Accuracy and Reliability of Malaria Diagnostic Techniques for Guiding Febrile Outpatient Treatment in Malaria-Endemic Countries

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Abstract. The main purpose of this study was to assess the accuracy of various techniques available for diagnosis of malaria. Blood samples were collected from 313 patients with clinical suspicion of uncomplicated malaria in 2 primary health centers in Madagascar. The presence of Plasmodium parasites was assessed by conventional microscopy, 2 rapid diagnostic tests (one HRP2-based test, PALUTOP+4, and one pLDH-based test, OptiMAL-IT), and real-time polymerase chain reaction (PCR), which was used as the “gold standard” method. The degree of agreement observed was very high for microscopy (0.99) and the HRP2-based test (0.93) and high for the pLDH-based test (0.82). Public-health implications are also discussed in this paper.

INTRODUCTION

One main element of global malaria control strategy for effective management is prompt and accurate diagnosis. However, in many endemic countries, such as Madagascar, the current approach to malaria diagnosis, especially in peripheral health centers, is entirely based on clinical diagnosis, which is of limited accuracy due to the poor specificity of symptoms and signs of malaria. Consequently, presumptive antimalarial treatment is widely administered for any fever with no obvious alternative cause, leading to significant overuse of antimalarial drugs, particularly throughout Africa. In addition, overdiagnosis of malaria in the formal healthcare sector often co-exists with underdiagnosis of malaria in the community.

With chloroquine (CQ) and sulfadoxine–pyrimethamine (SP) resistance becoming widespread and more effective but expensive antimalarial medicines, including artemisinin-based combination therapies (ACTs), being used in most African countries, there is a need to improve the diagnosis of acute febrile illness at various levels of the health care system; this is now a public health priority in Africa, as it would ensure that antimalarial drugs can be targeted to patients who need them.

The general view is that the “gold standard” method for malaria diagnosis is the detection of Plasmodium species by microscopic examination of blood films. This method is relatively simple and has low direct costs, but it is labor-intensive, time-consuming, and requires well-trained personnel who can differentiate between the different Plasmodium species. Alternative diagnostic tests for malaria and in particular rapid diagnostic tests (RDTs) have been developed over the past 20 years. These tests are fast, easy to perform, and do not require electricity or specific equipment. They include those based on histidine-rich protein 2 (HRP2) alone or a modified test format of HRP2 and parasite-specific aldolase enzyme (pan-malarial antigen), and those based on parasite lactate dehydrogenase (pLDH). The technical performances of these alternative techniques have already been assessed in various populations and epidemiologic settings. However, the main limitation of the majority of these studies was that microscopy was used as the “gold standard.”

The main purpose of this study was to assess the accuracy of different available techniques for the diagnosis of malaria, as a first step to improving malaria management at different levels of the health care system in Madagascar, where malaria is endemic. We designed our study to compare conventional microscopy, 2 RDTs (PALUTOP+4 and OptiMAL-IT tests), and real-time PCR using blood samples of patients with clinical suspicion of uncomplicated malaria.

METHODS

Field study. This prospective study was conducted between March and September, 2006, in 2 primary health centers in rural areas of Tsiroanomandidy on the west foothill areas of the Highlands in Madagascar. Malaria transmission in this area is low and predominantly seasonal. The main vector is Anopheles funestus, and the number of infective bites associated with Plasmodium falciparum is estimated to be 2.5 per person per year.

As part of the surveillance of antimalarial drug resistance in Madagascar, this study was reviewed and approved by the National Ethics Committee of the Ministry of Health and Family Planning of Madagascar (project no. 007-SANPF-2006).

