advantages over the current gold standard of laboratory based microscopy of thick and thin blood films.

Malaria POC tests can be grouped largely on the basis of the *Plasmodium* antigen detected. Some tests detect histidine-rich protein (HRP-2), which is solely produced by *Plasmodium falciparum*. Other tests detect aldolase, which is common to all *Plasmodium* species and therefore pan-specific. Yet other tests detect parasite lactate dehydrogenase enzymes (pLDH), which can be pan-specific, targeting a conserved pLDH element found in all *Plasmodium* species, or specific to particular *Plasmodium* species, targeting species unique regions of pLDH. A summary of available point-of-care malaria tests we identified can be found in the table in Appendix 1.

Patient Group and Use:

1) Ruling out malaria in travellers returning from malaria endemic regions with febrile illness.

2) Ruling out malaria in patients visiting the UK from malaria endemic regions presenting unwell to primary and/or secondary care.

Importance:

Light microscopy is considered the gold standard for malaria diagnosis (**4**). However, microscopic diagnosis of malaria requires time, trained personnel, and adequate laboratory facilities. In many parts of rural Africa in which malaria is most prevalent, access to such services is difficult or simply not possible. As such, there has been considerable interest in developing a new technology that could be used to rapidly diagnose malaria by non-skilled personnel (**5**).

Despite the burden of malaria being considerably less in the United Kingdom, there were 1501 cases of malaria in the UK in 2013 and 7 deaths (**6).** Prompt diagnosis and treatment of malaria could reduce morbidity and mortality. In the primary care setting, laboratory microscopic analysis of blood films is not possible. Implementation of a reliable malaria POC device could facilitate primary care diagnosis of malaria, allowing faster referral to secondary care, and more rapid administration of potentially life-saving treatment where appropriate.

Previous Research:

Accuracy compared to existing technology

Given the topical nature of malaria POC tests, a vast number of studies have examined their accuracy and potential utility. Below, we have focussed on the data from pertinent meta-analyses and other relevant studies.

<u>POC tests in malaria endemic regions</u>

A 2011 Cochrane review (**7**) analysed the use of POC tests in detecting clinical *Plasmodium falciparum* malaria in patients presenting to ambulatory healthcare centres in malaria endemic regions. The reference standard was defined as falciparum parasitaemia detected on microscopy, in conjunction with symptoms suggestive of malaria. Data from 74 studies described in 79 study reports were analysed. The POC tests were divided into seven different categories ('Type 1 tests' through to 'Type 7 tests') dependent on the test target antigen.

The vast majority of tests evaluated were 'Type 1 tests' evaluating HRP-2 specific POC tests. The authors identified 71 evaluations, in which 10 different brands of Type 1 POC tests had been verified with microscopy, encompassing 40,062 individuals. The sensitivities of the tests ranged from 42% to 100%, with specificities between 65% and 100%. The meta-analytical average sensitivity and specificity (95% confidence interval (CI)) were 94.8% (93.1% to 96.1%) and 95.2% (93.2% to 96.7%) respectively. Comparison of the 10 POC test brands analysed did not reveal statistically significant differences (p=0.18), however, substantial heterogeneity between studies was apparent.

There were 17 evaluations of 'Type 4' POC tests (identifying both *Plasmodium falciparum* specific and pan-specific pLDH antigens) verified with microscopy. The meta-analytical average sensitivity and specificity (95% CI) were 91.5% (84.7% to 95.3%) and 98.7% (96.9% to 99.5%), respectively. Upon comparison of the four brands of POC tests used in the type 4 tests evaluations, statistically significant (P=0.009) differences were noted. More precisely, Carestart Malaria Pf/Pan was found be more sensitive but less specific than OptiMAL, OptiMAL-IT and Parabank (sensitivity of 97.8% compared with 90.1%, 87.4% and 87.9%, respectively; specificity of 92.2% compared with 99.3%, 97.0% and 98.8%, respectively).

Statistical comparison was made between 'Type 1' and 'Type 4' tests with significant differences in test accuracy noted (p = 0.009). 'Type 4' tests were found to have a significantly higher specificity (p<0.001) than 'Type 1' tests in the comparisons based on all data, however, no significant difference was found between the sensitivity of these tests (p=0.34). The lower specificity of Type 1 tests may be due to the use of HRP-2 antibodies, which can give a false positive result in successfully treated cases of *Plasmodium falciparum* malaria, due to persistent antigenaemia. Thus, the choice of which test to employ in clinical practice would depend upon the prevalence of malaria in the affected region and additionally the goal of the test. In primary care, the intention would be to exclude malaria, and as such a test with high sensitivity would be desirable. Conversely, a highly specific test might be required in a secondary care setting to aid decisions regarding initiation of treatment.

A meta-analysis (**4**) examined the role of only the Parasight-F POC test (which had also been included in the Cochrane review) in the detection of *falciparum* malaria. 32 studies from 29 publications were evaluated, comprising 15,359 comprising 15,359 resident and non-resident subjects in a variety of malaria endemic and non-endemic countries. The included studies compared Parasight F against microscopy as a reference standard. Parasight-F demonstrated an overall meta-analytical sensitivity of 90.9% and specificity of 94.3%. The authors conclude that Parasight-F is a valid diagnostic tool that could be used stand-alone or in conjunction with microscopy. However, for any test it is important to recognise that the utility of the test is highly dependent upon the prevalence of malaria in a geographical region. Based on the pooled sensitivity and specificity data, in a region of 60% P. *falciparum* prevalence, the positive predictive value (PPV) would be 96%, with a negative predictive value (NPV) of 87%. However, in a region of 10% P. *falciparum* prevalence, the PPV would be much lower at 64%, conversely, the NPV would be 98%.

POC tests in Pregnancy

Plasmodium falciparum infection during pregnancy can result in severe illness and at worst death of mother and foetus (**8**). In pregnant women malarial parasites express an antigenic variant allowing them to sequester in the placenta, known as placental malaria, rendering microscopic diagnosis of peripheral blood inadequate (**9**). Placental histology is therefore the gold standard for diagnosis of placental malaria. However, placental analysis is only possible after delivery, and as such examination of peripheral blood during pregnancy is current standard practice.

A meta-analysis of 49 studies was performed to assess the accuracy of POC tests and PCR in diagnosis of malaria in pregnancy (**10**). Microscopic analysis of peripheral and placental blood was used as a reference standard, with the latter deemed the more accurate reference standard. The

sensitivity (proportion of microscopy positives in placental blood) detected by POC tests was 81%, versus 72% for peripheral blood microscopy and 94% for PCR analysis. The specificity (proportion of placental blood microscopy negative women) detected by POC tests was 94%, against 98% for peripheral blood microscopy and 77% for PCR.

POC tests in Non-immune travellers to malaria endemic regions

A meta-analysis (**2**) analysed the accuracy of POC tests in diagnosing malaria in non-immune travellers returning from malaria endemic countries, predominantly in Africa, Asia and South/Central America. Twenty-one studies were included, encompassing 5747 patients; eighteen of these studies were performed at regional or national tropical disease centres. The use of HRP-2 based tests and pLDH based tests was compared against microscopy and/or PCR as gold standards. Both two-band (detecting *Plasmodium falciparum* only) and three-band (detecting *Plasmodium falciparum* as well as *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium vivax*) HRP-2 tests were included in the analysis. Studies in which more than 10% of individuals were immune were excluded.

The negative likelihood ratio (LR-) was predefined as the primary measure of accuracy. This meta-analysis found that HRP-2 tests were statistically significantly more accurate than p-LDH based tests at ruling out *Plasmodium falciparum*, with LR-s of 0.08 and 0.13 respectively (p=0.019 for difference). For *Plasmodium vivax*, there was no statistically significant difference between the LR-for three band HRP-2 tests compared to parasite LDH tests (LR-s of 0.24 and 0.13 respectively; p=0.22), however, the available studies upon which these figures were based were few and heterogeneous in nature. The authors conclude that POC tests are a useful to rule out malaria when negative, but they should be used in conjunction with microscopy for species identification and confirmation when positive.

Summary

POC tests appear to be an accurate alternative compared to traditional microscopic analysis of blood films for malarial parasites. POC tests detecting HRP-2 antigens appear to have a higher sensitivity but lower specificity than POC tests detecting p-LDH. As such, the choice of which POC test to employ would largely depend upon the prevalence of malaria in the region of interest and the intended goal of the test. Given that the UK is a non-endemic region largely dealing with malaria in travellers and immigrants from endemic regions, and the aim of any rapid test would be to rule out. It is difficult to specify an optimal time-frame within which POC tests should be used given the varying incubation periods of *Plasmodium* species; in addition, latent blood infection with *Plasmodium* parasites can persist for years.

Impact compared to existing technology

A Cochrane meta-analysis (**11**) reviewed the utility of POC tests versus clinical diagnosis (relying on symptomatology and clinical signs alone) of malaria in febrile patients in rural African endemic settings, with a view to assessing whether this would reduce inappropriate use of anti-malarial drugs in patients with febrile illness not caused by malaria. Seven trials were reviewed, consisting of 17,505 febrile patients. Overall, POC tests did not reduce the number of unwell patients at day 4-7

post treatment; in those diagnosed with POC tests 2.8% to 9.3% remained unwell, versus a range of 4.1% to 10.8% remaining unwell in the clinically diagnosed group (Relative risk [RR[= 0.90, 95% CI 0.69-1.17).

Prescribing outcomes were very variable with high inter-study heterogeneity (I^2=98%); in one trial in Burkina Faso (**12**) 81% of patients with negative POC test results were prescribed anti-malarial drugs. As such, in this study and two others in which there was low adherence to prescribing in line with POC test results, no significant difference in anti-malarial prescribing was found between treatment groups (Risk ratio 0.90, 95% CI 0.68-1.20). However, in the four trials in which health workers adherence to prescribing in line with POC test results was high, a large reduction in anti-malarial prescribing was found, with a risk ratio of 0.44 (95% CI 0.29-0.67).

The safety of withholding anti-malarial drugs in patients with negative POC test results has been questioned (**13**). As afore-mentioned, in high prevalence areas of malaria transmission, a negative test result might carry a high false negative rate (**4**), meaning that some patients with malaria might be missed and therefore not treated on the basis of an inaccurate POC test result. As highlighted by the practice of healthcare workers in the study by Bisoffi *et al* (**12**), a POC test result may not necessarily lead to a change in practice if the clinical suspicions of the medical practitioner are different to the POC test result. Whilst the UK has a low prevalence of malaria, faced with a very unwell febrile patient with suspected malaria and a negative POC test, one might envisage empiric anti-malarial treatment being given until the definitive laboratory microscopic analysis result is available.

A prospective study was undertaken to determine the feasibility of non-immune travellers to Kenya between June 1998 and February 1999 to self-diagnose malaria using POC tests (**14**). Patients with fever (T>38 degrees Celsius) were asked to use an HRP-2 detecting POC test (ICT Malaria Pf) with assistance only from the device's accompanying manual and no prior training. A thick blood film was also performed on each patient. Of 98 patients with fever, only 67 (68%) were able to obtain a result. Of the 11 patients that had microscopically confirmed falciparum malaria, only one was able to produce a valid test result. Of those failing to obtain a test result, 87% cited that they were unable to interpret their test result, and 71% cited that they were unable to draw sufficient fingerprick blood for analysis. This would suggest that use of POC tests should be carried out by healthcare professionals, or at least those who have had basic training in their use.

In summary, malaria POC tests have the potential to reduce inappropriate use of anti-malarials in endemic regions, bypassing the time and expertise required for microscopic analysis. POC tests may also have a role in diagnosis of placental malaria. However, due to the possibility of obtaining a false negative result, the action taken in light of a negative result is likely to depend upon the prevalence of malaria in the region of use and the beliefs held by the clinician interpreting the result. Malaria POC tests should be used by healthcare professionals or those with adequate training in their use and interpretation.

Guidelines and Recommendations:

In the WHO guidelines for the treatment of malaria, it is stipulated that prompt confirmation of malarial parasite infection using microscopy or alternatively POC tests is advised in all patients with suspected malaria, prior to initiation of anti-malarial treatment (**15**). Whilst in the UK access to

microscopic diagnostics is readily available, in parts of rural Africa POC tests could be a giant step in the direction toward making the WHO edict a reality.

The guidelines for Malaria prevention in travellers from the UK, produced by Public Health England (PHE) **(16),** state that POC tests may be useful in the hands of medical personnel accompanying an expedition to a malaria endemic region, but not for self-diagnosis by lay people. Furthermore, this guidance cautions that in the UK POC tests are not a substitute for microscopy, but they may be used alongside blood films for diagnostic purposes.

Research Questions:

1) Trials in the primary care setting to help determine whether POC tests are a viable means of ruling out malaria, and hence improve targeted referral to secondary care when appropriate, as opposed to current practice of relying upon clinical suspicion.

2) Assessment of the cost:benefit ratio of implementing use of POC tests within primary care.

Suggested next steps:

1) Studies to determine the needs in different clinical situations and settings within primary care, e.g. urgent care/out-of-hours.

2) Studies to assess the utility and feasibility of training patients travelling to rural malaria endemic regions in use of malaria POC tests.

Expected outcomes:

The use of POC tests in diagnosis of malaria would be expected to lead to faster diagnosis of malaria in suspected cases, and therefore faster initiation of treatment for those affected. Conversely, prompt acquisition of a negative test result could help reduce inappropriate prescription of anti-malarial drugs, with consequent reduction of the morbidity that can be associated with adverse drug reactions, the ever-increasing problem of drug resistance, as well as reduction of the financial burden stemming from drug wastage. A negative test result should empower the clinician to investigate alternative differentials for febrile illness.

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Appendix 1: Table of available point-of-care malaria devices

Product	Manufacturer/ Location	Blood type analysed	Sample Volume (µl)	Analysis Time	CE Mark	FDA approved	Portable	Detection Range/Limit (parasites/ µl)	Positive result outcomes	Storage Temp. (Degrees Celsius)	Method Principle	Antigen detected
Paracheck-Pf	Orchid Biomedical Systems; India	Capillary Whole Blood	5 µl	20 mins	Yes	No	Yes	Unknown	*P. falciparum*	4-45	Immunochromato-graphic Assay	PfHRP-2
ParaSight - F	Becton Dickinson; Franklin Lakes, NJ, USA	Capillary Whole blood	50 µl	Unknown	Unknown	No	Yes	>100 parasites per microliter	*P. falciparum*	Unknown	Immunochromato-graphic Assay	PfHRP-2
ICT Malaria Pf/pv	Amrad-ICT Diagnostics; Sydney, Australia	Unknown	10 µl	Unknown	Unknown	No	Yes	Unknown	*1) P. falciparum* 2) Mixed infection	2-30	Immunochromato-graphic Assay	Aldolase and PfHRP-2
ICT Malaria PF	ICT Diagnostics; New South Wales, Australia	Capillary Whole blood/venous	5 µl	15 mins	Yes	No	Yes	> 200 parasites/ µl	*P. falciparum*	4-40	Immunochromato-graphic Assay	PfHRP-2
Rapid Malaria Pf/Pv	Accu-tell; New Delhi, India	Capillary Whole blood/Venous	10 µl	15 mins	Yes	No	Yes	Unknown	*1) P. falciparum* *2) P. vivax* 3) Mixed *P.falciparum* and *P. vivax*	2-30	Immunochromato-graphic Assay	PfHRP-2 and *P.vivax* pLDH
CareStart Malaria Pf/Pan	Access Bio; New Jersey, USA	Capillary Whole blood	5 µl	20-30 mins		No	Yes	Unknown	1) *P. falciparum* malaria or mixed 2) Non-falciparum malaria	4-30	Immunochromato-graphic Assay	PfHRP-2 and Pan-pLDH
Parabank	Zephyr Biomedicals; Verna, India	Capillary Whole blood	5 µl	20 mins	Yes	No	Yes	Unknown	Pan-specific	4-30	Immunochromato-graphic Assay	Pan-pLDH

ParaHIT-F	Span Diagnostics Ltd; Surat, India	Capillary Whole blood	5 µl	15 mins	Unknown	No	Yes	>100 µl	*P. falciparum*	4-40	Immunochromato-graphic Assay	PfHRP-2
BinaxNOW Malaria Test	Alere; Maine, USA	Capillary Whole blood/venous blood	15 µl	15 minutes	Yes	Yes	Yes	>310/ µl for P.falciparum >50/ µl for non-falciparum spp	1)*P. falciparum*/ mixed 2) Non-falciparum malaria	2-37	Immunochromato-graphic Assay	PfHRP-2 and aldolase
MAKROmed Malaria Test	MACROmed manufacturing, LTD; South Africa	Capillary Whole blood	Unknown	<20 mins	Unknown	No	Unknown	>100 µl	*P. falciparum*	Unknown	Immunochromato-graphic Assay	PfHRP-2
Visitect Malaria Pf	Omega Diagnostics LTD	Capillary Whole blood/Venous blood	5 µl	15 minutes	Yes	No	Yes	Unknown	*P. falciparum*	4-40	Immunochromato-graphic Assay	PfHRP-2
Visitect Malaria Combo Pan/Pf	Omega Diagnostics LTD	Capillary Whole blood/Venous blood	5 µl	15 minutes	Yes	No	Yes	Unknown	1) *P. falciparum* or mixed 2) Non-falciparum malaria	4-30	Immunochromato-graphic Assay	Pan pLDH and PfHRP- 2
DiaMed OptiMAL-IT	BIO-RAD; California, USA	Capillary Whole blood	10 µl	20 minutes	Yes	No	Yes	>50-100/ µl	1) *P. falciparum* malaria or mixed 2) Non-falciparum malaria	2-30	Immunochromato-graphic Assay	pLDH (*P.falci-parum* specific) and pLDH (pan-specific)
OptiMAL	DiaMed AG, Cressier, Switzerland	Capillary Whole blood	Unknown	20 minutes	Yes	No	Unknown	Unknown	1) *P. falciparum* malaria or mixed 2) Non-falciparum malaria	Unknown	Immunochromato-graphic Assay	pLDH (*P.falci-parum* specific) and pLDH (pan-specific)
Malaria-Ag CELISA	Cellabs, Australia	Capillary Whole blood or	100 µl	2 hours	Yes	No	No	>5-50 / µl	*P. falciparum*	2-8	Enzyme-linked Immunosorbent Assay	PfHRP-2

		serum										
Malascan	Zephyr Biomedicals; Verna, India	Capillary Whole Blood	5 µl	20 minutes	Yes	No	Yes	Unknown	1) *P. falciparum*/ mixed 2) Non-falciparum malaria	4-30	Immunochromato-graphic Assay	PfHRP2 and aldolase
PATH Falciparum Malaria IC test	PATH; Seattle, USA	Capillary whole blood	5 µl	Unknown	Unknown	No	Yes	>100 µl	*P. falciparum*	Unknown	Unknown	PfHRP-2
Determine Malaria Pf	Abbott Laboratories; Tokyo, Japan	(Capillary Whole blood)	2 µl	30 minutes	Unknown	No	Yes	Unknown	*P. falciparum*	Unknown	Immunochromato-graphic Assay	PfHRP-2
DiaSpot Malaria	Acumen Diagnostics Inc; USA	Capillary Whole Blood	10 µl	10 minutes	Unknown	No	Yes	Unknown	*P. falciparum*	Unknown	Immunochromato-graphic Assay	PfHRP-2
Hexagon Malaria	HUMAN Diagnostics, Germany	Capillary or venous whole blood	5 µl	15 minutes	Yes	No	Yes	Unknown	1) *P. falciparum*/ mixed 2) Non-falciparum malaria	2-30	Immunochromato-graphic Assay	PfHRP2 and aldolase
SD Malaria Antigen Bioline	SD Diagnostics; Korea	Capillary Whole Blood	5 µl	15-30 minutes	Yes	No	Yes	>50/ µl	1) *P. falciparum* or mixed 2) Non-falciparum malaria an-specific	1-40	Immunochromato-graphic Assay	PfHRP-2 and pan-pLDH
Parascreen Rapid Test for Malaria Pan/Pf	Zephyr Biomedical Systems; Verna, India	Capillary Whole blood	5 µl	20 minutes	Yes	No	Yes	Unknown	1) *P. falciparum* or mixed 2) Non-falciparum malaria	4-30	Immunochromato-graphic Assay	PfHRP-2 and pan-pLDH
First Response Malaria (pLDH/HRP2 combo test)	Premier Medical Corporation; Daman, India	Whole blood	5 µl	<20 minutes	Yes	No	Yes	>200/ µl	1) *P. falciparum* or mixed 2) Non-falciparum malaria	1-40	Immunochromato-graphic Assay	PfHRP-2 and pan-pLDH

Molecular tools for the diagnosis of malaria and monitoring of parasite dynamics under drug pressure

Mens, P.F.

Chapter 6

Molecular biology the best alternative for diagnosis of malaria to microscopy? A comparison between microscopy, antigen detection and molecular tests in rural Kenya and urban Tanzania

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Summary

The objective was to asses the agreement of different diagnostic methods for the diagnosis and confirmation of the clinical suspicion of *Plasmodium* infection in children in Tanzania and Kenya. Finger prick blood was collected from a total 338 children with the clinical suspicion of uncomplicated malaria in health clinics in Tanzania and Kenya. The presence of *Plasmodium* parasites was assessed with microscopy, rapid diagnostic tests (RDT's) and the molecular assays Quantitative-Nucleic Acid Sequence Based Amplification (QT-NASBA) and PCR. The results were compared and analysed for agreement. A high degree of agreement (88.6% - 100%) was observed between the different tests employed compared to microscopy. In rural Kenya, with a high incidence of malaria cases, the κ value was high ranging from 0.94 for RDT's to 0.76 for PCR. In urban Tanzania, where there was a low incidence of cases the κ value for RDTs was 1.0 but for PCR and NASBA only 0.25 and 0.33, respectively.

Malaria is overestimated if the diagnosis is based solely on clinical signs. Therefore, laboratory confirmation is essential. Microscopy is a reliable method in rural areas where malaria is often seen, but RDT's offer a good alternative with the advantage that it is an easy and rapid method. Molecular tests are more sensitive however difficult to implement in rural areas. In areas with lower incidence, molecular tests detect a significantly higher number of *Plasmodium* infections than RDT's or microscopy. Although implementation of molecular tools in the detection of malaria parasites can be difficult, the prospect of development of an easy and cheap detection system makes it promising tools for the near future.

