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Comparison of Two Commercial Assays with Expert Microscopy for Confirmation of Symptomatically Diagnosed Malaria

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Conventional light microscopy has been the established method for malaria diagnosis. However, recently several nonmicroscopic rapid diagnostic tests have been developed for situations in which reliable microscopy may not be available. This study was conducted to evaluate the diagnostic performance of a recently introduced ICT Malaria Pf/Pv test. This assay detects *Plasmodium falciparum* histidine-rich protein 2 antigen (PfHRP-2) for *P. falciparum* diagnosis and pan-malarial antigen for *P. vivax* diagnosis. In this study we compared the performance of ICT Malaria Pf/Pv with microscopy of Giemsa-stained blood films and with an OptiMAL test that detects *Plasmodium* lactate dehydrogenase (pLDH) antigen. A total of 750 clinically suspected malaria patients were examined at local health centers in Kuwait. Both the antigen tests had a high degree of specificity (>98%) for detection of malaria infection. However, they were less sensitive than microscopy. Compared with microscopy the ICT Malaria PF/pf test failed to detect malaria infection in 93 (34%) of 271 malaria patients (11% of patients with *P. falciparum* and 37% of patients with *P. vivax*) and the OptiMAL test failed to detect malaria infection in 41 (15%) of 271 malaria patients (7% of patients with *P. falciparum* and 13% of patients with *P. vivax*). The sensitivities of the ICT Malaria Pf/Pv and OptiMAL tests for detection of *P. falciparum* infection were 81 and 87%, and those for detecting *P. vivax* were 58 to 79%, respectively. The sensitivity of the ICT Malaria Pf/Pv and OptiMAL tests decreased significantly to 23 and 44%, respectively, at parasite densities of <500/μl. Both of the tests also produced a number of false-positive results. Overall, the performance of the OptiMAL test was better than that of the ICT Malaria Pf/Pv test. However, our results raise particular concern over the sensitivity of the ICT Malaria Pf/Pv test for detection of *P. vivax* infection. Further developments appear necessary to improve the performance of the ICT Malaria Pf/Pv test.

In malaria patients, a prompt and accurate diagnosis is the key to effective disease management. The two diagnostic approaches currently used most often, clinical diagnosis and microscopic diagnosis, however, do not allow a satisfactory diagnosis of malaria. Clinical diagnosis is the most widely used approach; however, the symptoms of malaria are very nonspecific and overlap those of other febrile illnesses (32). A diagnosis of malaria based on clinical grounds alone is therefore unreliable and, when possible, should be confirmed by laboratory tests. Microscopic examination of thick blood film is currently the standard method for malaria diagnosis. This method is relatively simple and has low direct costs, but its reliability is questionable, particularly at low levels of parasitemia and in the interpretation of mixed infection (17, 31).

Recently, rapid antigen detection methods have been developed for situations in which reliable microscopy may not be available. These tests are based on the detection of antigen(s) released from parasitized red blood cells (19). In the case of *Plasmodium falciparum*, these new methods are based on detection of *P. falciparum* histidine-rich protein 2 (HRP-2) (*Para*Sight F by Becton Dickinson, Cockeysville, Md., and the ICT Malaria Pf by ICT Diagnostics, Sydney, Australia) (2, 5, 26, 30; M. Garcia, S. Kirimoama, D. Marlborough, J. Leafasia, and K. H. Rieckmann, Letter, Lancet **347:**1549, 1996) or *Plasmodium*-specific lactate dehydrogenase (pLDH) (OptiMAL by Flow Inc., Portland, Oreg.) (12, 13, 18, 20). Species-specific pLDH isoforms have been used to develop a test for *Plasmodium vivax* (OptiMAL).