Febrile patients with typical malaria symptoms presenting at the participating health facilities were given full information concerning the trial and invited to participate in the study. Once informed consent had been given, about 3 mL of venous blood was collected from all patients with an axillary temperature ≥ 37.5°C or history of fever in the past 24 hours. Pregnant women and patients with signs of severe and complicated falciparum malaria according to the definition given by WHO in 2001 were excluded. Blood, freshly collected in an EDTA anti-coagulation tube, was first used by a trained technician to prepare both thick and thin blood films. The blood sample was then given to a second trained technician who performed each of the immunochromatographic tests according to the manufacturers’ instructions. The following immunochromatographic tests were included in the study: (i) the OptiMAL-IT test (DiaMed AG, Cressier sur Morat, Switzerland, www.diamed.com, batch number 46110.85.01, expiration date: February 2007) designed to detect pLDH of both P. falciparum and other plasmodia and (ii) the PALUTOP+4 test.
test (ALL.DIAG Strasbourg, France, www.alldiag.com, batch number 91014, expiration date: November 2007) designed to detect *P. falciparum*-specific histidine rich protein-2 ( PfHRP-2), *P. vivax*-specific pLDH, and pan-malaria-specific pLDH. All tests were transported and maintained at the study site at room temperature and were opened just before use to avoid humidity damage. The reader, blind to other tests performed, examined the RDTs independently. 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 invalid (test failure) if the internal control was not visible. The results of RDTs were reported to the health center physician. Patients with a positive result for the RDT were promptly treated according to National Malaria Policy, with a combination of artesunate and amodiaquine (Arsucam, Sanofi-Aventis, Paris, France).

**Laboratory procedures.** Blood, freshly collected in EDTA anti-coagulation tubes, and blood films were sent to the Malaria Unit laboratory within 24 hours in a controlled cool box at 4°C. Thick and thin smears were stained with 4% Giemsa at 4°C. A microscopist who was blind to the results of RDTs performed microscopy, examined the blood films for the presence of parasites and identified the parasite species under a high-quality microscope (HumaScope, Human, Wiesbaden, Germany) with an incandescent light source. Each smear required approximately 20 minutes to read. A minimum of 200 consecutive fields were counted in the thick blood film before a slide was classified as negative. According to the parasite density, parasites in thick blood films were counted against 200 or 500 white blood cells. The parasite density was estimated assuming 8,000 white blood cells/μL of blood.

Parasite DNA was extracted from 100 μL of blood by treatment with 0.1 M NaOH for 3 min at 100°C. The supernatant was collected and treated with 250 μL of lysis solution (0.1 M Tris-HCl, Triton X-100, 1 M NaCl, 10× SDS, 0.5 M EDTA) and 20 mg/mL proteinase K for 1 h at 37°C. It was then extracted twice with phenol/chloroform (1:1). DNA was precipitated with ethanol, resuspended in 100 μL of distilled water, and stored at −20°C. *Plasmodium* species carriers were detected by real-time PCR, as described by Mangold and others,23 with a RotorGene 3000 thermocycler (Corbett Life Science, Sydney, Australia) with technicians blind to the results of microscopy and RDT testing. Briefly, each 25-μL reaction mixture contained 2–5 μL of sample DNA, 12.5 μL of qPCR MasterMix Plus for SYBR Green I No ROX (Eurogentec S.A., Seraing, Belgium), and 0.6 mM of each primer (PL1473F18, 5′-TAACgAACgAGATCTTAA-3′; and PL1679R18, 5′-gTTCCCTTCAgAAgCTTT-3′). 