Introduction

Initiation of malaria treatment largely depends on good, laboratory-confirmed diagnosis. However, in many disease endemic countries, clinical diagnosis is the only method used to decide on treatments as laboratory techniques to confirm the clinical suspicion are considered to be too labour intensive or are not sensitive enough [4,8,9]. In general the screening of blood slides by microscopy is still considered to be the "gold standard". An experienced microscopist is said to have a detection limit of approximately 20 parasites/µl [8]. This method is cheap and simple but labour intensive, time consuming and requires well-trained personnel who can differentiate between the different *Plasmodium* species [15]. In recent years, a variety of rapid diagnostic tests (RDT's) have been developed for the diagnosis of malaria. These tests are fast, easy to perform and do not require electricity or specific equipment and currently around US$ 0.62/test [11,12,23]. These tests are based on the recognition of *Plasmodium* antigens in the blood circulation of the patient. The tests can detect less than 100 parasites/µl but with lower parasitaemia their sensitivity decreases, making these tests unsuitable for patients with low numbers of parasites [12,13]. Another drawback is the reported persistence of antigens, in particular Histidine Rich Protein II (HRP-II), in the blood circulation of the patient after parasite clearance, generating false-positive results when microscopy is used as a reference test [10,12,21]. However, other reports state that antigen persistence is not the problem but limitations of microscopy that miss a sub-set of the patient population [3]. This may be partly because of the skills of the microscopist or the setting in which microscopy is performed (e.g. a rural vs. an urban setting, endemic vs. non-endemic regions). Molecular techniques such as Polymerase Chain Reaction (PCR) and Nucleic Acid Sequence Based Amplification (NASBA), are much more sensitive than microscopy. For example PCR is said to have a lower detection limit of between 0,7 and 0,02 parasites/µl [2,6,18]. Quantitative Nucleic Acid Sequence Based Amplification (QT-NASBA) can detect parasites at a level as low as 0,02 parasites/µl blood, and allows for precise quantification of the parasite load over a range of 20-10^8 parasites/ml blood

[17,18,19]. This paper describes the comparison of conventional microscopy and two rapid diagnostic tests for the detection of *Plasmodium* species, based on the HRP-II or *Plasmodium* specific Lactate dehydrogenase, with QT-NASBA and PCR using blood samples of children with the clinical suspicion of uncomplicated malaria, visiting a local district hospital (Kenya) and an urban health clinic (Tanzania).

Materials and Methods

Study population

This prospective study was conducted in two areas in eastern Africa, the rural Kimbimbi sub-district hospital, (Mwea) a mesoendemic area in the Cental Province of Kenya, and in an urban setting in the Aga Khan Health Service, Dar es Salaam, Tanzania. Ethical approval for this study was obtained from the appropriate local authorities; i.e. Kenya Medical Research Institute (KEMRI) Kenya (SSC 947), The Aga Khan University Ethical Research Committee and Health Research Ethics Review Subcommittee, Tanzania. The samples were collected during the high transmission seasons of May 2005 (Kenya) and December 2005 (Tanzania). Children (6 months to 12 years) visiting the health centres, suspected to have uncomplicated malaria and willing to participate in the study after informed consent of their parents or guardians were included. Inclusion criteria were: suspicion of uncomplicated malaria, axillary temperature ≥ 37.5 ^{0}C or a history of fever in the past 24 hours. 149 children in Kenya and 154 children in Tanzania were enrolled in the study.

Microscopy

Finger prick blood was collected and Giemsa stained thick and thin smears were prepared according to WHO guidelines but because of local practice staining times varied from 5 -10 minutes instead of the recommended 20 minutes. Two independent experienced microscopists, who were blinded to the results of the other tests performed, examined the smears for the presence of parasites and

identified the observed parasite species. Parasitemia was determined from the thick smears by counting the number of parasites against 200 leukocytes assuming that there are 8000 leukocytes/µl blood.

Rapid diagnostic tests

Finger prick blood was also used to evaluate the rapid assays OptiMAL (Diamed AG, Switzerland) and Paracheck Pf (Orchid Biomedical Systems, India). In addition in Kenya the Parascreen test (Zephyr Biomedicals, Verna Goa, India) was also included in the study. All the tests were performed according to the manufacturers' instructions. Two readers, blinded from the other tests performed, independently examined the RDTs. An RDT was considered positive when both the internal control and the test band were stained (irrespective of the intensity of the staining). A test was considered negative if only the internal control was visible. The result of an RDT was considered not valid (test failure) if the internal control was not visible.

PCR and QT-NASBA

Blood was collected on Whatman 903 filter paper (Whatman international Ltd. Maidston, United Kingdom) and air-dried at room temperature for NASBA and PCR analysis. DNA and RNA was isolated as described by Boom *et al*.[5]. All molecular testing was done blinded from the results of microscopy and RDT testing. PCR for the detection of *Plasmodium* in the sample was performed on the 18S rRNA gene target as described previously [20]. Amplification products were detected on a 2% ethidium bromide stained agarose gel and visualized under UV light. QT -NASBA was performed on RNA of 18S rRNA gene on an IQ5 Real-Time analyser (Bio-RAD, The Netherlands) as described previously [19] with some modifications by Schneider et al.[18]. A generic *Plasmodium* reaction was used with the following primers: forward 5'-TCAGATACCGTCGTAATCTTA-3' and reverse 5'-AATTCTAATACGACTCACTATAGGGAGAAGGAACTTTCTCGCTTGCGCGAA-

3'. The reactions were performed with the Nuclisens Basic kit for amplification (bioMerieux, Boxtel, The Netherlands) according to the manufacturer's instructions with a KCl concentration of 80 mM. The reaction mixture (5 µl) containing the primers (100 pmol/µl), molecular beacon (20 µM) and template RNA (2.5 µl) was incubated at 65 ^{0}C for 2 minutes followed by 2 minutes at 41^{0}C. Thereafter 2.5 µl enzyme mixture from the basic kit was added to each reaction. Amplification was monitored for 90 minutes after which the results were analysed. In order to quantify the number of parasites in blood, a 10 fold serial dilution of 10^6 to 10 in vitro cultured parasites/ml was used as reference and processed and analysed as described above.

Statistical analysis

All data was recorded on separate case record forms. The agreement between microscopy, RDT testing and molecular analysis of blood samples was assessed using Epi Info version 6.04 (Centre for Disease Control and Prevention, Atlanta, GA, USA). Kappa (κ) value expressed the agreement beyond chance [1] and were calculated with a 95% confidence interval. A κ value of 0.21-0.60 is a moderate, a κ value of 0.61-0.80 a good and κ > 0.80 an almost perfect agreement beyond chance.

Results

Patient recruitment

In Kenya, in total 184 suspected cases of uncomplicated malaria were included in the present study during a 3-week recruitment period in May 2005. The mean age of the children was 77,5 months and the sex ratio male:female was 1:1. In Tanzania, 154 individuals (mean age: 70,6 months; sex ratio: 1,8:1) were recruited for the study during the 3 weeks the study was conducted (December 2005).

Microscopy

Expert microscopy in Kenya (see Table 6.1) revealed 60 slide-positive cases of malaria (32.6%). There were no discordant readings between the two independent microscopists who examined the slides. Species determination identified 58 slides with a mono-infection of *P. falciparum* ranging from 400 parasites/µl to 828,800 parasites/µl with a mean count of 18,680 parasites/µl and two slides with mono-infections with *P. malariae* (not counted). In 124 (63.4 %) slides *Plasmodium* parasite were not observed with microscopy. In Tanzania, only three slides (1.9%) were found *P. falciparum* positive (160, 280 and 1,000 parasites/µl) and in the remaining 151 slides no parasites were seen. There were no discrepant microscopic results obtained in Tanzania.

RDTs

The data of RDT testing are summarised in Table 6.1. The results of RDT testing in Kenya revealed the following: in total, 61 cases were found *Plasmodium falciparum* positive with the OptiMal test and two additional OptiMal tests were positive for *Plasmodium* non-*falciparum* (in total 34.2% of the OptiMal tests gave a positive result), 59 cases (32.0%) with Paracheck and 69 cases (37.5%) with the Parascreen test. Test failures, which were defined as tests that did not show a control band, were observed with all three RDTs; i.e. OptiMal four failures, Paracheck five failures and Parascreen one failure. In Tanzania only three cases (1.9%) were found positive with both RDTs evaluated which were the same as identified positive by microscopy. Only one test failure was observed with the OptiMal test and four failures were found for the Paracheck test. The total percentage of RDT test failures over all 338 cases examined was 1,5% for the OptiMal test and 2,7% for the Paracheck test.

Tabel 6.1: Comparison of five different diagnostic tests for malaria with microscopy in 338 blood samples from clinically suspected children, collected at two different endemic areas. The number of positive samples is indicated in each case.

	Kenya (n=184)			*Tanzania (n=154)*		
	Microscopy Positive (400-828800; 18680)[a] (n=60)	**Microscopy Negative (n=124)**	**K-value (Confidence-Interval 95%)**	**Microscopy Positive (160, 280, 1000)[b] (n=3)**	**Micoscopy Negative (n=151)**	**K-value (Confidence-Interval 95%)**
PCR	60	21	0.76 (0.667-0.855)	2	11	0.25 (-0.09-0.568)
NASBA	60	17	0.80 (0.716-0.891)	3	11	0.33 (0.015-0.648)
OptiMal®	58	3	0.94 (0.867-0.985)	3	0	1.0 (1.0-1.0)
Paracheck®	56	3	0.91 (0.851-0.975)	3	0	1.0 (1.0-1.0)
Parascreen®	60	9	0.89 (0.825-0.961)	n/a	n/a	n/a

(a) parasites/µl (range; mean)

(b) parasites/µl

(n/a) not applicable

Molecular Biology

Blood spots collected on filter paper at both study sites were analysed in the laboratory in the Netherlands for the presence of *Plasmodium* DNA/RNA with PCR and QT-NASBA, respectively (see Table 6.1). Forty-four percent (n=81) of the samples collected in Kenya were found positive with PCR and 41,8% was positive with NASBA (n=77), with parasite counts ranging from 16 – 10^8, with a mean count of 390,000 parasites/ml blood). Six of the PCR-positive samples were negative with all other tests including NASBA. Two NASBA-positive samples (parasite count 9 and 339 parasites/ml) were found negative with PCR as well as with the other tests employed in this study. Thirteen samples (8.4%) collected in Tanzania were found positive with PCR and 14 samples (9.1%) with QT-NASBA (parasite counts ranging from 9-1,370,000, mean count: 149,000 ml/ blood). All microscopy positive samples from Kenya were also found positive in PCR and NASBA. One microscopy positive sample from Tanzania was found negative by PCR but positive by NASBA. One sample from Tanzania was found positive by PCR but negative with the other tests employed.

Agreement between microscopy, RDTs and molecular tests

The degree of agreement, with a 95% confidence interval, observed between the different diagnostic test is presented in Table 1. The agreement beyond chance (κ value) varied in the different settings. High degree of agreement was observed between microscopy and RDT in Kenya. The number of positive cases found in Tanzania was too little to make a meaningful statement about agreement between the RDTs and microscopy. In Kenya molecular tests had also a high agreement for PCR and NASBA. In Tanzania, on the other hand, PCR and NASBA had only a moderate agreement beyond chance.

Quantitative Nucleic Acid Based Assay allowed for the sub-microscopic quantification of *P. falciparum* in blood samples. The mean parasite count with NASBA observed in the microscopy-negative samples from Kenya was 6,940 parasites/ml blood, with a range of 16 – 12,000 parasites/ml. In Tanzania, the

mean count of the NASBA-positive- microscopy-negative samples was 75,000 parasites/ml blood, over a range of 9 - 660,000 parasites/ml.

Discussion

Malaria can be a life-threatening disease, especially in children, when left untreated and therefore, it is important to have a quick and accurate diagnosis. However, even though malaria is a frequently encountered disease in many developing countries, it is difficult to impossible to make the right diagnosis relying on clinical signs only. To prevent unnecessary anti-malarial treatment, it is important to confirm clinical suspicions with a good laboratory test. In the light of the changing drug policies of many African countries, including Tanzania and Kenya, where the expensive Artimisinin based Combination Therapy (ACT) drugs are prescribed as first line treatment, good laboratory confirmation will also have its impact on the economical situation [8,14]. In this study we assessed which method is best suited for confirmation of the clinical suspicion of malaria in two areas: in Tanzania, with low prevalence of malaria, and Kenya with a high prevalence of malaria, where diagnosis relying on clinical signs only is always an overestimation of the true incidence. For this purpose blood samples of children presenting themselves with the clinical symptoms of malaria were analysed with microscopy, RDT or molecular biology. Almost all microscopy positive samples were found positive with the RDT's and molecular tests. However, the molecular tests found a substantial higher amount of positive samples compared to the RDT's and microscopy, confirming the higher sensitivity of QT-NASBA/PCR [19]. The mean number of parasites found in the QT-NASBA positive – microscopy negative samples in Kenya was below the accepted lower detection limit of 20 parasites/µl blood for microscopy [8]. It is noted however, that in Tanzania two samples were scored microscopically negative that had QT-NASBA parasite counts above this accepted limit. The shortened staining time of the blood slides may have caused missing these infections.

The sensitivity of the molecular tests identifies more children with low parasitaemia. As these children are symptomatic with the clinical suspicion of malaria the chances of feeling ill because of the submicroscopic infection with *Plasmodium* are real. However, the possibility of also having another cause for fever cannot be excluded. Nevertheless, the presence of parasites in the blood indicates a malaria infection that should be treated as such. Therefore, molecular tests would be preferred to microscopy and RDT testing for the confirmation of the clinical suspicion of malaria [16]. The drawback however is that many molecular tools and protocols are too cumbersome, too expensive, and not simple or rapid ore even not available at all because of limited resources such as electricity and inadequate laboratory infrastructures in developing countries [7]. In areas where incidence is low, the impact of molecular diagnostics is much higher than in high malaria incidence areas, therefore making molecular tools more important in the former. The more developed urban hospitals laboratories, such Aga Khan in Dar es Salaam, would greatly improve their diagnosis. Implementation of molecular methods as a quality control tool for microscopy diagnosis of *Plasmodium* infections would not only be an improvement for the developing countries but also in research settings and in the quality control programmes of routine diagnosis for malaria of developed countries where infections are less frequently encountered. The costs may be an obstacle, but in practice patients are at present treated on clinical grounds, often more unnecessarily than necessary. The presently advised ACT treatment is very costly. To avoid patient costs, proper diagnostic testing is favoured above prescription of medication on clinical grounds only. In the more underdeveloped areas, where the resources are poor, implementation of these techniques is a great obstacle. However in areas where there is a high incidence of malaria the lack of facilities undermines the benefit of molecular tools. RDT's, however, are in our hands as sensitive as microscopy and have a good agreement beyond chance. The simple format and quick results without the need of good microscopy equipment and electricity makes these test a good alternative to microscopy in these areas [11]. Nevertheless, the search for increased detection

sensitivity combined with a simple detection system is an ongoing challenge [22] and would ultimately lead to the feasibility of also introducing molecular tools in the developing areas where the need is immense but the facilities limited.

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COMPARISON OF HRP2- AND pLDH-BASED RAPID DIAGNOSTIC TESTS FOR MALARIA WITH LONGITUDINAL FOLLOW-UP IN KAMPALA, UGANDA

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Abstract. Presumptive treatment of malaria results in significant overuse of antimalarials. Malaria rapid diagnostic tests (RDTs) may offer a reliable alternative for case management, but the optimal RDT strategy is uncertain. We compared the diagnostic accuracy of histidine-rich protein 2 (HRP2)- and plasmodium lactate dehydrogenase (pLDH)-based RDTs, using expert microscopy as the gold standard, in a longitudinal study of 918 fever episodes over an 8-month period in a cohort of children in Kampala, Uganda. Sensitivity was 92% for HRP2 and 85% for pLDH, with differences primarily due to better detection with HRP2 at low parasite densities. Specificity was 93% for HRP2 and 100% for pLDH, with differences primarily due to rapid clearance of pLDH antigenemia after treatment of a previous malaria episode. RDTs may provide an effective strategy for improving rational delivery of antimalarial therapy; in Kampala, either test could dramatically decrease inappropriate presumptive treatments.

INTRODUCTION

Diagnostic capabilities are limited in Africa, and in most cases fevers are treated presumptively as malaria without laboratory-confirmed diagnosis. In many settings, presumptive treatment of all fevers as malaria results in extensive overuse of antimalarials and delays the diagnosis of other causes of fever.[1–4] With older antimalarial drugs, which were inexpensive, safe, and widely available, the potential benefits of early treatment of all fevers supported presumptive antimalarial therapy. However, in the era of increasing drug resistance, new combination therapies are being deployed that are much more expensive and have less established safety records.[5,6] In this setting, improved ability to diagnose malaria may prevent many unnecessary antimalarial treatments and should also allow prompt attention to other causes of fever when malaria is ruled out. Light microscopy, for decades the gold standard for malaria diagnosis, remains unavailable to most patients in Africa.[7,8] Malaria rapid diagnostic tests (RDTs), newer diagnostic modalities that identify circulating antigens of malaria parasites, may offer a reliable alternative for case management.

The most studied malaria RDTs offer simple identification of two parasite antigens: histidine-rich protein 2 (HRP2) and plasmodium lactate dehydrogenase (pLDH). HRP2 was the first antigen targeted by an RDT,[9] has been available in various commercial formats for several years, has shown good sensitivity in a variety of field settings, and is increasingly advocated as a diagnostic test where reliable microscopy is not available. A potential problem for HRP2-based assays is persistence of detectable circulating antigen for up to several weeks after parasites have been eradicated.[10–12] Persistent HRP2 antigenemia has not correlated with treatment failure, suggesting that this finding is not representative of persistent infection.[10,12] Persistent antigenemia thus may limit the usefulness of HRP2-based assays in areas of intense malaria transmission, where positive tests may commonly be due to prior infections that are no longer clinically relevant. pLDH-based RDTs appear to be slightly less sensitive than those detecting HRP2, but the antigen is rapidly cleared from the bloodstream, becoming undetectable at about the same time blood smears become negative after antimalarial therapy.[13–15] Thus, if sensitivity is adequate, the increased specificity of pLDH-based assays for acute malaria suggests that they may be better-suited for high-transmission areas, such as much of sub-Saharan Africa. With increasing advocacy for the implementation of RDTs, it is critical that optimal diagnostic strategies are identified. The true impact of the varied sensitivity and specificity of different tests is best compared with long-term follow-up to consider the impacts of prior infections and persistent antigenemia on test results. For this reason, we compared the diagnostic accuracy of HRP2- and pLDH-based RDTs, using expert microscopy as the gold standard, in a longitudinal cohort of children in Kampala, Uganda.

METHODS

Study population and longitudinal drug-efficacy trial. We evaluated two RDTs in a cohort of 601 children enrolled in an on-going longitudinal antimalarial treatment efficacy trial in Kampala. The trial began in November 2004, and is based at Mulago Hospital, Uganda's main public hospital. Participating children are residents of Mulago III parish, located within 2 km of Mulago Hospital. Households were randomly selected for enrollment into the trial after a census of the parish.[16] Children in the study cohort receive all their medical care free of charge at our study clinic. Participants are encouraged to come to the clinic promptly for any illness and to avoid any medications not administered by study clinic staff. Participants are seen at least monthly, either at the study clinic for evaluation of illness or for routine follow-up visits, or during home visits. Each time a participant presents to the study clinic with fever (documented tympanic temperature $\geq 38°C$ or history of fever within the previous 24 hours), a fingerprick blood sample is obtained for thick and thin smears and storage on filter paper. If the blood smear is positive, the child is treated with antimalarials and followed for 28 days; if the smear is negative, the child does not receive antimalarials and is treated according to standard clinical algorithms and

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the study physician's judgment. Parents/guardians gave informed consent for all study procedures, and the study was approved by the Uganda National Council of Science & Technology and by the institutional review boards of Makerere University and the University of California, San Francisco.

RDT study methods. At the time of the RDT evaluation, children in the cohort ranged in age from 1.5 to 11.5 years. From October 2005 to May 2006, each time a blood smear was done to evaluate fever in a study participant, except when the fever occurred within 3 days of a confirmed episode of malaria, a fingerprick blood sample was obtained for the two RDTs in addition to thick and thin smears and storage on filter paper (Figure 1). If a participant presented with repeated episodes of fever after diagnosis of a non-malarial illness, the RDT was repeated at the study physician's discretion. Clinical management was guided by microscopy results; RDT results did not influence patient care.

Thick and thin smears were stained with 2% Giemsa for 30 minutes and read by experienced laboratory technologists. Parasite densities were calculated from thick smears by counting the number of asexual parasites per 200 leukocytes (or per 500 leukocytes, if the count was < 10 asexual parasites/200 leukocytes), assuming a leukocyte count of 8,000/μL. Smears were considered negative if the examination of 100 high-power fields did not reveal asexual parasites. Gametocytemia was determined from thick smears and parasite species from thin smears. All smears were read a second time by study laboratory staff to confirm results, and discrepant readings were resolved by a third reader. If the first and second readers both reported a positive smear, but the second density report differed from the first by ≥ 2000/μL, the final density recorded was that of the third reader.