The sensitivity and specificity of each of these tests have been assessed in a range of clinical situations (2, 5, 12–14, 18–20, 26, 27; Garcia et al., letter). Although the overall sensitivity and specificity of all these assays for detection of *P. falciparum* infection are high (usually >90%), the sensitivity falls off at parasite densities of <350/μl. Both the ICT Malaria Pf and *Para*Sight F tests do not detect *P. vivax* infection. Recently, a new test kit capable of detecting antigen of *P. falciparum* and *P. vivax* has been introduced: ICT Malaria Pf/Pv (AMRAD ICT, Frenchs Forest, New South Wales, Australia) (3, 6, 10, 15). The performance of this test has yet not been fully assessed.

In this study we compared the sensitivities of the OptiMAL and ICT Malaria Pf/Pv tests in symptomatically diagnosed malaria patients against microscopy of thick or thin blood films.

MATERIALS AND METHODS

Patients. Seven hundred fifty patients with a clinical suspicion of malaria attending the local district hospitals and health centers participated in this study. The study was conducted during the period September 1999 to March 2002 in Kuwait. The majority of these patients were workers from tropical countries where malaria infection is endemic. There is no malaria transmission in Kuwait; however, each year more than 800 imported malaria cases are reported (11).

The symptomatic diagnosis of malaria was based on the presence of fever (temperature > 37.5°C) at the time of presentation to the health centers, or within the previous 48 h, coupled with a recent history of living in or visiting a malaria-prone country. Patients were between 2 and 50 years old. All patients who had been treated for malaria in the previous 4 weeks were excluded from the study. For each patient, a finger prick was made and the following was collected:

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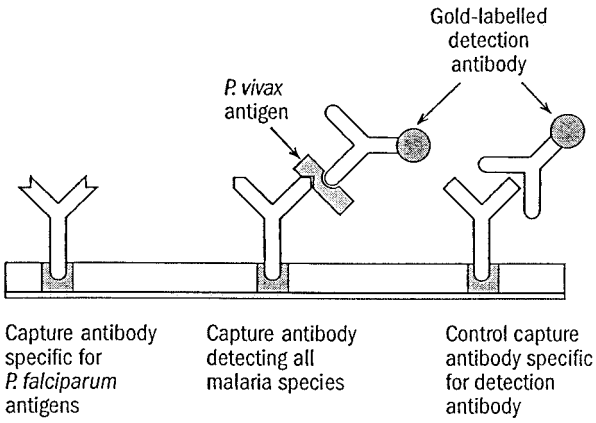

FIG. 1. Schematic representation of immunologic reaction on a positive strip (*P. vivax* infection).

50 μl of blood in a preheparinized Eppendorf tube for the ICT Malaria Pf/Pv and OptiMAL tests and one thick or thin blood smear for Giemsa stain microscopy. The microscopists, ICT reader, and OptiMAL reader were all blinded to each other's diagnoses. Informed consent to participate in the study was obtained from participants, and the Ethical Committee of the Faculty of Medicine, University of Kuwait, approved the study.

Microscopy of Giemsa-stained blood films. Thick and thin blood films were stained with 10% Giemsa stain for 10 min and examined by two experienced microscopists who had no knowledge of patient disease status or nationality to avoid any bias in blood film readings. The microscopist counted a minimum of 200 consecutive fields in the thick blood film before classifying a slide as negative.

Parasites in thick blood film were counted against 200 to 500 white blood cells. The parasite density was estimated assuming 8,000 white blood cells/μl of blood (19, 29, 31).

OptiMAL test. The OptiMAL test was performed following manufacturer's instructions. Briefly, 1 drop of whole blood was mixed with 2 drops of lysis buffer A, which disrupts the red blood cells and releases the pLDH, and the specimens were allowed to migrate to the top of the pLDH strip. After 8 min the strip was placed in washing buffer B, which cleared the hemoglobin from the strip. Interpretation of the test results was performed immediately. A negative control sample taken from an individual who had not been exposed to malaria for 3 years was included with each batch tested. In the pLDH assay there are two diagnostic zones of reaction containing different antibodies (Fig. 1). A monospecific antibody that recognizes only *P. falciparum* is present in the bottom reaction zone. A second, pan-specific antibody is present immediately above this zone. This monoclonal antibody recognizes the pLDH isoforms of *P. vivax*. A third reaction zone containing a pan-specific monoclonal antibody is present at the top of the test strip and serves as a procedural control for the assay.