**RESULTS**

**Patient recruitment.** Between March and September 2006, 313 patients, from age 6 months to 79 years (median age = 10.0 years, inter-quartile range 4–24.7 years), were recruited: 29.1% were < 5 years old, 34.3% between 5 and 14 years old, and 36.6% > 15 years old. The male/female ratio was 1.2:1. The mean axillary temperature was 37.8°C (SD ± 1.0°C, range 36–40.9°C). According to light microscopy, the mean parasite density on the 104 positive samples was 4,041 parasites/μL of sample DNA, 12.5 μL of qPCR MasterMix Plus for SYBR Green I No ROX (Eurogentec S.A., Seraing, Belgium), and 0.6 mM of each primer (PL1473F18, 5′-TAACgAACgAGATCTTAA-3′; and PL1679R18, 5′-gTTCCCTTCAgAAgCTTT-3′). PCR conditions consisted of an initial denaturation at 95°C for 10 min, followed by amplification for 40 cycles of 30 s at 95°C, 30 s at 56°C, and 90 s at 65°C, with fluorescence acquisition at the end of each extension step. Amplification was immediately followed by a melting program consisting of 2 min at 95°C, 2 min at 68°C, and a stepwise temperature increase of 0.5°C/s up to 90°C, with fluorescence acquisition at each temperature transition. The fluorescence data were analyzed using F1/F2 settings, which improved the detection of *P. falciparum*, and a cut-off of 40 cycles was used to define *Plasmodium*-positive samples.

**Data analysis.** Data were entered, processed, and analyzed using EpiInfo software (version 3.3.2, CDC, Atlanta, GA). The χ² test was used to compare the performance of the diagnostic methods. P values < 0.05 were considered to indicate statistically significant differences.

To assess sensitivity and specificity, results of microscopy and RDT were compared with real-time PCR results. The sensitivity was calculated as the proportion of positive test results obtained among samples scored as containing malaria parasites by real-time PCR; the specificity was calculated as the proportion of negative test results obtained among samples whose real-time PCR results were negative. Positive and negative predictive values were also calculated as the proportion of true-positive or true negative results among all samples scored as positive or negative by PCR, respectively. Kappa (κ) values, expressing the agreement beyond chance, were calculated. A κ value of 0.21–0.60 is a “moderate,” a κ value of 0.61–0.80 a “good,” and a κ value > 0.80 is an almost perfect agreement, beyond chance.24

### TABLE 1

<table>
<thead>
<tr>
<th>Field-study patients positive for <em>Plasmodium</em> spp. by real-time PCR, microscopy, PALUTOP&lt;sup&gt;44&lt;/sup&gt;, and OptiMAL-IT, Madagascar, March–September 2006</th>
<th>Microscopy</th>
<th>PALUTOP&lt;sup&gt;44&lt;/sup&gt;</th>
<th>OptiMAL-IT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Real-time PCR results</strong></td>
<td><strong>n (%)</strong></td>
<td><strong>Negative</strong>&lt;sup&gt;PF&lt;/sup&gt;</td>
<td><strong>Pv</strong>&lt;sup&gt;PF&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Negative</strong></td>
<td>205 (65.5)</td>
<td>205</td>
<td>0</td>
</tr>
<tr>
<td><strong>PF</strong></td>
<td>87 (27.8)</td>
<td>1</td>
<td>86</td>
</tr>
<tr>
<td><strong>Pv</strong></td>
<td>17 (5.4)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td><strong>Pf/Pv</strong></td>
<td>4 (1.3)</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>
Microscopy. The results of parasite detection by microscopy are shown in Table 1, and diagnostic performances are shown in Table 2.

Briefly, microscopy reported 104 slide-positive cases of malaria (33.2%). Species determination identified 86 slides with a monoinfection of *P. falciparum* ranging from 32 to 52,750 parasites/µL (mean count, 4,130 parasites/µL), 15 slides with a monoinfection of *P. vivax* ranging from 250 to 12,000 parasites/µL (mean count, 3,455 parasites/µL), 1 slide with a monoinfection of *Plasmodium malariae* (1,250 parasites/µL), and 2 slides with multi-infections of *P. falciparum/P. vivax* (440/640 and 1,500/7,250 parasites/µL). The *Plasmodium* parasite was not observed in 209 (66.8%) slides.

RDTs. PALUTOP™ testing gave the following results: in total, 90 cases were scored *P. falciparum*-positive; 16 cases *P. vivax*-positive; 2 cases *P. malariae*- or *Plasmodium ovale*-positive; and 3 cases both *P. falciparum*- and *P. vivax*-positive. OptiMAL-IT testing gave the following results: in total, 68 positive; and 3 cases both *P. falciparum*- and *P. vivax*-positive. 2 cases *P. malariae*-positive.