RDTs were selected for evaluation on the basis of ease of use (relatively few preparation steps and clear distinction between positive and negative results), safety (minimal exposure to blood during test preparation), completeness of packaging and labeling, appropriate packaging for transport and storage in tropical environments (each test individually wrapped in foil with plastic liner and desiccant), low market price, and reliability of supply. The RDTs studied were Paracheck (detection of HRP2, Orchid Biomedical Systems, Goa, India) and Parabank (detection of pLDH, Zephyr Biomedicals, Goa, India). RDTs were obtained directly from the manufacturers and stored in their original packaging at room temperature in the study clinic. Temperature and humidity of the storage area were monitored, but not controlled. Over the course of the study period, the temperature in the storage area ranged from 19 to 29°C, with a mean low of 24°C and a mean high of 27°C. The relative humidity ranged from 31% to 82%, with a mean low of 53% and a mean high of 69%. Prior to the beginning of the study, positive and negative control blood samples were obtained, and stored at −80°C for quality-control testing of RDTs throughout the study. Each batch of RDTs underwent quality-control testing when opened and at 8- to 12-week intervals thereafter. The two positive control samples had densities 84/μL and 5000/μL, respectively. All

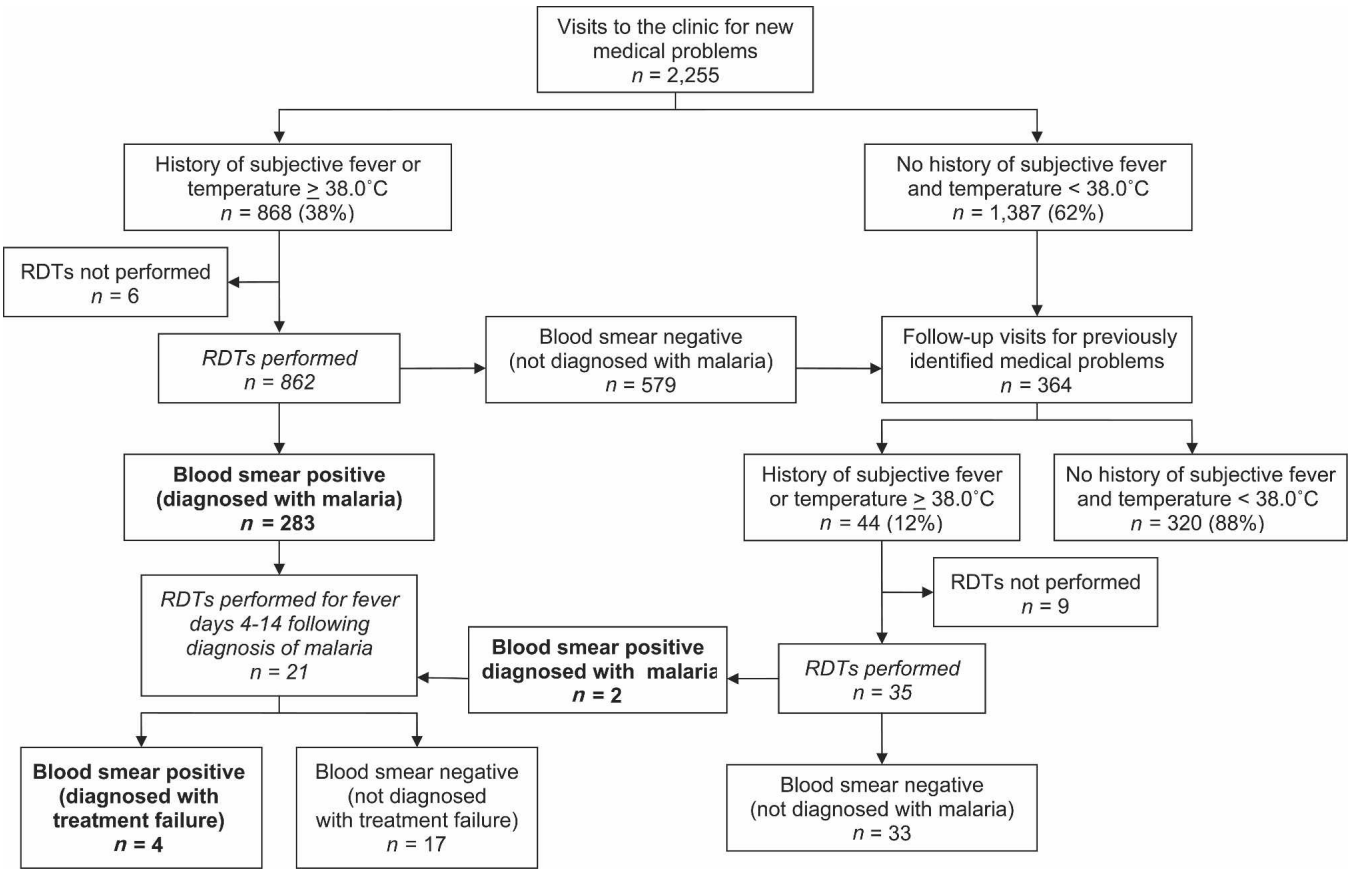

FIGURE 1. Trial profile showing clinic visits, blood smear results, rapid diagnostic tests (RDTs) performed (italics), and malaria episodes (bold). At the beginning of the RDT evaluation, 565 children were enrolled in the study cohort; 524 remained enrolled at the end of the evaluation.

HRP2 RDTs tested with quality-control samples were accurate; all pLDH RDTs tested were accurate for the negative and 5000/μL samples, but only 2 of 8 were accurate for the 84/μL sample.

RDTs were prepared and read by study physicians and then read by laboratory technicians. All readers were trained to perform the tests according to manufacturers' instructions. Study physicians interpreted and recorded RDT results after 15 minutes, at which time they were unaware of blood smear results. They were advised that if the background of the RDT test window remained pink (bloody) at the end of 15 minutes, they should allow the background to clear before reading the RDT. RDTs were then carried to the adjacent study laboratory, where they were re-read by laboratory technicians who were unaware of both the physician's interpretation and the patient's microscopy result. Readers recorded RDT results as either positive or negative; they were trained to consider faint test lines as positive.

Molecular methods. PCR was performed to identify parasite species in samples positive by microscopy but negative by RDT, as well as to detect subpatent infections in samples negative by microscopy but positive by RDT, and in a random sample of microscopy-negative and RDT-negative samples. DNA was extracted from filter paper samples using Chelex resin[17] and stored at −20°C until use. To detect *Plasmodium falciparum*, the block-3 region of merozoite surface protein-2 (msp-2) was amplified by nested PCR with primers corresponding to conserved sequences flanking this region[18] followed by primers to amplify the *IC3D7* and *FC27* allelic families, using conditions described previously.[19] In addition, to detect *P. falciparum, P. vivax, P. malariae*, and *P. ovale*, genus-specific followed by nested species-specific PCR of 18S small subunit ribosomal RNA[20] (ssu rRNA) for the four species (Malaria Research and Reference Reagent Resource Center, Manassas, VA) was performed, using oligonucleotide primers and conditions as described previously.[21] PCR products were analyzed by electrophoresis using 2% agarose gels.

Statistical methods. Data were entered using Epi-Info version 6.04 (Centers for Disease Control and Prevention, Atlanta, GA) and analyzed with Stata version 8.0 (Stata Corporation, College Station, TX). Sensitivity, specificity, and positive and negative predictive values were calculated by comparing the proportion of positive and negative results for each RDT with expert microscopy. Categorical variables were compared using χ^2 or Fisher's exact test. A P value of < 0.05 was considered statistically significant.

RESULTS

Overall RDT accuracy. We evaluated 918 episodes of fever over an 8-month interval in children from our cohort in Kampala (Figure 1). Over the 8-month period, 868 fevers were new fevers in a previously well child, 21 occurred 4–14 days after a diagnosis of malaria, and 44 occurred during follow-up of a non-malarial illness. RDTs were not performed in 15 episodes, in 9 at the discretion of the physician during follow-up of a non-malarial febrile illness, and in 6 because of protocol errors. Light microscopy identified positive malaria smears in 289 episodes (31%). Blood smear results served as the gold standard for comparison with RDT results. As RDT results are dependent on reader accuracy, we compared readings by two groups of clinic personnel: study physicians and laboratory technicians. In both cases, the sensitivity (> 92%) and negative predictive value (> 96%) were higher for the HRP2 assay, and specificity (> 99%) and positive predictive value (> 99%) were higher for the pLDH assay (Figure 2). First readers interpreted RDT results an average of 15 minutes after preparation, and second readers interpreted results an average of 7 minutes later. First and second test readings agreed in 98% of readings; they disagreed for 16 HRP2 tests and 13 pLDH tests. For 14/16 (88%) discordant HRP2 readings and 10/13 (77%) discordant pLDH readings, only second readings were positive.

Evaluation of false-negative results. Possible reasons for false-negative RDT results include low parasite density, non-falciparum parasite species, and interpreting the RDT before the test line has fully developed. HRP2 is produced only by *P. falciparum* parasites, while the pLDH assay evaluated here detects antigen from all human malaria parasites, although some reports suggest pLDH may be less sensitive for non-falciparum species.[22,23]

Of the 22 false-negative HRP2 results (based on first reading), 15 (68%) occurred in non-falciparum infections (Figure 3). Of the remaining 7 false negatives, 5 were interpreted as positive by the second reader. The 2 remaining false negatives occurred in a *P. falciparum* mono-infection with parasite density 48/μL, and a *P. falciparum* and *P. vivax* mixed infection with density 680/μL. Considering only *P. falciparum* infections, the sensitivity of the HRP2 assay at the second reading was 99% (272/274).

Of the 43 false-negative pLDH results, 12 (28%) occurred in non-falciparum infections; the remaining 31 were all *P. falciparum* mono-infections. Of these 31 false negatives, 9 were interpreted as positive by the second reader. For the remaining 22 false negatives, the geometric mean parasite density was 352/μL (range 16 to 26,080/μL). Considering only *P. falciparum* infections, the overall sensitivity of the pLDH assay at the second reading was 91% (250/274). The sensitivity for *P. falciparum* infections decreased from 98% (217/222) to 88% (28/32) to 25% (5/20) for parasite densities > 5000/μL, between 1000 and 5000/μL, and ≤ 1000/μL, respectively ($P < 0.0001$).

Evaluation of false-positive results. Possible reasons for false-positive RDT results include persistent antigenemia after antimalarial treatment, detection of gametocytes when asexual forms are not present, RDT detection of low-density microscopy-negative infections, or presence of antigenemia early in infection before parasites are detected by microscopy.

Of the 42 false-positive HRP2 results, 12 (29%) occurred within 14 days of a prior diagnosis of malaria, 26 (62%) within 28 days, and 32 (76%) within 42 days. In contrast, negative HRP2 results occurred as early as 7 days after initial diagnosis of a previous episode of malaria.

Gametocytes were detected by microscopy in only 12 of the 918 cases (1.3%). No HRP2 result was positive in a case where the smear showed gametocytes but not asexual parasites.

PCR was conducted to assess whether false-positive RDT results may have been associated with subpatent parasitemia. Of 40 evaluable false-positive HRP2 results, PCR was positive for *P. falciparum* in 8 (20%), compared with PCR positivity in 5/66 (8%) of control HRP2- and microscopy-negative samples ($P = 0.07$). Four of the 8 smear-negative, RDT- and

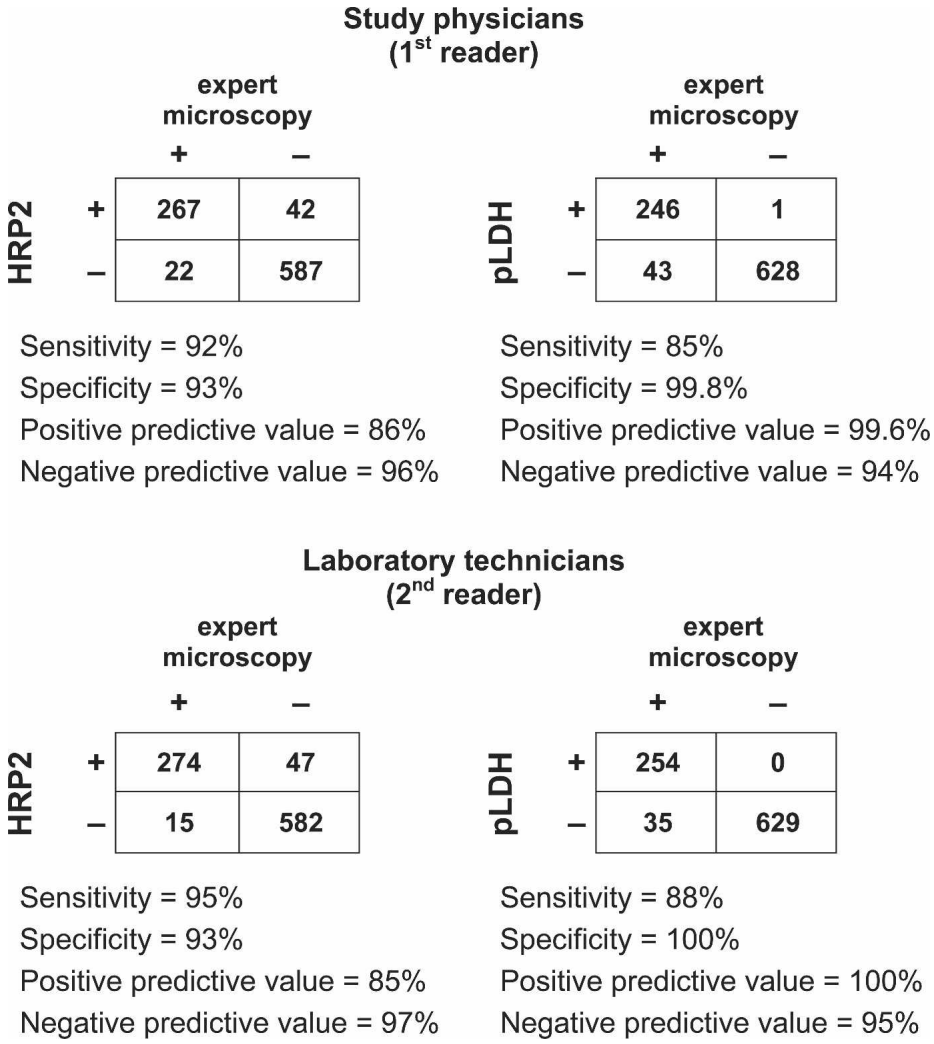

FIGURE 2. Point estimates of RDT accuracy. Blood smears were read by experienced microscopists in the study laboratory. All smears were read a second time by study laboratory staff to confirm results, and discrepant readings were resolved by a third reader. RDTs were read sequentially by study physicians and laboratory technicians, as described in Methods.

PCR-positive samples were obtained within 28 days of a prior episode of malaria.

Negative HRP2 results were recorded up to 3 days prior to an episode of malaria. Only one patient developed malaria within a week after a false-positive HRP2 result. The sample from the initial evaluation showed no asexual parasites or gametocytes but was positive for *P. falciparum* by PCR. The patient returned with persistent fever 5 days after initial evaluation, at which time the blood smear was positive, with parasite density 52,840/μL.

Only one pLDH test result was false-positive, in a patient who had no documented previous episode of malaria over 469 days of follow-up, and no malaria during the subsequent 2 months of study follow-up. No gametocytes were seen in the smear, the sample was negative by PCR for all four malaria species, and the second reading of the RDT was negative, strongly suggesting that this false positive was due to an error during the first test reading.

DISCUSSION

As compared with microscopy, both HRP2- and pLDH-based RDTs demonstrated acceptable sensitivity and specificity for the diagnosis of malaria in Kampala. The HRP2 assay showed superior sensitivity but inferior specificity compared with the pLDH assay. The longitudinal design of our study allowed us to clarify the relative importance of contributors to RDT false-negative and false-positive results. The difference in sensitivity between the tests was due mostly to better detection with HRP2 at low parasite densities. Non-falciparum infections contributed to false-negative results for both RDTs. In particular, in two-thirds of cases in which the HRP2 test was negative although microscopy detected parasites, the infection was caused by non-falciparum species. The higher specificity and positive predictive value of the pLDH assay was due to the fact that pLDH antigenemia closely mirrors parasitemia, while HRP2 commonly persists in the bloodstream weeks after successful treatment of malaria.[10,12] Subpatent parasitemia, as detected by PCR, pre-patent infections, and gametocytemia, did not appear to contribute importantly to false-positive results for either RDT. In summary, both studied RDTs accurately identified clinically relevant malaria infections but they differed importantly in sensitivity and specificity.

In Uganda, RDTs are increasingly available in the private

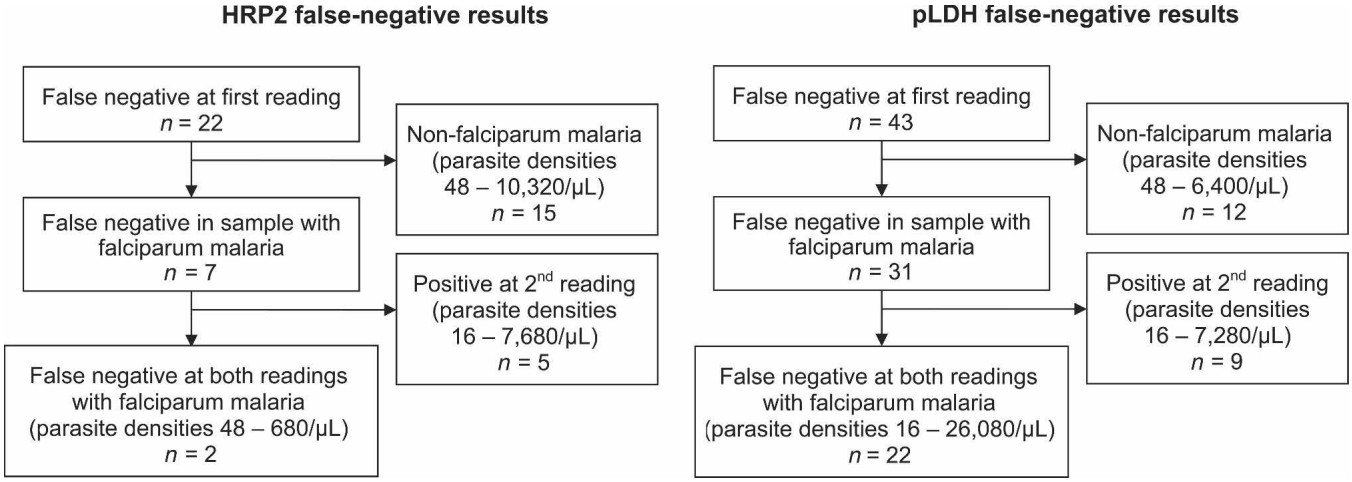

FIGURE 3. Factors associated with false-negative HRP2 and pLDH RDT results.

health care sector and are widely advocated for use in the public sector, though clear guidelines or algorithms for their use are lacking. In Kampala, both the HRP2 and pLDH tests showed a high negative predictive value and appeared to offer good reliability in ruling out malaria as the cause of a fever. Considering the potential values of RDTs, some limitations in both sensitivity and specificity may be acceptable. The lower specificity of the HRP2-based test, due to persistent antigenemia after recent infections, may lead to some inappropriate treatments, but many fewer than if all episodes of fever were treated as malaria. However, the lower specificity of HRP2 assays may be more problematic, with many more unnecessary malaria treatments, in regions with higher transmission intensity than Kampala. The lower sensitivity of the pLDH-based assay might also be a concern, but in Kampala, missed episodes were primarily of relatively low parasitemia, suggesting that, in immune populations, mostly mild or asymptomatic infections will be missed. Indeed, especially if technological innovations can improve the sensitivity of pLDH-based tests, they may well offer the optimal balance of sensitivity and specificity for the diagnosis of malaria in Africa.

To our knowledge, this study offers the first comparison of RDTs in a longitudinal format, allowing assessment of the importance of previous and future malaria infections in RDT accuracy. A number of other RDT evaluations have been conducted, though results have varied widely, likely due at least in part to different methodologies and locations. Two previous RDT studies in western Uganda compared HRP2-based tests with expert microscopy. One evaluation, using an older HRP2 assay, found a sensitivity of 99.6% for parasitemia > 500/μL and specificity of 92.7% in patients with fever.[24] The other study, using the same HRP2 test as in our evaluation, found a sensitivity of 97% and specificity of 88% for *P. falciparum* infections.[25] These estimates are similar to those for the HRP2-based test in our current evaluation. Our results also confirm the superior specificity of pLDH seen in a study in Tanzania,[26] although sensitivity of both tests was somewhat lower in our study.

Our results are not immediately applicable to fever case management across Africa. We obtained RDTs directly from manufacturers, and we used and stored kits as recommended by manufacturers; adherence to these guidelines may be challenging in rural settings, and test quality is likely to deteriorate if kits are less well maintained.[27] Our evaluation was performed in an area with relatively low malaria transmission. Because of the location and design of our study, our patients likely presented to the clinic earlier in the course of malaria than in non-research settings. Our staff was carefully trained in use of the two RDTs before initiation of our study; test accuracy may be lower in field settings, although a number of reports indicate that village health workers with minimal training are able to satisfactorily prepare and interpret RDTs.[28,29] Considering these limitations, how should the results of this evaluation influence malaria treatment policy? For Kampala, our results suggest that, in settings without access to microscopy, use of either HRP2- or pLDH-based RDTs could dramatically lower the use of inappropriate antimalarial therapy without missing many episodes of clinical malaria. However, it will be necessary to perform similar analyses in areas with different epidemiology to determine the predictive values of different RDTs in various settings. In addition, the issue of cost and cost-effectiveness of RDTs, compared with presumptive treatment and with diagnosis with microscopy, must be considered. In the era of artemisinin combination therapies, using RDTs to target treatment to confirmed cases of malaria may help to maximize the impact of these valuable resources.

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Review

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 (*Pf*HRP2), 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 *Pf*HRP2, 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, 2011b; 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

Abbreviations: DHFR, dihydrofolate reductase; FIND, Foundation for Innovated New Diagnostics; HDP, haem-detoxification protein; HRP2, histidine-rich protein 2; Hsp, heat-shock protein; LDH, lactate dehydrogenase; *Pf*HRP2, *Plasmodium falciparum* histidine-rich protein 2; pLDH, plasmodial lactate dehydrogenase; RDT, rapid diagnostic test; TS, thymidylate synthase; WHO, World Health Organization.

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 (*Pf*HRP2) 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

*Pf*HRP2

Three *Pf*HRPs, *Pf*HRP1, *Pf*HRP2 and *Pf*HRP3, have been identified (Howard *et al.*, 1986; Rock *et al.*, 1987; Parra *et al.*, 1991). *Pf*HRP1 is expressed in all knob-positive *P. falciparum* species and is associated with the cytoskeleton of infected red blood cells. *Pf*HRP2 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 *Pf*HRP2, which is a good marker for *P. falciparum* infection (WHO, 2003).

The water-soluble, heat-stable, 30 kDa *Pf*HRP2 protein is synthesized only by *P. falciparum* parasites (Stahl *et al.*, 1985; Howard *et al.*, 1986; Wellems & 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 HRP2 has been characterized (Scherf & Mattei, 1992), as well as a laboratory-induced mutant (Wellems *et al.*, 1988). In addition, some isolates from Africa and South America have been found lacking the HRP2 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. *Pf*HRP2 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 *Pf*HRP2 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). *Pf*HRP2 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). *Pf*HRP2 contains 35 % histidine, 40 % alanine and 12 % aspartate, but the percentages vary depending on the isolate expressing *Pf*HRP2 (Wellems & Howard, 1986). *Pf*HRP2 has multiple repeats of AHHAAD, with AHH and AHHAA 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 (Wellems & Howard, 1986; Panton *et al.*, 1989). Commercial assays that detect *Pf*HRP2 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). *Pf*HRP2-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; Kyabayinze *et al.*, 2011; Aguilar *et al.*, 2012). The *Pf*HRP2 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 *Pf*HRP2-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 *Pf*HRP2 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 *Pf*HRP2 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 *Pf*HRP2 as a good target for malaria detection in pregnant women compared with RDTs detecting *P. falciparum* LDH (Kattenberg *et al.*, 2012b).

Problems associated with *Pf*HRP2-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 *Pf*HRP2 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 *Pf*HRP2 sequence difference within and between 75 isolates of *P. falciparum* from 19 countries. The effect of *Pf*HRP2 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 *Pf*HRP2 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 *Pf*HRP2 antigen for detection by *Pf*HRP2-based RDTs was confirmed as the cause of diagnostic failure in a French visitor who returned to 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 *Pf*HRP2 can cause patient bloodstream infections and false-negative results, as reported in Mali by Koita *et al.* (2012). The presence of polymorphisms in the *Pf*HRP2 antigen (Mariette *et al.*, 2008), or the absence of the gene and thus the targeted antigen sequence, means that alternative RDTs other than a *Pf*HRP2-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 *Pf*HRP2-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). *Pf*HRP2-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 *Pf*HRP2 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 *Pf*HRP2 in the blood for long periods after parasite clearance, as determined by microscopy (Mharakurwa & Shiff, 1997; Mayxay *et al.*, 2001; Huong *et al.*, 2002; Iqbal *et al.*, 2002, 2004; Moody, 2002; Grobusch *et al.*, 2003; Mueller *et al.*, 2007; Houzé *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 *Pf*HRP2 persistence and malaria parasite density. Moreover, antigen persistence was found to be likely to be influenced by gametocytaemia (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 *Pf*HRP2 for up to 28 days after artemisinin-based combination therapy (Kattenberg *et al.*, 2012b). Using Paracheck-Pf, an RDT that specifically detects *Pf*HRP2, 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 *Pf*HRP2 after malaria treatment has been reported in many situations, suggesting that *Pf*HRP2 is unsuitable as a target for drug-susceptibility testing. Thus, a positive result with a *Pf*HRP2-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 *Pf*HRP2 and aldolase bands in blood with $\geqslant 40\,000$ 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 *Pf*HRP2-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 *Pf*HRP2 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 *Pf*HRP2 concentrations are determined in an ELISA format, there appears to be a relationship between patient plasma *Pf*HRP2 concentrations and severity of infection in moderate to high areas of malaria transmission (Hendriksen *et al.*, 2013). Plasma *Pf*HRP2 levels appear to predict disease progression to severe malaria, and thus there is a need for the development of a quantitative *Pf*HRP2 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 *Pf*HRP2 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 *Pf*HRP2-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 *Pf*HRP2 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 *Pf*HRP2 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 *Pf*HRP2 tests.

The *Pf*HRP2-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 *Pf*HRP2-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 *Pf*HRP2 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 erythrocytic 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; Heutmekers *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 *Pf*HRP2 detection or four-line tests that combine *Pf*HRP2, 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 *Pf*HRP2 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 *Pf*HRP2, 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 *Pf*HRP2 (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 *Pf*HRP2, 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/*Pf*HRP2 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 (Bioland) 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 Hellemond *et al.*, 2009). The OptiMAL-IT test detected *P. knowlesi* parasites in the blood of an individual returning to the Netherlands from Borneo (van Hellemond *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 (Hurdayal *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 *Pf*HRP2, 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 (Druilhe *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 *Pf*HRP2 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 *Pf*HRP2-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, mono- or 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 *Pf*HRP2- and pLDH-based tests. Aldolase in conjunction with *Pf*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 (*Pf*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 *Pf*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 Orania 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 *Pf*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/*Pf*HRP2 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 *Pf*HRP2 and aldolase, has an overall sensitivity of 100 % for *P. falciparum* and 83 % for *P. vivax* using finger-stick samples (Murray *et al.*, 2008). The CareStart RDT has been used for comparison of a new VIKIA Malaria Ag Pf/Pan test detecting *Pf*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 Hellemond *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) 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; Thézénas *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 (Morimoto *et al.*,

Table 1. Characteristics of current malaria RDT tests

Characteristic	HRP2	pLDH	Aldolase
Species detected	*P. falciparum* only	*P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*	*P. falciparum*, *P. vivax*
Persistence after parasite clearance (days)	>28	<10	<10
Genetic variation	Yes	None to date	None to date*
Repeat epitopes	Yes	None	None
Sensitivity for *P. falciparum* (%)	95†	93.2†	48–80‡
Sensitivity for *P. vivax* 2000 parasites μl^{-1} (%)	Not present	78.9–98.8‡	15–83‡
Specificity (%)	95.2§	98.5§	||
Monitor parasite clearance	No	Yes	Potential
Monitor drug efficacy	No	Yes	Potential
Prozone effect	Yes	No	No
Predict progression to severe malaria#	Potential	Not tested	Not tested
Diagnose severe malaria#	Potential	Not tested	Not tested

*Kim *et al.* (2012) found a point mutation in Korean isolates of *P. vivax*.
†Abba *et al.* (2011).
‡Summary of data in the current review.
§Specificity pLDH > HRP2 (Abba *et al.*, 2011).
||Insufficient data.
#ELISA-based data at present.

1994). 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-*Pf*Hsp70 IgM and anti-*Pf*Hsp90 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 *Pf*Hsp70 have been found to be higher than those of *Pf*HRP2 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 *Pf*Hsp70 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 *Pf*Hsp70 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 *Pf*HDP 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 bi-functional 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 *Pf*HRP2 (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. *Pf*HRP2- and pLDH-based RDTs are the most commonly used. *Pf*HRP2 RDTs appear to be more sensitive than pLDH RDTs, particularly at low parasite densities, although there are exceptions (WHO, 2011b). To date, both *Pf*HRP2 and pLDH RDTs are more sensitive than aldolase-based tests. *Pf*HRP2-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 *Pf*HRP2 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 *Pf*HRP2 antigen, *Pf*HRP2 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 *Pf*HRP2 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 *Pf*HRP2 RDT can have a prozone effect, which does not occur with pLDH or aldolase RDTs. The *Pf*HRP2 protein has been used to detect malaria infections in samples from Predynastic Egyptian mummies (Cerutti *et al.*, 1999). The concentration of *Pf*HRP2 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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Brief Communication

Malaria Rapid Diagnostic Test Product Lots Quality Evaluation in Ethiopia

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Abstract

As malaria rapidly lead to death, quick and accurate diagnose is important to manage patients. Malaria Rapid Diagnostic Test's (RDTs) has offered extension of diagnosis to remote and poorly resourced areas. However, the qualities vary between different products and batches/lots. Therefore, all production lots must be checked, either pre or post marketing to check lot-lot quality variations and to guarantee end users that RDT result saves lives by guiding the correct treatment. This laboratory evaluation was done in 72 product lots of *Plasmodium falciparum (Pf)*-only RDT lots tested against positive Pf, *Plasmodium vivax (Pv)* and negative sample panels. The results indicated that 69(95%) lot RDTs detected the parasite antigen to an acceptable threshold level whereas 3 lots (4.8%) showed inadequate sensitivity of the laboratory evaluation. Most product lots passed this laboratory evaluation while some lots showed inadequate sensitivity. Laboratory evaluation of each lot is important to identify and distribute good quality lot RDTs for malaria programme and to ensure the adequate performance of the test.

Keywords: Lot-testing; Malaria, RDTs

Introduction

Malaria is the most important public health problem in Ethiopia hence; the national malaria programme set its goal for control and elimination until 2020 through key strategies such as vector control, case management and environmental management. Therefore, malaria control or elimination strategies require effective patient management, quick and accurate diagnosis [1,2]. In Ethiopia, *Pf* accounts for 60% and *Pv* for 40 % of malaria cases. Malaria Rapid Diagnostic Tests (RDTs) is a test device that detects parasite antigen in the blood with >100p/µl and used for rapid diagnosis and patient management, avoid misdiagnosis of patients who became febrile due to other illness and treat target patients who really have malaria [3,4].

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However, the qualities of manufacturing RDTs greatly vary between different products and batches/lots "(each lot is usually identified by a number by the manufacturer and usually consists of 40,000-80,000 tests [2,5])". For this reason the quality of manufacturers RDT product will be evaluated every two years through product testing programme and WHO releases the result for countries procurement guide. But, since the quality of RDTs between lots of the same product varies due to different manufacturing practice, WHO recommends that all production lots be checked, either pre or post marketing through lot-testing programme [2]. Therefore, this laboratory evaluation of different RDT product lots carried out to assess the quality before or after purchase because lots of most products vary; to convince clinicians, users and regulatory authorities that the tests work and to ensure no damage has occurred during transport to a country (post-purchase testing) [5].

Materials and Methods

The laboratory evaluation was done in 72 lots of different RDTs (ICT malaria cassette test, Carestart combo, Paracheck Pf, Parascreen pan/Pf and First response Pf/pan). Malaria RDT's detects a parasite antigen of positive bloods with 100p/µl or more parasite density. However, with this laboratory evaluation 200p/µl of malaria positive blood used as a minimum threshold value and all tests needs to be positive to pass the evaluation [3]. Each lot- RDTs were tested with positive samples prepared at parasite density of minimum and maximum threshold (200 and 2000 parasite/µl) of *Pf* samples, 200, 500, 2000 parasite/µl of *Pv* samples and 10 malaria negative panels using WHO protocol [2,6]. The blood samples (10ml) collected from *Pf/Pv* malaria positive patients (consented) and the samples prepared to different parasite density (200,500 and 2000p/µl) by counting the parasite against white cell using microscopy and diluted with negative blood (prepared from Ab plasma and O^+ cell). The sample aliquots used for this evaluation was characterized using Polymerase Chain Reaction (PCR) tests for speciation and ELISA test for antigen quantification. The laboratory lot testing was carried out using good samples identified based on the characterization results.

Based on WHO protocol [2], *P. falciparum*-only RDTs were tested against four different quality control panels and 10 different negative quality control samples. For each of the four quality control *Pf* samples six RDTs were tested at an aliquot of 200 parasites per microliter and one RDT was tested at an aliquot of 2000 parasites per microliter. One RDT was tested with each of the 10 negative quality control samples. *P. falciparum* and pan/combination RDTs were tested against four different *P. falciparum* quality control panels, four *P. vivax* samples and 10 negative quality control samples. For each of the four *P. falciparum* quality control samples, six RDTs were tested at an aliquot of 200 parasites per microliter and one was tested at an aliquot of 2000 parasites per microlitre. For each of the four *P. vivax* quality control samples, six RDTs were tested at an aliquot of 200 parasites per microlitre, and one was tested at an aliquot of 2000 parasites per microlitre. For RDTs failed to detect *P. vivax* at 200 parasites per microlitre; it was re-tested with a *P. vivax* sample diluted at 500 parasites per microliter. One RDT was performed for each of the 10 negative quality control samples.

This study conducted after getting the ethical approval from Ethiopian Public Health Institute (EPHI) Scientific and Ethical Review Committee (SERC).

Results and Discussion

The results revealed that 69(95%) lot RDTs; tested with positive samples of different parasite density (200p/µl and 2000p/µl *P. falciparum* panels, 200/500p/µl and 2000p/µl *P. vivax* panels) and 10 negative samples; detect parasite antigen to an acceptable threshold level whereas 3 lots (4.8%) nearly equivalent to 120,000-240,000 tests failed to detect the parasite antigen to an acceptable threshold (Figure 1). Of all the product lot RDTs evaluated, all lots of CareStart Pf/Pan RDTs showed 100% accuracy and high test band intensity at both high and low parasite density. The lot evaluation results before and after distribution had no variation although the intensity of the reaction was become low with time.

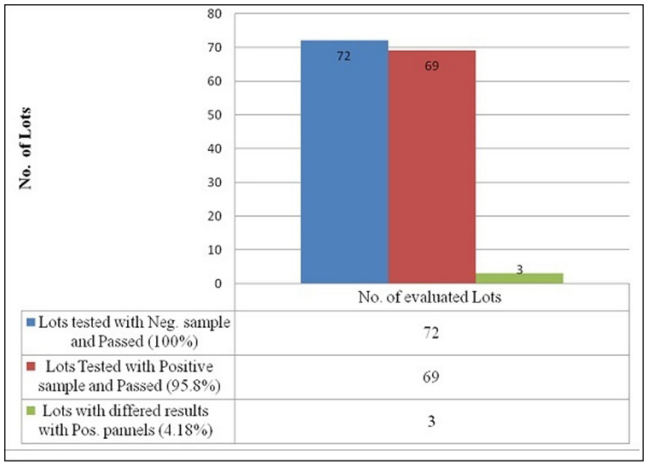

Figure 1: Lot Testing Results of Different Lots Tested with Negative and Positive Analysis.

Conclusion

The result revealed that most lots passed the laboratory evaluation. Therefore, it is important to ensure continued adequate performance of malaria RDTs lot on delivery and throughout the expected shelf life before and after introducing for national malaria programme. Countries must conduct RDT product lot evaluation to ensure its quality and only confirmed good quality lots should be distributed for the program. This will improve accurate diagnosis and proper treatment of malaria cases.

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Competing Interests

The authors declare that there is no any competing interest.

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STUDY OF PLATELET COUNT IN MALARIA PATIENTS AND THE CORRELATION BETWEEN THE PRESENCE AND SEVERITY OF PLATELET COUNT WITH TYPE OF MALARIA.

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ABSTRACT: BACKGROUND: Malaria remains one of the major health problems in the tropics with increased morbidity & mortality. Thrombocytopenia is a common finding in malaria, but its correlation with the type of malaria and prognostic implications in context with severity of low platelet count has not been evaluated in large studies. In view of paucity of data from Indian studies, we attempt to correlate the low platelet count with type of malaria and outcome. **AIM:** Study of platelet count in malaria patients and correlation between the presence and severity of platelet count with type of malaria. **MATERIAL & METHODS:** A total of 180 patients diagnosed to have Malaria over a period of two years admitted in Guntur Teaching and General Hospital attached to Guntur Medical College, Guntur were studied. All study subjects were identified positive for Malaria parasite on peripheral smear examination with conventional microscopy. Platelet count was done on a fully automated, quantitative analyzer. Daily platelet count was done for all those admitted with malaria. P.falciparum antigen test (PfHrp antigen test- Parascreen) was performed in subjects with P.vivax Malaria on the peripheral smear with a platelet count less than 20,000cells/cmm for more emphatic exclusion of associated P.falciparum infestation. P.falciparum antigen test was also performed in subjects with high index of clinical suspicion or multi organ involvement. **RESULTS:** a total of 180 patients were found to have malaria, 114(63.3%) were P.vivax, 62(34.4%) were P.falciparum and 4(2.7%) were mixed. 146(81.1%) patients had thrombocytopenia. 34(23.3%) developed complicated malaria. Severe thrombocytopenia was noted in 58.8% of complicated malaria with p<0.001. 20 patients persisted to have thrombocytopenia on 6th day even after adequate therapy. 14(70%) patients out of 20 recovered and 6(30%) died in which 2 was P.falciparum and 4 were mixed infection. **CONCLUSION:** Thrombocytopenia is a common association of malaria with incidence of 81.1%. Severe thrombocytopenia is commonly seen in P.falciparum. Platelet count <20,000 was seen in P. falciparum and P.vivax. But more commonly in P. falciparum. Out of 36 severe thrombocytopenia 34 developed complicated malaria with significant p value indicating that patients with severe thrombocytopenia at the time of admission are 8.5 times more prone to develop complications when compared to mild and moderate thrombocytopenia. Patients who persisted to have thrombocytopenia even after 6th day of therapy, their mortality increased by 30%.

KEYWORDS: Malaria; Thrombocytopenia; Complications.

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INTRODUCTION: Malaria is probably one of the oldest diseases known to mankind that has had profound impact on our history. For centuries it prevented any economic development in vast regions of the earth. It continues to be a huge social, economical and health problem, particularly in the tropical countries. History of malaria and its terrible effects is as ancient as the history of civilization, therefore history of mankind itself. The term malaria (From the Italian mala "bad" and aria "air") was used by the Italians to describe the cause of intermittent fevers associated with exposure to marsh air or miasma. The word was introduced to English by Horace Walpole, who wrote in 1740 about a "horrid thing called malaria that comes to Rome every summer and kills one." The term malaria, without the apostrophe, evolved into the name of the disease only in the 20th century. Up to that point the various intermittent fevers had been called jungle fever, marsh fever, paludal fever, or swamp fever. Malaria affects more than 2400 million people, over 40% of the world's population, in more than 100 countries in the tropics from South America to the Indian peninsula. The tropics provide ideal breeding and living conditions for the anopheles mosquito, and hence this distribution.[1] Every year 300 million to 500 million people suffer from this disease. WHO forecasts a 16% growth in malaria cases annually.[2]About 1.5 million to 3 million people die of malaria every year (85% of these occur in Africa), accounting for about 4-5% of all fatalities in the world. One child dies of malaria somewhere in Africa every 20 sec., and there is one malarial death every 12 sec somewhere in the world. It accounts for 2.6 percent of the total disease burden of the world.

AIMS AND OBJECTIVES:

1. To study the incidence of thrombocytopenia in Malaria.
2. To study the incidence of thrombocytopenia in relation to type of malaria.
3. To study and correlate the severity of thrombocytopenia in vivax and falciparum malaria.
4. To determine whether the initial platelet count is an independent prognostic marker for severity of malaria.

Study Design: Prospective study.

Study Period: From Jan. 2014 to May 2015.

Study Population: A total of 180 patients diagnosed to have Malaria admitted in Government general hospital attached to Guntur Medical College, Guntur included in the study.

MATERIAL AND METHODS: A total of 180 patients diagnosed to have Malaria admitted in Government general hospital attached to Guntur Medical College, Guntur included in the study. All study subjects were identified positive for Malaria parasite on peripheral smear examination with conventional microscopy. Platelet count was done on a fully automated, quantitative analyzer. Platelet count was the number of thrombocytes derived from directly measured platelet pulses, multiplied by a calibration constant and expressed in thousands of thrombocytes per microliter of whole blood. Repeat platelet count was done in subjects with marked thrombocytopenia until normal or near normal values was reached. Patients with thrombocytopenia were divided into 3 catgories[3] mild-0.5-1.5 lakhs cells/cu mm, moderate-0.2-0.5 lakhs cells/cumm and severe <0.2 lakhs cells/cu mm. P.falciparum antigen test (PfHrp antigen test-Parascreen) was performed in all subjects with malaria parasite positive on peripheral smear. P.vivax Malaria on the peripheral smear with a platelet count less than 20,000cells/cmm for more emphatic exclusion of associated P.falciparum infestation. P.falciparum antigen test was also performed in subjects with high index of clinical suspicion or multi organ involvement. Other investigation includes CBC, LFT, RFT, Chest X- Ray, Ultrasound Abdomen, if necessary Blood Culture, Urine Culture, Dengue serology.

P.falciparum was treated with either chloroquine or artesunate depending upon the clinical severity. P.vivax malaria was treated with chloroquine followed by two week course of primaquine.

Data was expressed on a excel spreadsheet and statistical analysis was performed. P values less than 0.005 were considered significant.

Inclusion Criteria:

- All patients above 14 years of age and either sex whose blood smear was positive for malaria are included in the study.
- Platelet count done before starting treatment.

Exclusion Criteria:

- Clinical diagnosis of malaria without positive blood smears.
- Platelet count done after starting treatment.
- Patients who received partial treatment outside the B.T.G.H and referred later to this hospital.
- Patients with known HIV positive.
- Patients with known case of chronic renal and liver disease.
- Congenital & Hereditary Thrombocytopenia Immune induced thrombocytopenia Drug induced thrombocytopenia.

Statistical Analysis: Chi square test or Fisher Exact test and student 'T' test has been used to find the significant association of study characteristics (Thrombocytopenia) with type of malaria.

RESULTS: A total of 180 subjects who diagnosed to have Malaria over a period of one and half years were studied. The mean age of patients was 40.13±14.10 years. The study included 75.6% males and 24.4% females. Typical paroxysms were observed in 40 patients of P.Vivax and 10 patients of P.Falciparum. Under atypical manifestations like vomiting was seen in 26 patients of P.Falciparum and 10 patients in P.Vivax 4 in mixed infection, headache in 30 patients of P.Falciparum and 16 in P.Vivax, jaundice in 20 patients of P. falciparum,4 P.vivax and 4 mixed infection. altered sensorium in 16 patients of P.Falciparum and none in P.Vivax, pain abdomen in 12 patients of P.Falciparum and none in P.Vivax, cough and breathlessness in 16 patients of P.Falciparum and none in P.Vivax, joint pain in 6 patients in P.Falciparum 4 in P.Vivax. Commonest atypical symptom being headache and vomiting. Common clinical sign in decreasing order are splenomegaly (86%), pallor (46.6%), Icterus (13.3%), hepatomegaly (10%), altered sensorium (8%), petechiea (6%). A total of 180 subjects who had malaria, 114 were P.vivax and 62 were P.falciparum. Incidence of P.vivax 63.3% and P.falciparum 34.4%. Incidence of Thrombocytopenia was 146(81.1%), with mild Thrombocytopenia 54(30%), moderate Thrombocytopenia 56(31.1%) and 36(20%) with severe Thrombocytopenia. Normal platelet count was observed in 34(18.9%) of patients.

Mean platelet count overall was 0.95±0.80 lakhs. Mean platelet count in mild thrombocytopenia was 1.23±0.11 lakhs. Mean platelelet count in moderate thrombocytopenia was 0.37±0.07 and Mean platelet count in severe thrombocytopenia was 0.16±0.04 lakhs, indicating that thrombocytopenia is a common association in malaria. 146 out of 180 who had thrombocytopenia were taken up, to study its prognostic implication. Mild Thrombocytopenia was observed in 36(42.8%) cases of P.Vivax and in 18(31%) cases of P.Falciparum. Moderate Thrombocytopenia was noted in 32(38%) cases of P.Vivax and in 24(41.4%) cases of P.Falciparum. Severe Thrombocytopenia was noted in 16(19.1%) cases of P.Vivax and in 16(27.5%) cases of P.Falciparum and all 4 cases (100%) of mixed malaria. Mean platelet count in Falciparum species overall was 0.62±0.56 lakhs with range from 0.07-2 lakhs when compared to 1.16±0.85 lakhs with range from 0.1-3 lakhs in Vivax species.

Mean plaelet count in mild thrombocytopenia in Falciparum was 1.12±0.14 lakhs when compared to 1.24±0.11 lakhs in vivax. Mean platelet count in moderate thrombocytopenia in Falciparum was 0.36±0.07 lakhs when compared to 0.39±0.06 lakhs in vivax. Mean platelet count in severe thrombocytopenia in Falciparum was 0.14±0.05 lakhs when compared to 0.17±0.04 lakhs in vivax.