Diagnostic performances of the two RDTs were evaluated using real-time PCR as the “gold standard” (Table 2): for detection of monoinfection of *P. falciparum* malaria, the PALUTOP™ test was significantly more sensitive (95.4% versus 75.8%, *P* = 0.0001) and had a higher NPV (98.0% versus 90.6%, *P* = 0.0002) than the OptiMAL-IT test; for detection of non-*P. falciparum* malaria, the PALUTOP™ test was also significantly more sensitive (94.1% versus 85.7%, *P* = 0.005) and had a higher PPV (100.0% versus 94.7%, *P* = 0.0015) than the OptiMAL-IT test.

The sensitivity of each RDT was compared with that of the microscopy for the detection of both *P. falciparum* and *P. vivax* (Table 3). As expected, both RDTs had lower sensitivity for lower parasitemia. For *Plasmodium falciparum* samples with parasite density < 500 parasites/µL, the PALUTOP™ test was significantly more sensitive than the OptiMAL-IT™ test (for parasite density < 100 parasites/µL, *P* = 0.008, and parasite density 100–500 parasites/µL, *P* = 0.03).

Agreement between real-time PCR, microscopy, and RDTs. The performances of the various malaria diagnostic tests were compared (Table 4). With real time PCR, the degree of agreement was very high for microscopy (0.99) and the PALUTOP™ test (0.93) and high for the OptiMAL-IT™ test (0.82).

**DISCUSSION**

In light of changing drug policies in many African countries, where the expensive ACT drugs are now recommended as first-line antimalarial treatment, accurate laboratory confirmation of malaria would obviously be economically beneficial. We therefore assessed which method is best suited for guiding febrile outpatient treatment in malaria-endemic countries, such as Madagascar. Indeed, diagnosis relying only on clinical signs, as is frequent in Madagascar, always leads to

### Table 2

<table>
<thead>
<tr>
<th>Infection type</th>
<th>Malaria diagnostic technique</th>
<th>n</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
<th>PPV (95% CI)</th>
<th>NPV (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mono-infection with <em>P. falciparum</em></td>
<td>Microscopy</td>
<td>292</td>
<td>98.8 (94.4–99.9)</td>
<td>100.0</td>
<td>100.0</td>
<td>99.5</td>
</tr>
<tr>
<td></td>
<td>PaluTop™</td>
<td>292</td>
<td>95.4 (89.3–98.5)</td>
<td>97.1</td>
<td>93.3</td>
<td>98.0</td>
</tr>
<tr>
<td></td>
<td>OptiMAL-IT</td>
<td>292</td>
<td>75.8 (66.1–4.0)</td>
<td>99.0</td>
<td>97.1</td>
<td>90.6</td>
</tr>
<tr>
<td>Mono-infection with non-<em>P. falciparum</em></td>
<td>Microscopy</td>
<td>222</td>
<td>88.2 (66.2–97.9)</td>
<td>100.0</td>
<td>100.0</td>
<td>99.0</td>
</tr>
<tr>
<td></td>
<td>PaluTop™</td>
<td>222</td>
<td>94.1 (74.2–99.7)</td>
<td>100.0</td>
<td>100.0</td>
<td>99.5</td>
</tr>
<tr>
<td></td>
<td>OptiMAL-IT</td>
<td>226</td>
<td>85.7 (65.9–96.2)</td>
<td>99.5</td>
<td>94.7</td>
<td>98.5</td>
</tr>
<tr>
<td>Multi-infection with <em>P. falciparum/P. vivax</em></td>
<td>Microscopy</td>
<td>209</td>
<td>50.0 (9.4–90.5)</td>
<td>100.0</td>
<td>100.0</td>
<td>99.0</td>
</tr>
<tr>
<td></td>
<td>PaluTop™</td>
<td>209</td>
<td>75.0 (24.2–98.7)</td>
<td>100.0</td>
<td>100.0</td>
<td>99.5</td>
</tr>
</tbody>
</table>