According to the revised WHO guidelines of 2000 patients who had Thrombocytopenia were grouped into complicated and uncomplicated. In our study 34 cases had complicated malaria and 112 cases had uncomplicated malaria. In complicated malaria 24 patients had Hemoglobin <5gm% in which 20(83.3%) were P.Falciparum and 4(16.7%) were P.Vivax, 18 patients had s.creatinine >3mg% in which 8(44.4%) were P.Falciparum and 6(33.3%) were P.Vivax, 24 patients had T.Bilirubin >3mg% in which 20(83.3%) were P.Falciparum and 4(16.7%) were mixed, 22 patients had metabolic acidosis (ph<7.2), 16(72.7%) were P.Falciparum and 2(9.1%) were P.Vivax, and 4 mixed(18.2%). 12 patients had spontaneous bleeding with DIC in which 10(83.3%) were P.Falciparum and 2(16.7%) in mixed, 6 patients had coma for >30min, in which 2(33.3%) were P.Falciparum and 4(66.7%) were mixed, 8 patients had hyperparasitemia in which 4(50%) in P.Falciparum and 4(50%) in mixed, 2 patients had hypoglycemia which was mixed infection, 24 patients had prostration in which 16(66.7%) were P.Falciparum,4(16.7%) P.Vivax and 4(16.7%) mixed, 8 patients had ARDS in which 4(50%) were P.Falciparum and 4(50%) were mixed, 12 patients developed shock in which 4(33.3%) were P.Falciparum, 4(33.3%) were P.Vivax and 4(33.3%) were mixed. Complications were commonly seen in P.falciparum and mixed compared to P.vivax of which anemia and hyperbilirubinemia being the most common. Relationship of degree of thrombocytopenia to severity of malaria.

Out of 112 uncomplicated malaria, mild thrombocytopenia was noted in 48(42.9%), moderate thrombocytopenia in 48(42.9%), and severe thrombocytopenia in 16(14.3%). Out of 34 cases of complicated malaria mild thrombocytopenia was noted in 6(17.6%), moderate thrombocytopenia in 8(23.5%) and severe thrombocytopenia in 20(58.8%). P value <0.005, was noted in severe thrombocytopenia. Daily platelet count was done for all patients from the day of admission to day of discharge, and underwent specific treatment. On an average 6th day was considered as last day. On day one 146 patients had low platelet count, on day two 122 patients had low platelet count, on day three 116 patients had low platelet count, on day four 90 patients had low platelet counts, on day five 56 patients had low platelet counts, on day six 20 patients persisted to have low platelet count despite of adequate therapy.

Out of 146 cases 6 died with the overall mortality of 4.1%.20 patients who persisted to have thrombocytopenia at 6th day 14 recovered and 6 died in which 2 was P.falciparum and 4 were mixed infection with an increase in mortality rate of 30%.

DISCUSSION: A total of 180 malaria cases were studied. The mean age of the patients was 40.13± 14.10. This study is comparable with other studies like Gayatri K et al in their study, they found that mean age was 42.13±16.12.[4] Another study conducted by by Jadhav UM et al, in their study, they found that mean age was 44.17±13.13.[5] Older age groups are more susceptible to infection due to lack of immunity.

This study includes 136 male and 44 female patients. In the present study males are more commonly involved due to the fact that most of the patients had recent history of travel to endemic areas. This findings are comparable to other studies conducted by Gayatri K et al, Trampuz A et al,[6] and Patel U et al,[7] who found that males were more commonly involved than females since most of the male patients had a recent history of travel to endemic areas. The commonest clinical manifestation was fever with chills and rigors (100%), headache (25.5%)'vomiting (22.2%) jaundice (15.5%).[8] Commonest sign being splenomegaly (86.7%) followed by pallor (46.7%) and icterus (13.3%)· These findings are comparable with other studies like Oh MD et al, Song HH et al Giha HA et al. Grobusch MP et al Trampuz A et al who found that fever with chills was the most common symptom found followed by headache, vomiting and jaundice. They also found that spleenomegaly was the most common sign followed by pallor and icterus. A clinical spectrum of fever, splenomegaly and pallor is most often associated with malaria.

In the present study 146 subjects out of 180 malaria cases had thrombocytopenia. Incidence of thrombocytopenia being 81.1%. Thrombocytopenia is a common feature of acute malaria and occurs in both P.falciparum and P.vivax infection regardless of severity of infection. Thrombocytopenia in a patient with febrile illness increases the possibility of malarial infection.[9] Figures of the present study are comparale to studies done by other investigators eg, Jain M et al.[10] in their study, they found that thrombocytopenia was present in 70% of the patients. Another study conducted by Jadhav UM et al, in their study, they found that thrombocytopenia was found in 79.4% of the patients. Another study conducted by Sharma K. et al, in their study, they found that thrombocytopenia was present in as high as 90% of the patients. Sheraz jamal khan et al,[11] in their study, they found that significant thrombocytopenia was seen in more than half of the patients. Another study conducted by Lathia et al.[12] in that study they found that thrombocytopenia alone was a predictor for malaria and thrombocytopenia in a patient with acute febrile illness increases the probability of malarial infection.

Out of 180 cases 114 had P.vivax malaria, 62 patients had P.falciparum, and 4 had mixed infection. Incidence of P.vivax malaria is 63.3% and P.falciparum 34.4%.[13,14] Prevalence of P.vivax malaria is common in India, because of variation in climatic condition, breeding places of mosquito and genetic resistance of P.falciparum., These figures are comparable to studies done by other investigators as Sheraz jamal khan et al, in their study, they observed that thrombocytopenia was seen in malaria patients but more common in vivax type contrary to the belief that thrombocytopenia is very common in falciparum.[15] Another study conducted by Jain M et al, in that study they found that thrombocytopenia[70%] was common in malaria patients in which 67.3% were falciparum which was more common than the vivax type. Another study conducted by Jadhav

UM et al,in their study, they found that thrombocytopenia was common in malaria patients in which falciparum was more common than vivax type. Another study conducted by Horstmann et al, [16]in their study, they found thrombocytopenia in 85% of falciparum and 72% of vivax malaria.

In this study, mild and moderate thrombocytopenia was statistically insignificant when compared to severe thrombocytopenia. We noticed that severe thrombocytopenia was commonly associated with P.falciparum (27.5%) as compared to P.vivax (19%).These figures are comparable to the studies done by other investigators as Abdul Rauf Memom et al,in their study, they found that mild to severe thrombocytopenia was observed in hospitalized malarial patients, in which falciparum was found to be the most common. Another study conducted by Jadhav UM et al, in their study, they found that absence of thrombocytopenia is uncommon in malaria, its presence is not a distinguishing feature between the two types. Severe thrombocytopenia can occur in both but more commonly in falciparum malaria. Another study conducted by Kaur D et al,[17] in their study, they found that severe thrombocytopenia was seen in vivax malaria.

The mechanism of thrombocytopenia is uncertain. Immune mediated lysis, sequestration in the spleen and a dyspoietic process in the marrow with diminished platelet production have all been postulated. Abnormalities in platelets structure and function have been described as consequences of malaria, and in rare instances platelets can be invaded by malarial parasite themselves.[18]

Thrombopoietin (TPO) is the key factor for platelet production and is elevated in state of platelet depletion. TPO serum levels have been shown to be significantly higher in subjects with severe malaria, normalizing within 14 - 21 days of therapy. Two types of changes in platelet dysfunction are seen in malaria. Initially there is platelet hyperactivity; this is followed by platelet hypoactivity. Platelet hypoactivity results from various aggregating agents like immune complex, surface contact of platelet membrane to malarial red cells damage to the endothelial cells. The injured platelets undergo lysis intravascularly. The release of platelet contents can activate the coagulation cascade and contribute to DIC. Transient hypoactivity is seen following this phase and returns to normal in 1-2 weeks. In many studies undertaken, the significance of haemostatic abnormalities as a consequences of malaria has been difficult to assess as a result of the presence of various associated complications such as liver dysfunction, uremia.[19,20]

In this study, we found that DIC was the commonest cause for severe thrombocytopenia in 16 cases of P.falciparum. 14 recovered with adequate medical therapy within 7 to 10 days, 2 patients died of severe metabolic acidosis and multi organ dysfunction. 16 cases of P.vivax malaria had severe thrombocytopenia. All the 16 cases recovered within a week. These findings are comparable to the study done by other investigators as Krishnan et al,[21] in their study, they found that about 19 patients had thrombocytopenia and DIC, noticed that malaria is an important cause of multi organ failure in India and mortality rate was less when one or few organs were involved as compared to two or more organs involved. Another study conducted by Koulmann P et al,[22] in their study, they found that failure of one or more organ systems and development of several metabolic disorders secondary to the presence of falciparum malaria.

146 patients who had thrombocytopenia were categorized into complicated and uncomplicated malaria based on WHO guidelines. Among 146 cases 34(23.3%) had complicated malaria and 112(76.7%) had uncomplicated malaria. Among 34 cases of complicated malaria, 22 were P.falciparum, 8 were P.vivax and 4 had mixed infection. Complicated malaria is common in P.falciparum infection[23,24] These findings are comparable to the study conducted by other investigators such as Dharmesh Kumar N Patel et al,[25]in their study, they found that complicated malaria was more commonly caused by falciparum malaria and was rarely caused by other malarial parasites.

The mechanism for complicated malaria is complex. In P. falciparum infection, membrane protuberance appears on the erythrocyte surface towards the end of the first 24hrs of asexual cycle. These "knobs" extend high molecular weight antigenically variant, strain specific, adhesive protein (PfEMP1) that mediate attachement to receptors on venules and capillary endothelium an event termed cytoadherence. Several vascular receptors are identified, of which intracellular adhesion molecule1 is probably the most important in the brain and CD36 in most other organs. Thus, the infected erythrocytes stick inside the small blood vessels. At the same stage, these P.falciparum infected RBCs may also adhere to uninfected RBCs to form rosettes. The process of cytoadherence, rosetting and agglutination are central to the pathogenesis of P.falciparum malaria.[26]

Among 34 cases of complicated malaria, 24 patients had severe anemia (Hb<5gm%) with hepatic dysfunction and 22(64.7%) had metabolic acidosis. 16 patients had altered sensorium. CSF analysis was done which was normal. 6 Patients developed coma and were put on mechanical ventilators, who died within 2 weeks of hospitalization, which shows high mortality rate in cerebral malaria.[27] These findings are comparable to other studies done by authors such as Trampuz A et al, in their study, they found that complications involve the nervous, respiratory, renal, and/ hematopoietic system. Metabolic acidosis and hypoglycemia were common systemic complications. These complications are commonly caused by falciparum malaria.

Out of 10 complicated P.vivax malaria 4 had severe anemia(Hb<5gm%), 6 developed ARF secondary to severe vomiting and dehydration, Renal impairments is common among adults with severe P.falciparum malaria. Studies also suggested that P.vivax can also cause renal dysfunction.[28,29] This study is comparable to other studies done by authors such as Prakash et al, in their study, they found that acute renal failure can occur in both vivax and falciparum malaria but more commonly in falciparum. Another study done by Kaur et al found that unusual presentation of vivax malaria with severe thrombocytopenia and acute renal failure. The pathogeneses of renal failure is unclear but may be related to erythrocyte sequestration interfering with renal microcirculatory flow and metabolism. Clinically and pathologically this syndrome manifests as acute tubular necrosis, although cortical necrosis never develops. In survivors, urine flow resumes in a median of four days, and serum creatinine levels return to normal in a mean of 14 days.

In this study all the 6 patients who developed ARF, their serum creatinine returned to normal by 2nd week. 2 patients required dialysis and other 4 were treated conservatively. There were no deaths in complicated vivax malaria. This study is comparable to other study such as Prakash et al,in which he found that vivax malaria can cause acute renal failure but the prognosis of it in vivax malaria is favourable.

When thrombocytopenia is co-related with severity of malaria, severe thrombocytopenia was commonly associated with complicated malaria (58.8%) as compared to uncomplicated malaria (14.3%). Maximum thrombocytopenia occurred on third and fourth day of infection and gradually returned to normal by fifth to sixth day. Those persisted to have severe thrombocytopenia beyond 6th day, their mortality and morbidity increased despite adequate therapy. This study is comparable to other studies done by investigators such as Beale PJ et al,[30] in their study, they found that lowest platelet count was found between the day of diagnosis and the fourth day of treatment, thereafter they returned to normal values. Another study conducted by Horstmann RD et al,who found that thrombocytopenia was a common feature in human malaria and in all the patients the platelet count rose to threefold the initial values with the clearance of parasite. Another study conducted by A Kumar et al,[31] found that platelet count which was decreased, reverted to normal values on treatment.

Patients who had severe thrombocytopenia at the time of admission are 8.5 times more prone to develop complications when compared to mild and moderate thrombocytopenia based on student 'T' test. In this study 20 patients had severe thrombocytopenia beyond 6th day, 14 recovered within 7 to 10 days, 6 died with an increase in mortality rate from 4.1% to 30%[32], of which 2 was P.falciparum and 4 were mixed infection.

CONCLUSIONS:

- Thrombocytopenia is a common association of Malaria
- Thrombocytopenia is seen in both P.vivax and P.falciparum.

- Severe thrombocytopenia (Platelet count<20,000) is seen in P.falciparum & P.Vivax malaria but more common in P.falciparum.
- Severe thrombocytopenia is a good predictor of poor prognosis than mild and moderate thrombocytopenia.
- Patients who present with severe thrombocytopenia are 8.5 times more prone to develop complications than mild and moderate thrombocytopenia. This indicates that initial platelet count is an independent prognostic marker for severity of malaria.
- In patients with low platelet count, platelet count returned to normal values in more than 80% of patients after starting treatment within 6 days.

If severe thrombocytopenia persists for more than six days despite of adequate therapy, mortality rate increases from 4.1% to 30%.

Early diagnosis and prompt treatment of complications reduces the global burden of malaria. Severity of thrombocytopenia is a better predictor of outcome but it does not help in early diagnosis of complicated malaria. Hence further studies should be conducted on thrombocytopenia in malaria, rate of fall in platelet count which may help in early diagnosis of complications in malaria.

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Age in years	Number of Patients	Percentage
21-30	54	30.0
31-40	48	26.7
41-50	44	24.4
51-60	22	12.2
61-70	12	6.6
Total	**180**	**100.0**
Mean ± SD	40.13 ± 14.10	

Table 1: Age Distribution

Sex	Number of Cases	Percentage
Male	136	75.6
Female	44	24.4
Total	**180**	**100.0**

Table 2: Sex distribution

Symptoms	P.Falciparum	P.Vivax	Mixed Infection
	(n=62)	(n=114)	(n =4)
Typical Paroxysum	10(16.1%)	40(35.1%)	-
Vomiting	26(32.3%)	10(8.8%)	4
Headache	30(48.4%)	16(14.1%)	-
Jaundice	20(32.3%)	4(3.5%)	4
Altered sensorium	12(25.8%)	0	4
Pain abdomen	12(19.4%)	2(1.8%)	-
Cough & Breathlessness	8(12.9%)	0	-

Joint pain	6(9.7%)	4(3.5%)	-

Table 3: Distribution of Symptoms

Signs	Number of Cases (n=180)	Percentage
Splenomegaly	156	86.7
Pallor	88	48.8
Icterus	24	13.3
Hepatomegaly	20	11.1
Altered sensorium	16	8.9
Petechiea	6	3.7

Table 4: Distribution of signs

Type of Species	Number of Cases (n=180)	Percentage
P.Falciparum	62	34.4
P.Vivax	114	63.3
Mixed	4	2.7

Table 5: Type of species

Incidence of Thrombocytopenia	Number of Cases (n=180)	Percentage
Normal (>1.5 lakh)	34	18.9
Mild (0.5-1.5 lakh)	54	30.0
Moderate (0.20-0.50 lakh)	56	31.1
Severe (<0.2 lakh)	36	20.0

Table 6: Incidence of thrombocytopenia

Thrombocytopenia	Species			Total
	P. Falci	P. Vivax	Mixed	
Mild	18(31%)	36(42.8%)	-	54
Moderate	24(41.3%)	32(38%)	-	56
Severe	16(27.5%)	16(19%)	4(100%)	36
Total	**58**	**84**	**4**	**146**

Table 7: Association of Thrombocytopenia with Species

Severity of Malaria	Number of Cases (n=146)	Percentage
Uncomplicated Malaria	112	76.7
Complicated Malaria	34	23.3

Table 8: Severity of Malaria

Criteria	Number of Patients	Species		
		P. Falciparum	P.Vivax	Mixed
Hb<5 gm/dl	24	20(83.3%)	4(16.7%)	-
S.Creatinine >3mg%	18	8(44.4%)	6(33.3%)	4(22.2%)
		20(83.3%)	-	4(16.7%)
M. acidosis.ph<7.2	22	16(72.7%)	2(9.1%)	4(18.2%)
Spt bleeding and DIC	12	10(83.3%)	-	2(16.7%)

Coma >30min	6	2(33.3%)	-	4(66.7%)
Hyperparasitemia>5%	8	4(50.0%)	-	4(50.0%)
B.sugar<40mg%	2	-	-	2(100.0%)
Prostration	24	16(66.7%)	4(16.7%)	4(16.7%)
ARDS	8	4(50.0%)	-	4(50.0%)
Systolic BP<80mmhg	12	4(33.3%)	4(33.3%)	4(33.3%)

Table 9: WHO guidelines for complicated malaria

Thrombocytopenia	Severity of Malaria		Total
	Uncomplicated	Complicated	
Mild (0.5-1.5 lakh)	48 (42.9%)	6(17.6%)	54
Moderate (0.2-0.5 lakh)	48(42.9%)	8(23.5%)	56
Severe (<0.2 lakh)	16 (14.3%)	20(58.8%)	36
Total	**112 (100.0%)**	**34(100.0%)**	**146**

Table 10: Association of Thrombocytopenia with severity of malaria

Species	Severity of Malaria		Total	P value
	Uncomplicated	Complicated		
P Falciparum	38(33.9%)	20(58.8%)	58	0.066+
P. Vivax	74(66%)	10(29.4%)	84	0.007**
Mixed	-	4(11.7%)	4	0.062+
Total	**112(100%)**	**34(100%)**	**146**	**-**

Table 11: Association of malaria species with severity of malaria

Platelet Counts	Number of Patients with thrombocytopenia (n=146)	Percentage
1st day	146	100.0
2nd day	122	83.6
3rd day	116	79.5
4th day	90	61.6
5th day	56	38.4
6th day	20	13.7

Table 12: Number of patients had thrombocytopenia

Species	Outcome		Number of Patients
	Died	Recovered	
P. Falciparum	2(3.4%)	56(96.6%)	58
P. Vivax	-	84(100.0%)	84
Mixed	4(100.0%)	-	4
Total	**6(4.1%)**	**140(95.9%)**	**146**

Table 13: Association of Species with outcome

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Clinical benefits to pregnant women on the use of rapid diagnostic test to microscopy in malarial diagnosis in Jigawa State, Nigeria

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The study was aimed at comparing rapid diagnostic test kits (RDTs) and microscopy in detecting sequestered placental malaria or deep tissue malaria from pregnant women and the clinical benefits that can be derived. About 300 pregnant women were enrolled in the study. Five (5) ml of venous and placental blood was collected into an ethylenediaminetetraacetic acid (EDTA) tube, respectively. The blood samples were tested for malaria using microscopy and parascreen (RDTs). The hemoglobin (Hb) concentration was estimated by Hb color scale method. Out of the 300 enrolled, a total of 250 (82.5%) were positive with microscopy while the RDTs detected 300 (100%). Comparing the sensitivity, RDTs had 100% while microscopy had 88.3% and both had 100% specificity. Comparing the age group with frequency of infection, the 21 to 25 years age groups were the most vulnerable with 134 (45.54%). With parity, secundgravidae (1+1) had the highest with 104 (34.32%) and ≥ 4 parity had the least with 50 (16 50%). Those with Hb values ≤ 9 g/dl had the highest incidence with 245 (80.85%), 10 to 11.4 g/dl had 51 (16.86%) while ≥ 11.5 g/dl had the least with 4 (1.32%). About 16.5% were RDTs positive which might have been lost if only microscopy was done.

Key words: Rapid diagnostic tests (RDTs), microscopy, pregnancy, anaemia, parity, sequestration.

INTRODUCTION

Malaria is an infectious disease caused by *Plasmodium species*. They are transmitted from person to person through the bite of an infected female anopheles mosquito (Fernandez, 2006). Malaria generally is a disease of major public health concern in African region, with 562 million people at high risk (World Health Organization (WHO), 2013). It was estimated that there were 166 million clinical cases of malaria in 2012 and up to 90% malaria deaths of world total was from sub Saharan Africa. 77% of the deaths in the African region was among children < 5 years (WHO, 2013). Each year, 25 to 30 million women become pregnant in malaria-endemic areas of Africa, and similar numbers are exposed to malaria in Asia, Oceania, and South America. Malaria is an important cause of severe anemia in pregnant African women, and by this mechanism malaria

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causes an estimated 10,000 maternal deaths each year. Moreover, malaria infections result in 75,000 to 200,000 low birth weight babies each year, due to combinations of preterm delivery and fetal growth restriction (Guyatt and Snow, 2004). The yearly exposure of at least 50 million pregnancies to malaria infection makes it the most common and recurrent parasitic infection directly affecting placenta (Federal Ministry of Health (FMOH), 2006).

In Africa, perinatal mortality due to malaria is at about 1500/day. In areas where malaria is endemic, 20 to 40% of all babies born may have a low birth weight, hence making malaria in pregnancy one of the priority areas of Roll Back Malaria strategy. It affects more than 3 million pregnant women per year in developing countries, where it commonly causes poor birth outcome and maternal anemia (WHO, 2004). To revert malaria in Africa, there have to be tremendous efforts from all angles to curtail it. Concurrently, there has to be a shift away from the concept of eradication of malaria using indoor house spraying to integrated vector control approaches (WHO, 2006). Efforts to control the disease are as well hampered by the resistance to drugs shown by the *Plasmodia*, to the insecticides by the vectors and the lack of an effective vaccine (Elizabeth et al., 2005).

Malaria in pregnancy is an obstetric, social and medical problem requiring multidisciplinary and multidimensional solution. It is a debilitating, infectious disease characterized by chill, shaking and periodic bouts of intense fever. Pregnant women constitute the main adult risk group for malaria and 80% of deaths due to malaria in Africa occur in pregnant women and children < 5 years (Worts et al., 2006a).

Parasitaemia level and number of peripherally-detected malaria infections, but not the presence of fever, are associated with adverse birth outcomes. Hence, prompt malaria detection and treatment should be offered to pregnant women regardless of symptoms or other preventive measures used during pregnancy, and with increased focus on mothers living in remote areas.The physiological changes of pregnancy and the pathological changes due to malaria has a synergistic effect on the course of each other, thus making the life difficult to the mother and the child (Kakkilayer, 2006; Reyburn et al., 2007). In Africa, malaria in pregnancy is responsible for 400,000 cases of severe maternal anaemia and 200,000 newborn deaths each year. Placental infection, premature birth and low birth weight (a significant factor in infant mortality) are also caused by maternal malaria. In addition, severe maternal anemia increases the risk of perinatal complications.

Plasmodium falciparum causes three specific changes in the placenta. Infected erythrocytes (IE) containing mature trophozoite and schizont parasite stages accumulate in the intervillous spaces (the lake-like structures through which maternal blood circulates), sometimes to high densities. High placental parasitemia has been associated with preterm delivery (PTD). Placental malaria may be accompanied by intervillous infiltrates of monocytes and macrophages, some containing malaria pigment (hemozoin). High-density monocyte infiltrates are especially common in first pregnancy, and are associated with low birth weight (LBW) and anemia (Brabin et al., 2004; Rogerson et al., 2003).

The problems in the new born include low birth weight, prematurity, intrauterine growth retardation (IUGR), malaria illness and mortality. The pathogenesis of placental malaria is only partially understood, but it is clear that it leads to distinct epidemiological pattern of malaria during pregnancy (Worts et al., 2006b). An integrated understanding of the epidemiological, immunological and pathological processes must be achieved in order to understand how to control malaria in pregnancy. In pregnant women, parasitological and both hematological and biochemical changes should be promptly investigated as part of good clinical practice to improve the differential diagnosis of fever and any possible derangements. This may also reduce the unnecessary prescription and use of anti malaria drugs, many of which are of questionable safety. During pregnancy, *P. falciparum* is squestered in placenta, often without being detected in the peripheral blood (Moody, 2002).

Rapid diagnostic tests have considerable potential as a tool to improve the diagnosis of malaria. Several commercially available tests are sensitive, specific, and stable under operational conditions. Although microscopy remains the gold standard for diagnosis of malaria, its accuracy under operational condition in Africa is often low. Result of RDTs are rapidly available, less liable to the theoretical risk of being falsely negative due to parasite sequestration, and accessible to both prescriber and patient and can restore confidence in the laboratory (Reyburn et al., 2007). Although RDTs are significantly more costly than the traditional routine microscopy in hospital settings, they are potentially cost effective (Reyburn et al., 2007).

Diagnosis of malaria involves identification of the malaria parasite or its antigens/products in the blood of the patient. Although this seems simple, the efficacy of the diagnosis is subject to many factors. The different forms of the four species; the different stages of erythrocytic schizogony; the endemicity of different species; the population movements; the inter relation between the level of transmission, immunity, parasitemia, and the symptoms; the problem of recurrent malaria, drug resistance, persisting viable or non-viable parasitemia, and sequestration of the parasites in the deeper tissues; and the use of chemoprophylaxis or even presumptive treatment with the basis of clinical diagnosis can all have an impact on the identification and interpretation of malaria parasitemia on a diagnostic test (Bates et al., 2006b). The Jigawa State Ministry of Health in collaboration

Table 1. Baseline characteristics of patients for slides (microscopy) and RDTs.

Characteristic	Microscopy/RDTs
Mean age (years)	30±15
Fever in last 48 h	280
Low hemoglobin level (≤9 g/dl)	235
Previous use of antimalarial in current illness	215
Parity stages	4±3
Previously diagnosed positive	300
HIV status	Negative

Table 2. Statistical analysis depicting sensitivity, specificity and predictive values of RDTs and microscopy (n=300).

Methods	Positive S/C	Negative S/C	Sensitivity	Specificity	PPV	NPV
RDTs	300	0	100	100	100	100
Microscopy	250	50	83	100	100	83
P-Value	<0.05	<0.05	<0.05	>0.05	>0.05	<0.05

RDTs = rapid diagnostic tests, PPV = Positive predictive Value, NPV = Negative Predictive Value, S/C = Slide/Cartridge.

with PATHS deployed RDTs to formal health care system of rural areas as part of intensifying the need to avoid missed diagnosis especially to pregnant women and children < 5 years.

MATERIALS AND METHODS

Sample collection

A total of 300 pregnant women were recruited, all attending antenatal clinic at General Hospital, Ringim of Jigawa State.
Informed consent was sorted from each participant for the study. At delivery, 5 ml of maternal and placental blood were collected into separate EDTA tubes for thick films, RDTs and hematological assessment.

Making and staining of thick films

Thick films of 2 cm in diameter were made from well mixed blood on a clean grease free microscope slide. The films were stained using Field's stain rapidly (Bates et al., 2006a). All films were later reviewed by the State Malaria Microscopy Quality Control Officer. Parascreen RDT kit was used according to manufacturer's procedures to test for the presence of malarial antigen in the blood samples.

Hemoglobin concentration estimation

Hemoglobin (Hb) color scale technique was used to estimate the Hb concentration of the blood samples in g/dl (Bates et al., 2006a).

Statistics

The results were analyzed using SPSS 15.0 statistical package.

RESULTS

Table 1 shows the baseline characteristics of the participants at commencement of the study, with mean age of 30 ± 15 years. Those with fever within the 48 h were 280, those detected with low haemoglobin level (≤ 9 g/dl) were 235 and those on drugs were 215. Parity ranged between 1 to 4, they were all previously diagnosed malaria positive and non reactive to human immunodeficiency virus (HIV). Table 2 shows the result of sensitivity, specificity and predictive values using both Microscopy and RDTs. Microscopy and RDTs both had 250 vs 300 of positive S/C, Negative S/C of 50 vs 0, Sensitivity 83 vs 100, Specificity 100 vs 100, PPV 100 vs 100, and NPV 83 vs 100, respectively. A significant value ($p < 0.05$) was observed in all but specificity and PPV. Table 3 shows the distribution of malarial infection among the different age groups ranging between 15 to 45 years of age. The age group of 21 to 25 years has the highest infection rate, followed by 15 to 20 age group. From 26 to 30 age group, the infection rate decreases down to 41 to 45 age group in both the diagnostic methods. Table 4 shows the relative malarial infection in association with the number of parity by the mother. Secundigravidae has the highest infection rate followed by those with first time pregnancy. Third with the high rate were those with third time pregnancy while those with the least infection rate were those with four and above parity status. Table 5 shows the hemoglobin distribution among the malaria infected pregnant women. Those with hemoglobin level of 9 g/dl and below have the highest populations followed by those with 10 to 11.4 g/dl while those with hemoglobin11.5 g/dl and above have the least number of

Table 3. Age groups compared to the rate of malaria infection.

Age groups (years)	Microscopy n (%)		RDTs n (%)	
	Positive	Negative	Positive	Negative
15-20	78 (26.20)	13 (4.29)	91 (30.03)	0 (0)
21-25	104(34.67)	34 (11.33)	138(45.54)	0 (0)
26-30	28 (9.33)	4 (1.33)	32 (10.56)	0 (0)
31-35	24 (7.92)	1 (0.33)	25 (8.25)	0 (0)
36-40	6 (1.98)	4 (1.33)	10 (3.30)	0 (0)
41-45	3 (0.99)	1 (0.33)	4 (1.32)	0 (0)

Table 4. Showing association of parity status and malaria infection.

Parity	Total examined (n)	Microscopy n (%)	RDTs n (%)
		Positive	Positive
1+0	83	72 (24.00)	83 (27.67)
1+1	104	78 (25.74)	104 (34.32)
1+2	63	55 (18.15)	63 (20.79)
≥4	50	45 (14.85)	50 (16.5)

n = sample size, 1+0 = First pregnancy, 1+1 = Second pregnancy, 1+2 = Third pregnancy, ≥4 = Fourth pregnancy and above.

Table 5. Depicting association of hemoglobin content and malaria infection.

Hemoglobin (g/dl)	Total examined (n)	Microscopy n (%)	RDTs n (%)
		Positive	Positive
≥ 11.5	4	4 (01.33)	4 (01.33)
10-11.4	49	46 (15.33)	49 (16.33)
≤ 9.00	247	200 (66.67)	247 (82.33)

infected population.

DISCUSSION

In Jigawa state, the natural event of pregnancy puts women at greater risk of death at a higher rate than expected. An average of 1500 to 2000 pregnancies out of 100,000 live birth will end in the death of the mother, child or both. In some part of the world in developed countries, the number of pregnancies is fewer than 100 per 100,000 live birth (Department for International Development (DFID), 2006).

The challenges for diagnostic laboratory in Jigawa and most of the African regions which include defective microscope, intermittent power, poor supply of consumables, and time limit to examine slides are well known both to the laboratory managers and to their consumers. To improve these to the standard and comparable sensitivity and specificity of RDTs is not simple or easy to sustain. RDTs if embarked upon will supplement as a tool to offer improvement in accurate and precise diagnosis of malaria in our local setting were competent and other basic requirements are lacking. In most of the request made to the laboratories in syndrome manner, the findings in most cases with respect to malaria parasites request in most cases turnout negative even in severe infections. This may be explained by sequestration of parasites into deep vascular beds. Other possibilities that may affect sensitivity of microscopy in our settings may include work overload, shortage of staff and substandard Romanowsky's stain that flooded our chemical stores throughout the nation. From the study it was observed that the routine may fail to indicate the presence of malaria parasites as a result of tissue sequestration in the placenta. Therefore, recognizing the increasing importance of accurate diagnosis in an era of negative clinical benefits experiencing by pregnant women, government should be encouraged by experts to place substantial orders for RDTs as guide to treatments of febrile illness (Reyburn et al., 2007).

Prompt detection and treatment with effective anti-malaria should be offered, irrespective of symptoms and use of other preventive measures in pregnancy. While frequent screening was associated with improved birth outcome, reaching mothers living in remote areas to

prevent late attendance and low number of visits at antenatal care is essential. What this study has added is that, the parasites in some patient might be sequestered or missed diagnosis in the placenta in about 16.6% (50) cases in the pregnant women attending this comprehensive hospital of the locality. This may contribute significantly in preventing the pregnancy complication due to plasmodiasis among this great population. In Cameroon, 20.1% of pregnant women in a similar study were detected by HRP-2 based RDT and therefore rescued from missed diagnosis using microscopy (WHO, 2004).

CONCLUSION AND RECOMMENDATION

Public enlightenment through the local media radio stations and traditional town criers will ultimately help in reducing the risk by attending clinic in the early stage of the pregnancy. Public/community sanitation should be enforced so as to clear away the harboring areas that proliferates the mosquitoes. Those attending antenatal clinic should be told on the risk of abandoning their routine drugs in relation to their health and the fetus. They should also be given a free set insecticide treated nets (ITN) as part of the Federal government effort on Roll Back Malaria program. The RDTs test kits should also be supplied free or at well reduced price to the reach of less privileged.

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Conflict of interest

The authors have no conflict of interest

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Evidence for additive and interaction effects of host genotype and infection in malaria

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The host mechanisms responsible for protection against malaria remain poorly understood, with only a few protective genetic effects mapped in humans. Here, we characterize a host-specific genome-wide signature in whole-blood transcriptomes of *Plasmodium falciparum*-infected West African children and report a demonstration of genotype-by-infection interactions in vivo. Several associations involve transcripts sensitive to infection and implicate complement system, antigen processing and presentation, and T-cell activation (i.e., *SLC39A8*, *C3AR1*, *FCGR3B*, *RAD21*, *RETN*, *LRRC25*, *SLC3A2*, and *TAPBP*), including one association that validated a genome-wide association candidate gene (*SCO1*), implicating binding variation within a noncoding regulatory element. Gene expression profiles in mice infected with *Plasmodium chabaudi* revealed and validated similar responses and highlighted specific pathways and genes that are likely important responders in both hosts. These results suggest that host variation and its interplay with infection affect children's ability to cope with infection and suggest a polygenic model mounted at the transcriptional level for susceptibility.

host response | parasite load | eQTL | eSNP | genotype-by-environment interactions

Accumulating evidence has converged on the recognition that the onset of disease implicates complex biological processes. Susceptibility to infection, like any other complex trait, is multifactorial and has a significant heritable component. Genome-wide association (GWA) approaches have been extended to mapping the genetic architecture underlying susceptibility to infectious diseases (1–5), but only hemoglobin mutations and a handful of other loci conferring risk or protection to malaria have been identified (5–8). There has also been no explicit effort to characterize the effects of host regulatory variation, polygenic inheritance, and genotype-by-infection interactions on malaria phenotypes in vivo.

Host transcriptional response to malaria infection takes place in several organs. We set out to uncover the heritable and infection-response components of host immunity to malaria infection in whole blood of a sample of West African children (*SI Appendix*, Figs. S1 and S2). Whole blood constitutes a reservoir of circulating immune and nonimmune cells that respond to signals from the parasite while incorporating information from host genotype and play important role in controlling the course of infection. Blood is also a readily accessible system to capture these effects in regions of the world where malaria is endemic. Nonetheless, key transcriptional events in response to infection take place in other organs such as spleen, liver, and bone marrow, the signature of which may not be well preserved in blood. Also, correcting for the effects of differences of cell type proportions on differential expression can be challenging. Here, we test the hypothesis that malaria infection, host regulatory variation, and their interplay generate significant transcriptional variation that affects key immune response mechanisms. First, we uncover the magnitude at which malaria infection and parasite load impact transcript abundance and identify the immune processes influenced by these effects. Second, we identify the genetic factors that influence transcript abundance and test their dependence on infection status. Finally, we use joint analysis of genotypic and gene expression data to identify genes and mechanisms likely affecting the course of infection.

Results

Influence of Infection on Human Transcriptome. By using unbiased unsupervised statistical analysis, we first evaluated the consistency of the expression profiles between cases and controls (i.e., the combined dataset) and across the range of the parasite load within the infected sample alone (i.e., cases). Clustering of gene expression profiles based on similarity (Fig. 1 *A* and *C*), as well as principal component (PC) analysis of the genome-wide gene expression correlation matrix (Fig. 1 *B* and *C*), suggest that individuals cluster largely based on their infection status and parasite load. This analysis revealed the presence of strong correlation structure in the data such that expression PC1 (ePC1) explains 19.6% and 17.5% of total variation in the combined dataset and in the cases, respectively.

Supervised multiple regression and variance component analyses accounting for sex, hemoglobin genotype, location, total blood cell counts, and ancestry confirmed the strong effect exerted by malaria infection and parasite load on the transcriptome. The majority of variation captured by the first ePCs is explained largely by malaria infection status (74% of total variation in the combined dataset; $P < 10^{-5}$) and by parasitemia class (47% of total variation within the cases; $P < 10^{-4}$) when modeled as a function of sex, hemoglobin genotype, location, total blood cell counts, and ancestry (*SI Appendix*, Fig. S3). To estimate the effect of parasite load independently of the hemoglobin

Author contributions: Y.I., P.G., M.C.R., A.S., and P.A. designed research; Y.I., J.Q., J.B., and E.G. performed research; V.B., T.d.M., J.-C.G., S.G., P.G., and P.A. contributed new reagents/analytic tools; Y.I. and J.-P.G. analyzed data; M.C.R. and A.S. supervised recruitment and sample collection in Benin; P.A. oversaw the genomic analysis; and Y.I. and P.A. wrote the paper.

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Data deposition: The gene expression data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE34404).

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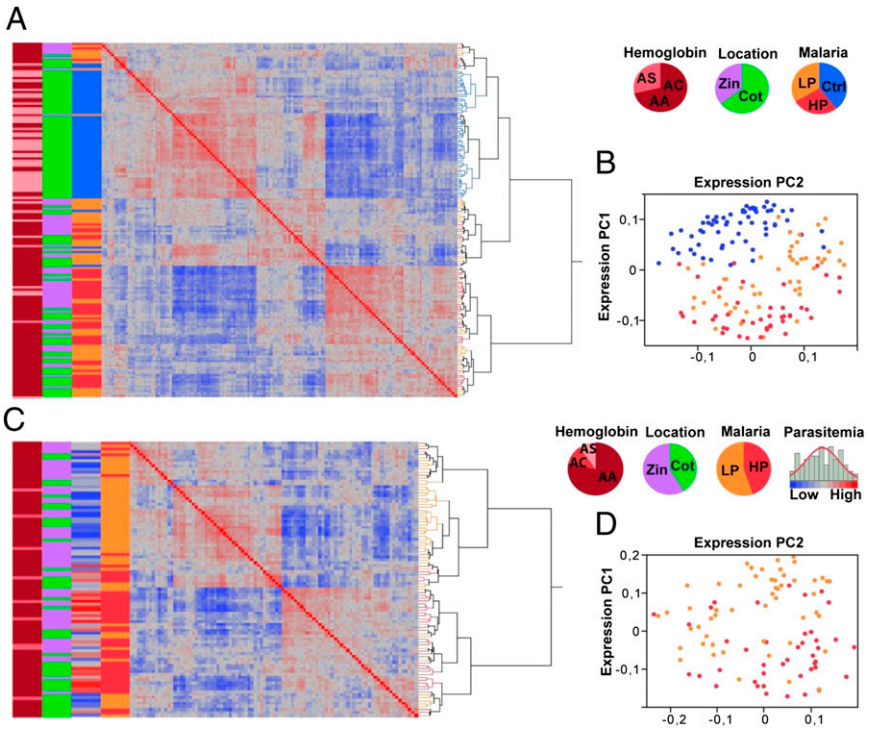

Fig. 1. Malaria infection impacts gene expression genome-wide. Correlation structure in whole-transcriptome data for the combined dataset of 155 cases and controls (*A* and *B*) and for the 94 cases alone (*C* and *D*). (*A* and *C*) Hierarchical clustering of whole-genome gene expression correlation matrix. The colored bars from left to right indicate the following phenotypes in the proportions displayed in the pie charts: hemoglobin genotype (AA, AC, or AS), location (Cotonou and Zinvié), and malaria infections status (control and high and low parasitemia groups). Parasite load or log2 parasitemia (low to high) is shown only in *C*. (*B* and *D*) PC analysis of the correlation matrix. The two major expression PCs (ePC1 and ePC2) are shown and individuals are labeled to indicate their infection status (controls, blue; high parasitemia, red; low parasitemia, orange).

genotype, we rerun PC analysis on 73 infected individuals who are AA homozygotes for the hemoglobin locus. The expression profiles again strongly correlate with parasitemia class explaining 39% ($P < 10^{-4}$) of the variance of ePC1–3.

Next, we evaluated the magnitude and significance of differential expression of individual transcripts first between cases and controls, and second between the controls, the high and low parasitemia groups. ANOVA (accounting for location, sex, hemoglobin genotype, and infection status) and analysis of covariance (ANCOVA; accounting also for total blood cell counts and ancestry) revealed a strong effect of infection status on whole-blood transcriptome. A statistical significance threshold at 1% false discovery rate (FDR; per Benjamini and Hochberg) was applied to all tests of differential expression. A total of 3,334 transcripts (23%) were differentially expressed between cases and controls, whereas 3,177 and 3,154 of these transcripts remained differentially expressed even after accounting for total blood cell counts and ancestry, respectively (Table 1). Breaking down the ANOVA into pair-wise comparisons, we observed that the effect of malaria infection on differential expression of individual transcripts is highest when comparing controls vs. the high parasitemia group (4,085 transcripts), and less so when comparing controls vs. the low parasitemia group (2,377 transcripts), with evidence for a within malaria-infected sample differentiation (2,078 transcripts; Table 1, Fig. 2 *A* and *B*, and Dataset S1).

Gene Set Enrichment Analysis. Pathway analysis (9) of the differentially expressed genes implicates divergence in core immune processes. We particularly note a strong signature of induced innate immunity (up-regulation of IFN-inducible genes, neutrophil-associated modules, and markers of FcGR-mediated phagocytosis) and suppression of several adaptive immune processes (down-regulation of MHC genes, T cells, B cells, and cytotoxic T cell signaling pathways) in the cases relative to controls (Fig. 2 *C* and *D*). Few studies that report whole blood or peripheral blood mononuclear cell (PBMC) transcriptional signatures associated with malaria infection in African populations have been carried out (10–12). Among these studies, Griffiths et al. (10) detected two main signatures in whole blood related to neutrophil and erythroid activity differentiating acutely ill and

Table 1. Number of transcripts differentially expressed

Effect	ANOVA	ANCOVA I	ANCOVA II
Malaria			
Parasite load	2,971	3,014	1,990
Cases:controls	3,334	3,177	3,154
High parasitemia:control	4,852	4,402	4,085
Low parasitemia:control	2,493	2,438	2,377
High parasitemia:low parasitemia	2,772	2,601	2,078
Three-way comparison	6,178	5,856	5,180
Location			
Village:city	1,089	310	30
Sex			
Female:male	40	48	43

All contrasts shown in this table are from analyses performed on the cases and controls combined dataset (155 individuals), except the parasite load effect, which was estimated by analyzing the 94 cases alone. ANOVA accounts for the infection status effect, sex, location, hemoglobin genotype and pair-wise interactions. ANCOVA I and ANCOVA II additionally account for total blood cell counts and significant gPCs (gPC1-3; Tracy–Widom statistic < 0.01), respectively. The FDR was evaluated by using the Benjamini and Hochberg method.