### Table 3

<table>
<thead>
<tr>
<th>Parasite detection by microscopy</th>
<th><em>P. falciparum</em></th>
<th><em>P. vivax</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Parasite density (trophozoite/µL)</td>
<td>n</td>
<td>No. positive by PaluTop™ (%)</td>
</tr>
<tr>
<td>&lt; 100</td>
<td>14</td>
<td>11 (78.6)</td>
</tr>
<tr>
<td>100–499</td>
<td>9</td>
<td>9 (100)</td>
</tr>
<tr>
<td>500–4,999</td>
<td>45</td>
<td>45 (100)</td>
</tr>
<tr>
<td>&gt; 5,000</td>
<td>18</td>
<td>18 (100)</td>
</tr>
</tbody>
</table>
overestimation of the true incidence. We collected blood samples from patients with the clinical symptoms of malaria and analyzed them by microscopy and RDTs and compared the findings to the results real-time PCR used as the “gold standard.”

Overall, we found that microscopy examination performed better than RDTs (HRP2/pan-malarial antigen or pLDH dipsticks) for detecting malaria infections. This observation was consistent with the limit of detection reported in previous studies, 5–20 parasites/μL for conventional microscopy of Giemsa-stained peripheral blood films versus 100 parasites/μL for RDTs based on the detection of HRP-2 or pLDH antigens.

Our study also suggests that RDT based on HRP2/P. vivax-specific LDH/Plasmodium-specific LDH lactate dehydrogenase (PALUTOP) have a better degree of agreement with real-time PCR than those based on P. falciparum-specific LDH/Plasmodium-specific LDH lactate dehydrogenase (OptiMAL-IT). Like the WHO, we also observed that the HRP2-based test (PALUTOP) was more reliable and accurate than the pLDH-based test (OptiMAL-IT test) for detection of P. falciparum or non-P. falciparum infections because of (1) its better sensitivity (95.4% versus 75.8% for detection of P. falciparum infections and 94.1% versus 85.7%, for detection of non-P. falciparum infections) and (2) its low frequency of false negatives (0.96% versus 6.7%, P = 0.0004). Moreover, a recent report has shown that performance of malaria RDTs was adversely affected at high ambient temperature, as is prevalent in rural tropical zones, and the pLDH-based RDTs were more susceptible to heat-induced damage than HRP2-based RDTs.

It is obvious that the choice between HRP2-based RDTs and microscopy, the two best methods for malaria diagnosis, will depend on local circumstances, including available skills, electricity, and laboratory facilities, and also the use of microscopy for the diagnosis of other diseases. In Madagascar, the National Malaria Control Program (NMCP) will promote the improvement of the malaria microscopy in all district hospital settings. Use of RDTs and HRP2-based RDTs should be favored over pLDH-based RDTs, and should be considered as a means of extending parasite-based diagnosis only to areas where good microscopy cannot be guaranteed or is not available, such as at primary health care facilities or at the community level. Implementing this policy is likely to require a series of laboratory-focused interventions, including making high-quality microscopes available, regular training or re-training of laboratory technicians, strengthening routine supervision, establishing currently non-existent national quality-control systems (microscopy quality control and quality control of RDT batches), team building between laboratory and clinical staff, regular consensus reviews, and surveillance and trend analysis for laboratory-confirmed malaria.

Moreover, in the light of recent reports, significant efforts should made by policy makers to improve the use of antimalarial treatment in Madagascar: (1) by providing non-ambiguous national guidelines for febrile outpatient treatment in malaria-endemic zones, and especially for treatment of patients with negative tests; and (2) by changing the long-standing malaria diagnostic behavior of clinicians to encourage them to use laboratory results for malaria diagnosis and stop them over-prescribing antimalarial drugs.

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