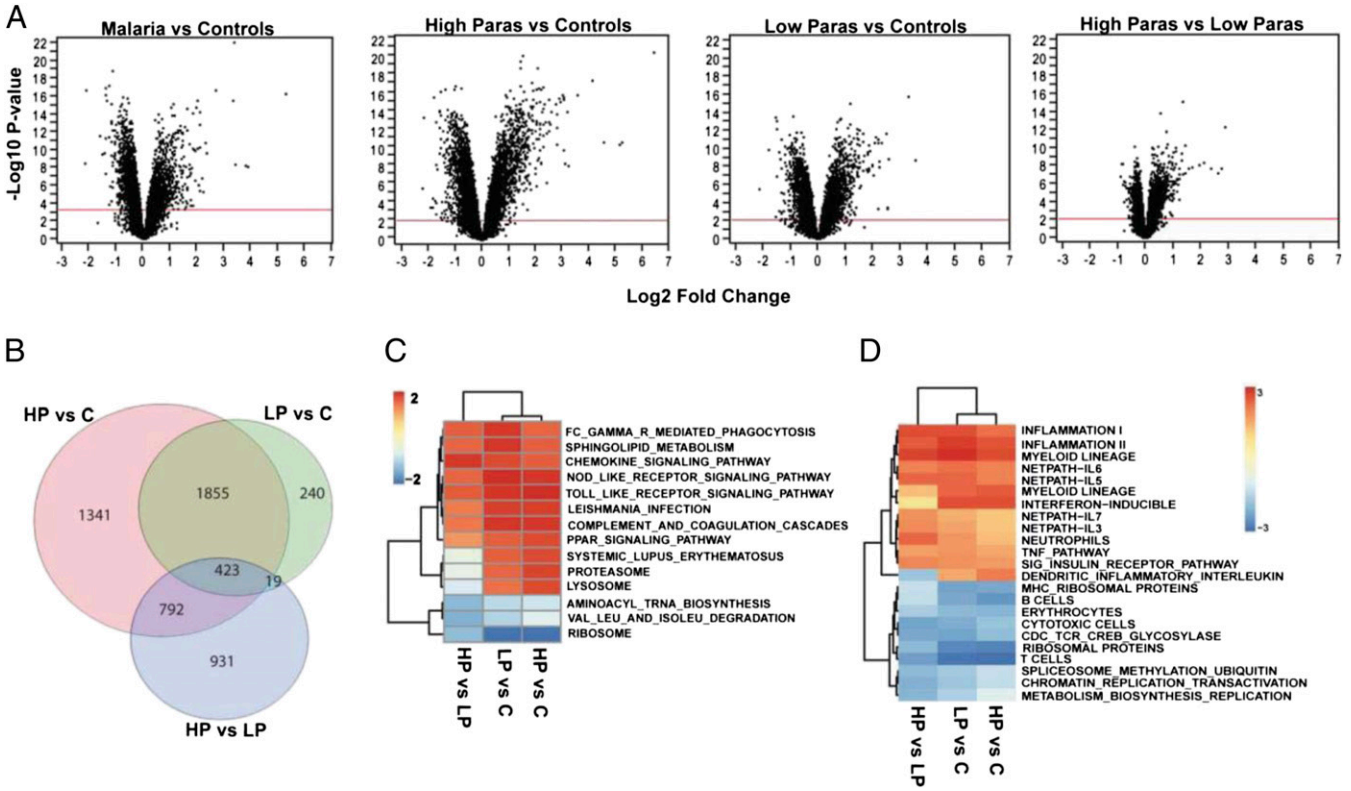

Fig. 2. Differential expression in whole-blood transcriptome. (*A*) Volcano plots of statistical significance vs. magnitude of differential expression for the two-way contrasts between the controls (marked as "C") and high parasitemia (HP) and low parasitemia (LP) groups. For each transcript, significance is shown as the –log10 *P* value on the *y* axis, and the log2 of magnitude of mean expression difference is on the *x*-axis. The red horizontal line indicates the 1% FDR threshold. (*B*) Venn diagram shows numbers of differentially expressed transcripts for each comparison and the overlaps between them. For each contrast, GSEA was performed for KEGG pathways (*C*) and the C2, C3, and C5 collections of the Molecular Signatures Database (*D*) as previously described (9, 16). Only pathways and modules significantly enriched (Bonferroni-adjusted $P < 0.05$) from at least one contrast are shown. Colors in the heat map indicate the enrichment score from the GSEA analysis.

convalescent Kenyan children. The authors reported a list of genes implicated in these two processes as being differentially regulated between the two groups. We highlight the replication of the expression patterns of the following loci: *C1QB* (Hochberg and Benjamini q-value = 8.72×10^{-19}; fold change, 11.15), *MMP9* (q-value = 1.12×10^{-12}; fold change, 11), *C3AR1* (q-value = 5.8×10^{-7}; fold change, 1.33), IL18R (q-value = 7.96×10^{-7}; fold change, 2.83), and *HMOX1* (q-value = 1.1×10^{-8}; fold change, 2.08). These genes seem a promising target for focused evaluation as circulating biomarkers of malaria infection. Several other genes that paralleled the intensity of the infection in our dataset have been reported by others (13, 14), but a systematic comparison with these reports is difficult given differences in study design and the different in vitro cell populations profiled.

A fraction of the expression differences detected for the parasite load effect after accounting for total cell counts is likely caused by average differences in the proportions of subtypes of PBMCs (15). To infer these effects in our sample, we used the genomic signature of flow cytometry-sorted immune cell types (16) in which cell type-specific modules are constructed based on transcript abundance of each gene relative to each other cell type in the PBMC mixture. These expression signatures are constructed from healthy individuals and therefore can be used as a reference panel. We computed Pearson correlation between parasite load and average transcript abundance of each module across all 94 infected individuals (*SI Appendix*, Fig. S4). This analysis shows a significant effect of parasite load on the six cell type-specific expression profiles investigated (B cells, T cells, myeloid dendritic cells, plasmacytoid cells, natural killer cells, and monocytes; $P < 10^{-7}$) that can result from modulation of cell type-specific transcription, a shift of cell type mixture in the bloodstream, or a combination of both. Particularly, we note that parasite load is positively correlated with average transcript abundance of myeloid antigen-processing cells and negatively correlated with average transcript abundance of B and T cells, along with the other innate immunity cell types (*SI Appendix*, Fig. S4).

Contrasting Host Whole-Blood Response in Humans and Mice. Animal models represent a valuable companion to the study of human clinical material for understanding host–parasite interactions in malaria (17). In particular, mouse models allow detailed characterization of pathogenesis and host response in an experimental framework in which the genetic contribution of the host and environmental factors (including parasite type and infectious doses) are carefully controlled. To test the role for some of the genes and pathways uncovered in our human study in host response to malaria, we infected mice (C57BL/6J) with *Plasmodium chabaudi* AS (10^6 parasitized erythrocytes, i.v.), and blood from infected mice was collected 4 d (3.6 ± 0.9% parasitemia) and 6 d (32.8 ± 2% parasitemia) postinfection. Globin-depleted total RNA was prepared, and gene expression profiles were generated by hybridization to microarrays (MouseWG-6 BeadChips; Illumina).

ANOVA revealed 1,783 transcripts differentially expressed (1% FDR) in at least one of the pair-wise contrasts, with the effect of infection being highest in the uninfected mice vs. high parasitemia comparison (1,575 transcripts; Dataset S2 and *SI*

Appendix, Fig. S5*A*). Gene set enrichment analysis (GSEA; 5% FDR) revealed a strong induction of IFN response, antigen processing and presentation, and the proteasome modules, along with a suppression of the B-cell module, which were all consistent with the human signature (*SI Appendix*, Fig. S5*B*). Next, we compared the gene lists derived from the ANCOVA II and ANOVA analyses of the human and mouse datasets, respectively. This contrast was limited to genes significantly regulated (1% FDR) in both hosts, with 47 genes showing fold change greater than two in the human dataset. Thirteen genes were significantly regulated when specifically comparing the high parasitemia group vs. controls (*SI Appendix*, Fig. S6*A*). Of these genes, 11 show the same pattern of response in both hosts, notably for three Fc receptors (*FCER2*, *FCGR3B* and *FCRLA*), indicating the importance of FcGR-mediated phagocytosis in host whole-blood response to malaria infection (*SI Appendix*, Fig. S6*B*).

Uncovering the Genetic Basis for Gene Regulation in Children Infected with Malaria. Next, we uncovered the genetic basis of gene expression variation in malaria by performing a GWA test of transcript abundance in the human host. We applied Bonferroni correction for all associations performed in this study. Each of 544,672 SNPs was tested for association with each of the 18,876 expressed transcripts, and a genome-wide Bonferroni correction for multiple testing accounting for the number of SNPs and loci was applied. This analysis gave rise to (*i*) a genome-wide Bonferroni threshold of 4.86×10^{-12} [$0.05/(18{,}876 \times 544{,}672)$] for distal associations, which is likely to be conservative given the linkage disequilibrium structure across the genome; and (*ii*) a genome-wide Bonferroni threshold for local associations considering the number of SNPs within the region spanning from 100 kb upstream to 100 kb downstream of the transcript (including the transcript itself) and accounting for the number of loci tested. This analysis revealed 263 peak local SNP-probe associations at $P < 1.3 \times 10^{-8}$ and five peak distal SNP–probe associations at $P < 4.86 \times 10^{-12}$ in the combined dataset (Fig. 3, *SI Appendix*, Fig. S7*A*, and Dataset S3). The threshold $P = 1.3 \times 10^{-8}$ is the most conservative threshold for local associations and corresponds to a test against 196 markers [$P = 1.3 \times 10^{-8}$, or $(0.05/(18{,}876 \times 196))$]. The effect sizes of regulatory variation in our dataset are more than an order of magnitude larger than typical SNP–disease associations (*SI Appendix*, Fig. S7*C*), thus providing sufficient power to uncover these associations at genome-wide significance. Applying the same global association test of gene expression to the cases alone revealed 149 and six peak local and distal associations, respectively (*SI Appendix*, Fig. S7*B* and Dataset S4). In total, both analyses revealed 265 local and eight distal peak SNP–gene associations.

We observed significant overlap between these associations and those reported in 13 published expression quantitative trait locus (eQTL) studies of various tissues, including peripheral blood and its derivatives at nominal P values $>10^{-7}$ and 10^{-12} for local and distal associations, respectively. A total of 147 of 272 genes (54%) are replicated, including one distal association with

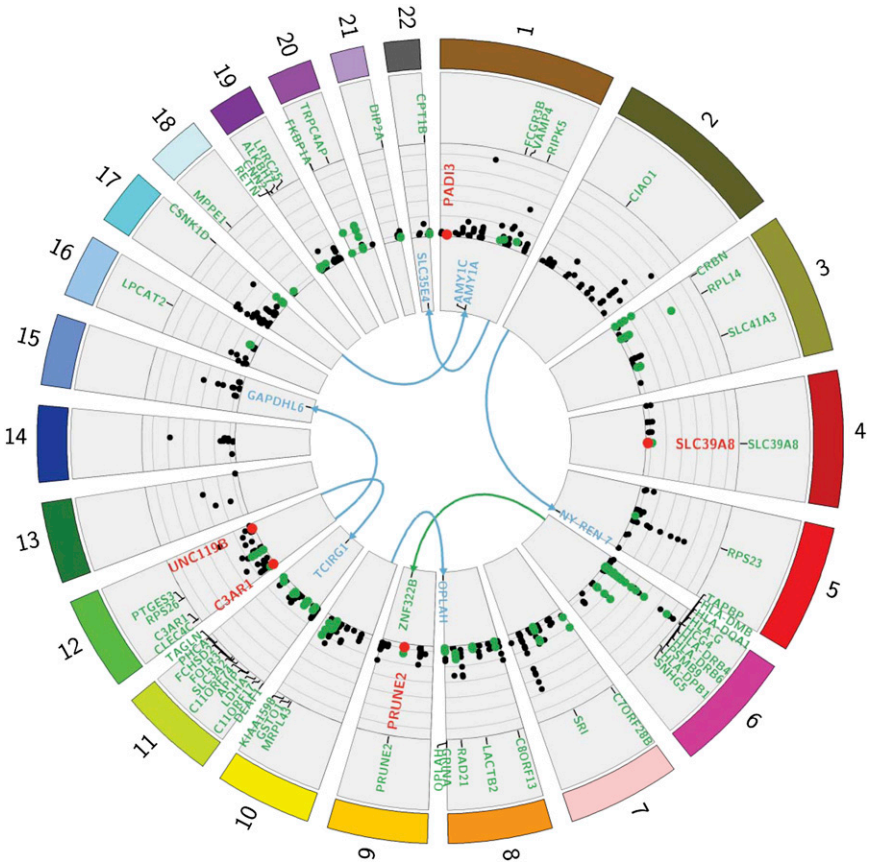

Fig. 3. Genome-wide eSNP map in malaria-infected children. Circos plot displaying all genome-wide significant associations detected in the combined dataset of cases and controls and in the cases alone. Each chromosome is shown in a different color. Distal associations are displayed in the center of the plot, with the links indicating target transcripts. Circularized Manhattan plot displays local associations and their respective significance (–log10 *P* value). Associations significant for the genotype-by-infection effect are shown in red, and those implicating genes differentially expressed at 1% FDR in at least one of the two-way contrasts among control and high and low parasitemia groups (Table 1) are shown in green.

AMY1A. Approximately half of these associations (76 of 147) are exact, namely implicating the same SNP–gene pair and most of the remaining report a SNP in the same linkage group. The other associations in our dataset are novel, of weaker strength in the 13 eQTL studies, or might have been reported in other studies.

Joint Action of Host Genotype and Infection on Gene Expression. To test for genotype-by-infection interactions, we ran a model that accounts for SNP, infection, SNP × malaria status, sex, location, RBCs, and WBCs. This analysis identified five peak local genotype-by-infection interactions at Bonferroni significance: *PRUNE2* ($P = 4.17 \times 10^{-9}$), *SLC39A8* ($P = 8.37 \times 10^{-7}$), *C3AR1* ($P = 1.07 \times 10^{-6}$), *PADI3* ($P = 1.61 \times 10^{-6}$), and *UNC119B* ($P = 2.15 \times 10^{-6}$; Fig. 3 and *SI Appendix*, Table S1). The associations implicating *C3AR1*, *PADI3*, and *SLC39A8* are shown in Fig. 4, and the remaining associations are shown in *SI Appendix*, Fig. S8. These findings demonstrated the existence of genome-wide significant interactions in malaria, and our data also suggest that interaction effects are pronounced for several associations beneath genome-wide significance.

Our survey of the sources of gene expression variation revealed dozens of genes under statistically significant joint effects of malaria infection and host genotype. The genes for which the infection effect is highly dependent on host genotype translate into statistically significant interactions. These genes show a substantial expressed SNP (eSNP) effect in the infected group or the control group but not in both, or show the effect in opposite directions in the two different groups. Other genes subject to interaction effects beneath genome-wide significance show different magnitudes of eSNP effects between the two groups and likely have important roles in modulating the course of infection, and several of them have previously been associated with malaria (i.e., *FCGR3B*, *PSMB9*, and *GSTO1*) (18–20). In addition, we discovered several associations implicating key immune processes, particularly antigen processing and presentation, plasmacytoid dendritic cell activation, and T-cell activation and expansion (i.e., *RAD21*, *LRRC25*, *CLEC4C* *SLC3A2*, and *TAPBP*) (21–25). The genes that are associated with an eSNP and that are differentially regulated by the infection are shown in green in Fig. 3 (Datasets S3 and S4 provide further details). We also note that expression of five genetically regulated HLA (*HLA*) class II loci is negatively correlated ($r^2 = 0.31$) with parasite load and with key immune effectors such as *IL18R1*, *TLR4*, *TLR5*, *IFNGR1*, and *IFNGR2* ($P < 10^{-4}$), indicating an impairment of antigen processing and likely of subsequent priming of host immune response.

A number of studies surveyed transcriptional genotype-by-environment interactions in humans and reported dozens of response eQTLs in vitro under a variety of environmental challenges such as radiation (26) and treatment with various agents (27–29). The number of interacting loci in response to malaria infection in our in vivo study is lower than the number of response eQTLs reported in these studies despite the fact that similar sample sizes were used. This is likely because of a combination of factors, notably the strong induction of transcriptional response in vitro, the homogeneity of the cell population investigated, and the differences in the experimental design and statistical thresholds applied. Nonetheless, our results are consistent with the concept that transcriptional genotype-by-environment interactions are pervasive in human populations and can be detected in vivo.

Other eSNP associations deserved attention, but the case of *SCO1*, which encodes an inner mitochondrial membrane metallochaperone, stands out. This gene was implicated in the second top GWA hit by Jallow et al. (5) (rs6503319; $P = 7.2 \times 10^{-7}$; 10 kb from the TSS of *SCO1*), and, here, we detected two genome-wide significant local eSNP associations for this locus. The strongest eSNP we detected (rs201621; $P = 8.91 \times 10^{-14}$) is located 4 kb upstream of the *SCO1* transcription start site in a strong enhancer (30, 31) (*SI Appendix*, Fig. S9). This finding implicates allelic variation of rs201621 in the effect captured by the malaria GWA study likely through contribution of differential expression of *SCO1* to detoxification pathways of reactive oxygen species (32).

Discussion

Joint analysis of gene expression and genotypic data demonstrated that malaria infection and host genotype alters immune gene expression genome-wide in additive and multiplicative manners. The interactions we report here show the existence of robust interactions in vivo in an infectious disease. One of these associations implicates the *SLC39A8* locus, which encodes a zinc transporter protein highly up-regulated in response to primary T-cell activation, especially in the presence of low concentrations of zinc (33). Several studies and initiatives have proposed zinc supplementation as a strategy to help reduce the risk of malaria episodes (34, 35), and our data implicate a gene whose action is potentiated by zinc but also clearly and robustly conditioned by host regulatory variation. The interaction implicating *SLC39A8* illustrates a robust in vivo genotype-by-infection effect that is directly linked to the key process of T-cell development.

Our data also suggest the scenario of the presence of interactions for higher-level malaria phenotypes in the absence of robust genotype-by-infection interactions for transcription (36). The case of *GSTO1*, which encodes a protein involved in the metabolism of a broad range of xenobiotics, illustrates this scenario (Fig. 4). Supposing only individuals with a transcript abundance of >12.0, indicated by the horizontal line (Fig. 4), have an

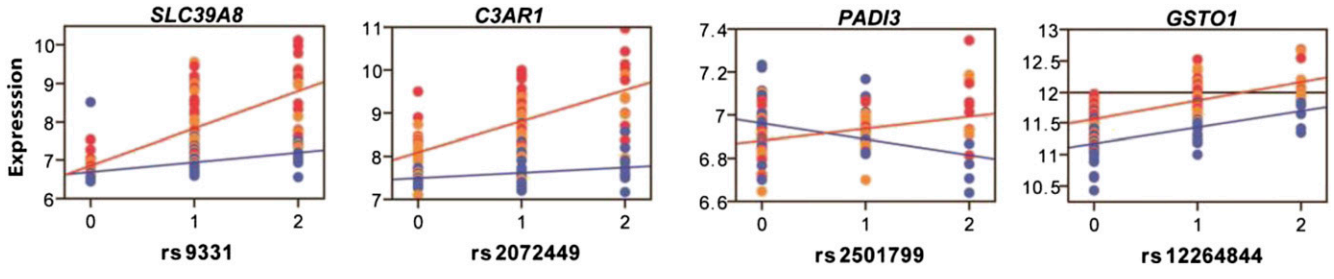

Fig. 4. Transcriptional additive and multiplicative effects in malaria. Examples of transcriptional interaction effects implicating the genes *SLC39A8*, *C3AR1*, and *PADI3*. The case of *GSTO1* illustrates the scenario of an interaction effect for a disease phenotype in the absence of a transcriptional interaction. This example illustrates how the effect of the gene is conditional on genotype with only the minor allele homozygote individuals shifting to the resistance zone (transcript abundance >12.0 indicated by horizontal line) when infected, giving rise to an interaction effect for the disease phenotype. Genotypes on the *x*-axis are labeled to indicate the number of minor alleles and individuals are labeled to indicate their infection status (controls, blue; high parasitemia, red; low parasitemia, orange). The *y* axis shows normalized expression values.

efficient detoxification capacity, certain individuals will have a greater capacity for parasite clearance and subsequently show resistance to malaria. Although hypothetical, the example of *GSTO1* illustrates how such effects can be conditional on genotype, with only the minor allele homozygote individuals shifting to the resistance zone when infected, giving rise to an interaction effect for the disease phenotype. A corollary of these interactions might mask associations of genotype with disease if the exposure increases disease risk in one genotype group and decreases it in another to yield an overall null effect.

In summary, we have provided a genome-wide picture of host in vivo regulatory variation events in malaria-infected whole-blood transcriptome and highlighted the implication of regulatory variation and interactions in modulating host immune response. The underlying genetic variation of such effects would predispose to how children mount an effective immune response to infection and likely to immunization. We also demonstrate that a systems genetics approach interrogating whole blood as one of the disease tissues can facilitate mapping of susceptibility genes and pinpoint causal mechanisms. Although challenging, it is equally important to extend this approach to investigate the key in vivo transcriptional events in malaria control that take place in other organs such as spleen, liver, and bone marrow. Last, we believe this approach is promising to uncover the genetic basis of response to infection and to immunization in vivo, particularly in African populations in which GWA studies are typically underpowered.

Materials and Methods

Study Population. The human study was approved by the Ethical Review Committee of Sainte-Justine Research Center and by the Faculté des Sciences de la Santé of the University of Abomey-Calavi in Benin. A total of 94 malaria-infected children under the age of 10 y (median age, 3.7 y) and 61 age-matched control subjects were sampled under informed consent (Dataset S5). Cases were children admitted to a secondary level hospital in Cotonou, the cosmopolitan city of the Republic of Benin, and in a rural primary level health care center in the village of Zinvié, located 36 km from Cotonou. Cases were sampled within a period of 10 wk in spring 2010.

After an initial assessment by a pediatrician, children with fever and who were diagnosed as having uncomplicated acute malaria were considered for the study. Children whose malaria infection status was confirmed by using the Parascreen *P. falciparum* malaria rapid diagnosis test and standard thick blood smear analysis were enrolled. Children presenting symptoms for other diseases or with known history of HIV were not included. Following blood sampling, all cases received antimalarial treatment and had an uneventful course of the disease, except for two children who underwent transfusion at D+1 and D+2 for worsening anemia. Age-matched controls were from the city of Cotonou and were siblings of a large cohort of children with sickle-cell disease registered at the health clinic of the National Center of Sickle Cell Disease in Cotonou. Hemoglobin testing was done by thin-layer agarose isoelectric focusing (Pharmacia LKB Biotechnology) on dried blood collected on Guthrie paper, and S-hemoglobin genotypes were confirmed by genotyping the sickle cell mutation (rs334) using the Sequenom assay. None of the control subjects have sickle-cell disease, and only those without clinical signs of malaria and who tested negative on both malaria detection tests were retained. All children recruited in our study were of a similar age and sampled within similar geographic and hence environmental settings.

Sampling and Genomic Profiling. The same collection protocol was followed for all samples to minimize heterogeneity for technical reasons. Peripheral blood samples were collected between 9:00 AM and 2:00 PM and stored at −30 °C until shipping to Montreal at −20 °C. Approximately 4 mL of blood was collected: 3 mL for RNA work collected in Tempus Blood RNA Tubes (Life Technologies) in which blood cells are immediately lysed after collection and total RNA stabilized, 0.5 mL stored in EDTA tubes for DNA work, and the remaining blood for thick smear analysis and total cell counts work with the use of an automated KX-21 blood cell analyzer (Sysmex). Total RNA was extracted by using a Tempus Spin RNA Isolation kit (Life Technologies) followed by globin mRNA depletion by using a GLOBINclear-Human kit (Life Technologies). Total RNA samples were quantified and quality-checked with the RNA 6000 Nano LabChip kit and the 2100 Bioanalyzer (Agilent). Only samples of high RNA quality (Agilent RNA Integrity Number >7.5) were retained for expression profiling. HumanHT-12 BeadChips (48k probes; Illumina) were used to generate expression profiles following the manufacturer's recommended protocols. To minimize chip and batch effects, the order in which the samples were processed was randomized across all fixed effects in the sample at the extraction, cDNA synthesis, and hybridization steps.

Hybridization was performed on two different dates, and five samples from the first batch were rehybridized with the second batch. Clustering of these technical replicates with themselves indicated negligible batch effects in our data. This was confirmed by testing for batch effect in the probe-by-probe ANOVA. Only well annotated probes (RefSeq) were retained for the analysis. Furthermore, 472 probes aligning to more than one location in the African reference genome or overlying SNPs reported in dbSNP Build 135 and with minor allele frequency (MAF) >5% in the Yoruba sample were removed. Expression intensities were log2-transformed and quantile-normalized by using JMP Genomics version 5.0 (SAS) after an outlier filtering procedure (37) was applied to provide further quality control. The distribution of the probe-level expression data was assessed for normality by using a Levene test, and those that showed deviation from normality ($P < 0.01$) were removed from the analysis. The probes with expression greater than background levels averaged across all of the arrays were retained for further analyses as previously described (38). These probes correspond to 23,826 and 27,546 features in the combined dataset of cases and controls and in the cases alone, respectively.

For the mouse experiment, ten 9-wk-old female C57BL/6J mice were injected i.v. with 10^6 *P. chabaudi* AS parasites to model blood-stage malaria infection. Animal research has been approved by McGill University review board and all mice were maintained at the Animal Care Facility according to the guidelines of the Canadian Council on Animal Care. Parasitemia was monitored by microscopy of Hemacolor (Harleco)-stained thin blood smears, and mice were euthanized by CO_2 inhalation followed by cardiac puncture to exsanguinate at day 4 (low parasitemia, $n = 5$) and day 6 (high parasitemia, $n = 5$). Blood was also collected from age- and sex-matched uninfected controls. For each condition, blood was pooled in Tempus tubes (Life Technologies). Total RNA was extracted by using a Tempus Spin RNA Isolation kit (Life Technologies) followed by globin mRNA depletion by using a GLOBINclear-Mouse kit (Life Technologies). RNA samples were quantified and quality-checked with the RNA 6000 Nano LabChip kit and the 2100 Bioanalyzer (Agilent). MouseWG-6 v2 BeadChips (Illumina) were used to generate expression profiles by using three technical replicates for each condition. The replicates started at the stage of the RNA sample at which equal quantities of input RNA from the original stock were subject to the entire procedure. Expression intensities were log2-transformed and quantile-normalized.

Genome-wide genotyping data were generated by using OmniExpress arrays (733k SNPs) and extracted with the Genotyping Module in BeadStudio software (Illumina). Only samples with call rates >99% were retained, and all SNPs that had a cluster separation value below 0.3 or call frequency below 99% were removed. The process of quality-control checks resulted in retention of 544,672 SNPs (MAF >10%) in 151 individuals for the population structure analysis and eSNP analysis. Global genotypic variation and ancestry was inferred by using Eigenstrat (39), retaining the first three eigenvectors [genotypic PCs (gPCs) 1–3) according to the Tracy–Widom test statistic ($P < 0.01$). gPC1–3 scores are used to account for ancestry in the analysis detailed later.

Statistical Analysis of Gene Expression Data. All statistical analyses on the gene expression data were performed by using JMP Genomics version 5.0 and SAS 9.3 (SAS). Two datasets were subject to the analyses described later: (*i*) the combined dataset (94 cases and 61 controls for the gene expression data-alone analysis, or 92 cases and 59 controls for the joint genotypic and gene expression data analysis), and (*ii*) the cases alone (94 cases for the gene expression data-alone analysis, or 92 for the joint genotypic and gene expression data analysis). The malaria effect was considered in three different ways: (*i*) cases vs. controls, (*ii*) log2-scale transformed parasitemia counts as a quantitative measure of infection severity, and (*iii*) high vs. low parasitemia groups using the median value of the log2 parasitemia counts as a cutoff. PC analysis, PC variance analysis, and multiple regression analyses were performed such that the first three ePC are modeled simultaneously or individually as a function of various effects in the data: malaria infection status, log2 parasitemia, location, hemoglobin genotype, sex, pair-wise combination of fixed effects, total cell counts (RBCs and WBCs), and ancestry (gPC1–3).

SAS GLM was used to evaluate the magnitude and significance of differential expression of individual expressed probes. Variance was partitioned

among the malaria effect, sex, location, hemoglobin, pairwise contrasts, total cell counts, and ancestry as covariates. Batch effect, age, and pair-wise contrasts (i.e., malaria × location, malaria × sex and sex × location) were evaluated and found to be insignificant. Results from the following full ANCOVA model (ANCOVA II in Table 1) for each malaria effect contrast were used for GSEA and for the contrast with genotypic effects:

$$Expression = \mu + \text{malaria status} + \text{location} + \text{sex} + \text{Hb} + WBC_S + RBC_S + \text{gPC1} + \text{gPC2} + \text{gPC3} + \varepsilon$$

The malaria effect was considered in the ways indicated in Table 1 and the error ε was assumed to be normally distributed with a mean of zero. For the mouse dataset, ANOVA was used to evaluate the magnitude and significance of differential expression among controls and high and low parasitemia groups. Orthology was inferred by using the Ensembl Biomart tool. A statistical significance threshold at 1% Benjamini and Hochberg FDR was applied to each term in all tests of differential expression.

GSEA. Enrichment analysis for each contrast (high parasitemia vs. controls, low parasitemia vs. controls, and high vs. low parasitemia) was performed by using GSEA (9). The analysis was performed on the C2, C3, and C5 collections of MsigDB database (http://www.broad.mit.edu/gsea/msigdb). Appended to C2 canonical pathways are curated signaling pathways from NetPath (40), molecular signature gene sets of sorted PBMC cell types (16), and gene sets collected from transcriptional analyses of PBMC samples (41). The resulting *P* values from the GSEA were adjusted for multiple testing by using a Bonferroni correction ($P < 0.05$). Pearson correlations were computed between parasitemia and average transcript abundance of each module of genes from six PBMC cell type subsets obtained from Nakaya et al. (16) across all 94 infected individuals.

GWA of Gene Expression. Marker properties and association tests were performed by using JMP Genomics version 5.0 and SAS 9.3 (SAS). Regression tests for association of gene expression levels with each numeric genotype (coded as 0, 1, or 2, with each number representing the number of copies of the minor allele) were performed. Only autosomal SNPs with an MAF >10%, with missing data <1%, and in Hardy–Weinberg equilibrium ($P < 0.01$) were retained for the GWA tests. Tests of association were carried out with two models for each dataset (the combined dataset and cases only) separately. We distinguished between local and distal associations based on the location of the genotype and the associated transcript. We applied Bonferroni correction for all associations performed in this study. Each of 544,672 SNPs was tested for association with each of the 18,876 expressed transcripts. This analysis gave rise to (*i*) a genome-wide Bonferroni threshold of 4.86×10^{-12} [0.05 / (18,876 × 544,672); (−log10[*P*] > 11.3) for distal associations and (*ii*) to a genome-wide Bonferroni threshold of 2.65×10^{-6} to 1.3×10^{-8} for local association [−log10(*P*) > 5.57–7.88], considering the number of SNPs within the region spanning 100 kb upstream and 100 kb downstream of the transcript. Only linkage disequilibrium block tagging SNPs (based on $D' > 0.90$) were used in the full model testing for the interaction effects. The analysis on the infected sample was performed by using 535,838 SNPs (with no more than one missing genotype per parasitemia group) and 18,974 probes.

First, a model in which *m* is the mean measure of transcript abundance, and the error ε is assumed to be normally distributed with a mean of zero was used (model 1):

$$\text{Expression} = m + \text{SNP} + \text{malaria status} + \varepsilon \quad [1]$$

The results from this model provided a list of significant associations that we compared with the associations reported in 13 published eQTL studies of peripheral blood or its derivatives at nominal *P* values > 10^{-7} and 10^{-12} for local and distal associations, respectively. These published associations were accessed by using the eQTL Browser (http://eqtl.uchicago.edu/cgi-bin/gbrowse/eqtl/), and we also included the results of our own eQTL study of the leukocyte transcriptome in the Moroccan population (36).

To test for genotype-by-infection interactions, we ran a model on the combined dataset (544,672 SNPs and 18,876 expressed transcripts) that accounts for SNP, malaria status, SNP × malaria status, sex, location, RBCs, and WBCs, where *m* is the mean measure of transcript abundance, and the error ε is assumed to be normally distributed with a mean of zero (model 2):

$$\text{Expression} = m + \text{SNP} + \text{malaria status} + \text{SNP} \times \text{malaria status} + \text{sex} + \text{location} + RBC_S + WBC_S + \varepsilon \quad [2]$$

Because testing for multiplicative SNP effects between the control and the infected group might be sensitive to differences in the representation of each group within each genotype class, we applied an additional filter to the list of SNPs in model 2 and excluded all SNPs not in Hardy–Weinberg equilibrium and with a MAF <10% within each of the subgroups tested. ENCODE data (30, 31) retrieved from the University of California (Santa Cruz), browser was used to facilitate the interpretation of the detected eSNP signal for the *SCO1* gene.

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Performance Evaluations

CE

ISO 13485:2003

EXTERNAL EVALUATIONS

Parascreen®

Rapid test for Malaria (Pan/Pf)

Study Report

Evaluation of Parascreen tests

Rural Kenya, May 2005

P.F. Mens

KIT Biomedical Research

(Koninklijk Instituut voor de Tropen / Royal Tropical Institute)
AMSTERDAM
The Netherlands

Context

Malaria is one of the leading parasitic diseases in the world. Annually 2-3 million people die from the disease mostly children, from resource pour countries, under the age of 5. Prompt and accurate diagnosis is essential for treatment of the patient. For years microscopy has been used as the method of choice for the diagnosis of malaria and is by most still considered to be the golden standard. This technique is however labour intensive and requires expert microscopy and good equipment. Therefore the search for good and simple diagnostics is still ongoing. In recent years several antigen detection tests have been developed; so called Rapid Diagnostic Tests. The simple system and ability of the tests to give a result in less than 20 minutes makes these tests convenient for field use. There is however a great need for good evaluation of the tests since more and more companies develop and distribute RDT's.

Objective

Compare and valuate the performance of Parascreen compared to the golden standard microscopy.

Methodology

The study was conducted in a mesoendemic area in the Cental Province of Kenya in during the transmission season of May 2005.

One hundred eighty-four samples were obtained from children between 6 months and 12 years with the clinical suspicion of uncomplicated Malaria. Finger prick blood was taken from these children and Rapid diagnostic tests were preformed on the spot under field conditions (ambient temperature) and interpretated within the time indicated by the manufacturer and. Another drop of finger prick blood was used to make thin and thick films for microscopy which were examined by experienced microscopists for 300 fields at 1000X magnification

Results

Of the 184 sampels 124 samples tested negative in microscopy and 60 samples positive for *Plasmodium* of which three samples were identified as having a *P. malariae* infection. The Parascreen tests correctly identified all 57 microscopically *P. falciparum* samples. One test tested positive for *P. malariae* even though in microscopic examination there were only *P. malariae* parasites found. 2 other tests correctly identified *P. malariea* infections as a non-falciparum positive test. Eight microscopically negative samples were found positive in the Parascreen tests (see table 1) 1 test failure (no control line visible) was observed in the study.

Tabel 1 Parascreen results compared to microscopy

Microscopy	Parascreen		
	Negative	positive	total
Negative for *P. falciparum*	*116*	*9*	*125*
Positive for *P falicparum*	*0*	*57*	*57*
Total	116	66	182

Conclusion

In our hands the sensitivity compared to microscopy is 100% with a specificity of 92.8% In order to estimate the agreement between the performed test and microscopy a kappa value was calculated; 0.89 which is a very good level of agreement beyond chance with a 95% confidence interval of 0.819- 0.961

The results will be published in **Tropical Medicine and International Health.** *Molecular biology the best alternative for diagnosis of malaria to microscopy? A comparison between microscopy, antigen detection and molecular tests in rural Kenya and urban Tanzania. (in press)*

Report on the field test of Malaria RDT "PARASCREEN" made by Zephyr Biomedials, India

From the 15 boxes (25 pcs tester per box) of RDT received from the Indonesian Red Cross Society headoffice (Jakarta), a field test was conducted in the County of East SUMBA, Province of East Nusa Tenggara consuming 10 boxes (250 tests) from 16 November 2005 to 1 December 2005. Balance of 5 boxes will be kept as bench mark in case it is necessary to do tests on same RDT but of different batches in the future. The location data are as follows:

Province:	East NUSA TENGGARA (NTT)		
Kabupaten	East SUMBA		
Kecamatan	PANDAWAI		KARERA
Desa (Village)	Kawangu	Kambatatana	Nggongi
Total Tests	74	132	44

Test Method:

Tests were done on 250 patients who suffered from cycles of high fever and chills and were suspected to be infected by malaria parasites. Blood samples of each patient was taken for both microscopic examination and RDT test. The test results are summarized as follows:

	Microscopic test results	RDT Parascreen test results		Remarks
P.Falciparum	47	39 pos	8 neg	6 : parasite count < 100 2 : parasite count = 299 & 315
P.Vivax	10	4 pos	6 neg	5 : parasite count < 100 1 : parasite count = 111
P.Falciparum +P.Vivax	3	3 pos	-	
Total	60	46	14	

1. On 11 blood samples where parasite counts were $< 100/\mu l$ in microscopic examination, the presence of parasites could not be detected by **Parascreen RDT** (Parascreen RDT showing negative results).
2. On 3 blood samples where parasite counts were $> 100/\mu l$ in microscopic examination (parasite count were 315, 299 and 111 respectively), the presence of parasites were not detected by **Parascreen RDT** (Parascreen RDT showing negative results).
3. On 46 blood samples where parasite counts were $> 100/\mu l$ in microscopic examination, the presence of parasites were detected by **Parascreen RDT** (Parascreen RDT showing positive results).

SUMMARY:

The accuracy of **Parascreen RDT** in detecting malaria parasites with parasite count ≥100 µl (by microscopic examination method) is 46/49 (94%) which is quite satisfactory.

Jakarta, 6th December 2005
Laboratory Officer-in-charge

/S/ Sri Suprijanto
Civil Servant ID No. 140065674
Staff of the Malaria Subdirectorate
Ministry of Health

Evaluation of Parscreen and Paramax malaria diagnostic tests Manufactured by M/S Zephyr Bio Medicals, Verna, Goa.

Testing Laboratory:
Malaria Research Centre (ICMR),
DHS Building, Campal, Panaji, Pin-403 001,
Goa, India.

Name of the Product: Parascreen and Paramax

Type of Product: Immunochromatic Rapid Diagnostic kit for malaria parasites *Plasmodium vivax* and *Plasmodium falciparum*

Principle Of diagnostic tests:
1. Parascreen: Immunochromatographic test capable of detecting pan malaria specific pLDH and Pf specific PfHRP-2.

A *P. falciparum* positive test will show both PfHRP-2 and pLDH detection lines.

P. vivax, P. malariae and *P. ovale* will show pLDH positive reaction only. In this case, the judgment of species could be made on the basis of local epidemiological situation and known Plasmodium species in the area. The limitation of the test is that the cases of mix infection of *Pv+Pf* can not be identified.

2. Paramax: Immunochromatographic test capable of detecting PfHRP-2, pan specific pLDH and *P. vivax* specific *Pv*LDH

A *P. falciparum* positive test would show both PfHRP-2 and pLDH detection lines.

A *P. vivax* positive case will show pLDH specific and *P. vivax* specific pLDH lines.

In case only pan specific LDH (pLDH) line appears and test is negative for PfHRP-2 and *P. vivax* specific LDH, then there could be either *P. malariae* or *P. ovale* but again the judgment of the third species could be made based on local distribution of Plasmodia.

The Paramax test is capable of detecting mix infection of *Pf* + *Pv* and

Pf + Po/Pm

Period of study: 8th March, 2004 to 29th March, 2004

Patients enrolled: 197 routine fever cases visiting for malaria test.

Type of blood sample used: Fresh whole blood directly from finger prick of fever cases in passive collection and detection facility. Thick and thin blood smears were simultaneously prepared for microscopy.

Time of reading of test: 15 minutes after test was applied as prescribed by the manufacturer.

Gold Standard Used for Comparison: Blood smear stained with Giemsa stain. Blood slides blinded and read by 3 qualified Laboratory Technicians independently.

Results: Results of the testing of the kit have been summarized in Table 1 and 2 given below.

Table. 1. Comparison of malaria diagnosis with microscopy with Parascreen and Paramax diagnostic tests

Nos.	Microscopy	Parascreen	Paramax
Total Tested (%+ve)	197 (68.0)	197 (68.0)	197(68.0)
Pv (% +ve)	68 (34.5)	68 (34.5)	68(34.5)
Pf (% +ve)	7 (3.55)	7 (3.55)	7(3.55)
Pv+Pf (% +ve)	0 (-)	0 (-)	0 (-)

Table: 2. Shows results of evaluation of Parascreen and Paramax malaria diagnostic tests

	P. falciparum (N=7)	*P. vivax* (N=68)	Overall
Sensitivity (%)	100	100	100
Specificity (%)	100	100	100
PPV (%)	100	100	100
NPV (%)	100	100	100
Efficacy (%)	100	100	100

PPV = Positive Predictive Value

NPV = Negative Predictive Value

Parasitaemia: In thick blood film parasites counted against 200 WBCs to work out parasitaemia / micro litre of blood taking 8000 WBCs per micro litre as standard

Parasitaemia Range :

1. *P. falciparum*: 400 - 22720 parasites/µl of blood

2. *P. vivax* : 520 - 33600 parasites /µl of blood

Inference: As table 2 reveals both Paramax and Parascreen diagnostic tests are of standard quality for the diagnosis of malaria showing absolute sensitivity, specificity and efficacy.

Officer – In – charge
Malaria Research Centre (I.C.M.R.)
Field Station
Directorate of Health Services Building
CAMPAL, PANAJI, GOA - 403001.

Malaria RAPID DIAGNOSTIC TEST
Quality Control Report
(101018)

Institut Pasteur du Cambodge

Report prepared by:	Seiha YEN and Dr Frédéric ARIEY
of (*Institute*): in cooperation with the World Health Organization,	Institut Pasteur du Cambodge **(IPC)**
for the attention of: *(name and institution)*	Dr. Christine Brandt and Dr. Thuy Huong Ha, UNICEF and Dr David BELL, WHO WPRO.

Date report prepared:	25/11/2005 dd/mm/yyyy

Summary of results

Testing Interval	Date tested dd/mm/yyyy	***Product (lot): 101018***	
		Result	Other Observations
0 months	24/11/2005	**PASS**	No comment

Interpretation of results: see next page.

Details of RDTs tested:

Name of RDT:	**Parascreen**
Manufacturer:	Zephyr Biomedicals
Lot no:	101018
Expiry: mm/yyyy	10.2007
Transport Method: From manufacturer.	DHL, Bangkok Airway.
Previous storage conditions:	N/A
Condition of RDTs on receipt:	The RDTs are in sealed boxes and appeared to be in good condition.

Initial RDT receipt details:

Sent from:	**Zephyr Biomedical as ordered by UNICEF**
Transport method/ condition to testing institute:	DHL
Quantity received:	6 kits (25 tests/kit)
Place received:	**IPC**
Date received: dd/mm/yyyy	24/11/2005

5, Boulevard Monivong, BP 983-Phnom Penh, Cambodia
Tel: (855) 12 802 011, (855) 12 445 164; Fax: (855) 23 725 606
farlev@pasteur-kh.org ; seihayen@pasteur-kh.org

Method:

1. **QC testing Method:**

   RDTs were tested with frozen QC samples based on the algorithm described in SOP 4.3 of the WHO Quality Control Methods Manual for Malaria RDTs. For a lot of RDTs to pass the QC assessment, all quality control dilutions must be positive (100%) and the negative control must be negative. RDTs which do not meet these criteria will be forwarded to a second laboratory for confirmation.

   The RDT lots will be retained in this laboratory for long term Quality Control. A further report will be issued after the next QC assessment.

2. **Samples used for QC testing:**

   Quality Control (QC) samples of dilutions from wild-parasites prepared according to SOP 5.2 of the WHO Quality Assurance Methods Manual for Malaria RDTs. Samples are stored at -80°C.

   Samples used include:

   a) Negative control: 0 parasites/µl of *Plasmodium sp.*
   b) Low Positive Control: 200 parasites/µl of *Plasmodium falciparum*
   c) High Positive Control: 2000 parasites/µl of *Plasmodium falciparum*
   d) Low Positive Control: 200 parasites/µl of *Plasmodium vivax*

3. **RDT preparation method:**

   RDTs were tested as per manufacturer instructions, using micropipette for blood transfer.

Details of RDT QC testing results: [0] month testing

Table 1: First Testing

Quality control dilutions		***Product (lot): 101041***		
Sample ID	(parasites/µl)	RDTs Tested	RDTs Positive	% Positive
C2F21	200	2	2	100
	2000	2	2	100
C2F3	200	2	2	100
	2000	2	2	100
C2V4	200	2	2	100
C2V8	200	2	2	100
		RDTs Tested	RDTs negative	% negative
negative control	0	1	1	100

Interpretation of results:

For a lot of RDTs to pass the QC assessment, all quality control dilutions must be positive (100%) and the negative control must be negative.

Interpretation of results:

• **PASS:** This RDT lot passed the quality control test and the sample assessed is SUFFICIENTLY SENSITIVE FOR USE in the field.

Note:
This RDT lot will be retained for long term Quality Control. A further report will only be issued after the next scheduled assessment (0 months).

This assessment is performed in collaboration with the World Health Organization, Regional Office for the Western Pacific. The report is prepared for the confidential information of the institution that submitted the rapid diagnostic tests (RDT) for assessment. The results are for use of the institution that submitted the RDTs for assessment as evidence that the stored samples of the particular lot of RDTs tested performed with sufficient sensitivity for use. They must not be used for purposes of advertising or otherwise promoting a product, or as evidence of formal approval or recommendation of a product, without the written permission of the testing institution and World Health Organization. Other than confirmation of sufficient sensitivity of the sample of the tested lot, the results listed here do not indicate endorsement of the RDT product by the World Health Organization or the

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testing institution. While the results indicate that the RDTs tested are sufficiently sensitive against the QC parasite samples used for testing, they do not necessarily reflect actual sensitivity in the field where local storage conditions, variation in parasite antigen, and host factors may affect operation. Recommendations on use and storage of RDTs in the field can be obtained from the WHO website www.wpro.who.int/rdt, or by email from mal-rdt@wpro.who.int

Signed:

Seiha YEN
Technician

Frédéric ARIEY
Laboratory head

Copies of report:
Include email copy to:

Procurer.
WHO (mal-rdt@wpro.who.int *or* belld@wpro.who.int)

Hard copy to be retained by testing institute.

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