ICT Malaria Pf/Pv test. The procedural principle of the ICT Malaria Pf/Pv test is the same as that shown in Fig. 6. This test is based on detection of the *P. falciparum*-specific HRP-2 antigen and a pan-malarial antigen (*P. vivax*). The test was performed according to the manufacturer's instructions. Briefly, 10 μl of whole blood was added to a sample pad. A buffer reagent was added to induce cell lysis and allow *P. falciparum* HRP-2 antigen (PfHRP-2) and pan-malarial antigens to bind to colloidal gold-labeled antibody. Additional buffer caused the blood and immune complexes to migrate up the test strip and cross monoclonal antibody lines. Finally, more buffer was added to clear hemoglobin from the strip and facilitate reading. Tests were counted as valid if a control line was observed. They were considered *P. falciparum* positive if PfHRP-2-specific and pan-malarial antigen lines were visible or if only PfHRP-2-specfic lines were seen. If only the control and pan-malarial antigen lines were observed, the sample was counted as positive for a malaria parasite other than *P. falciparum*.

Statistical analysis. Data were collected and analyzed using the SPSS statistical program. For sensitivity and specificity the test kits were compared with Giemsa stain-microscopy results. The sensitivity was calculated as the proportion of positive test results obtained among samples containing malaria parasites by microscopy; the specificity was calculated as the proportion of negative test results obtained among samples whose thick blood films were negative. Positive and negative predictive values were calculated as the proportion of true-positive results among all positively reacting samples and as the proportion of true-negative results among all negatively reacting samples, respectively.

RESULTS

Among the 750 symptomatically diagnosed malaria patients included in this study, 187 (25%) patients presented with microscopically confirmed vivax malaria. A further 54 (7%) were infected with *P. falciparum*. Twenty-four patients had mixed infections with *P. falciparum* and *P. vivax*, two were infected with *P. malariae*, and four patients had *P. ovale* infections (Table 1).

The results of the detection of malaria by the two rapid antigen detection tests in comparison with microscopy are shown in Table 1. The ICT Malaria Pf/Pv test detected malarial antigen in 178 patients: 48 patients with *P. falciparum* infection, 118 with *P. vivax* infection, and 12 with mixed (*P. falciparum* and *P. vivax*) infection. The OptiMAL test detected malarial antigen in 230 patients: 50 patients with *P. falciparum* infection and 162 patients with *P. vivax* infection (Table 1). *P. malariae* and *P. ovale* were not detected by either of the antigen assays. Overall, the OptiMAL test was a better test than the ICT Malaria Pf/Pv test for detecting both *P. falciparum* and *P. vivax* infection. Using microscopy as the standard test, the ICT Malaria Pf/Pv test failed to detect malaria infection in 93 (34%) of 271 malaria patients (11% of patients with *P. falciparum* and 37% of patients with *P. vivax*), and the OptiMAL test failed to detect infection in 41 (15%) of 271 malaria patients (7% of patients with *P. falciparum* infection and 13% of patients with *P. vivax*). Compared with microscopy the performance of the ICT Malaria Pf/Pv test for *P. falciparum* and *P. vivax* parasites was as follows: sensitivity, 81 and 58%; specificity, 99 and 98%; positive predictive value, 92 and 92%; negative predictive value, 98 and 88%, respectively. The comparative performance of the OptiMAL test for *P. falciparum* and *P. vivax* parasites was as follows: sensitivity, 87 and 79%; specificity, 99 and 97%; positive predictive value, 94 and 91%; negative predictive value, 99 and 93%, respectively.

The results of sensitivity testing according to parasitemia are shown in Table 2. As expected, both the antigen tests performed suboptimally at parasitemias of <500 parasites/μl (ICT Malaria Pf/Pv test, 23% performed suboptimally; OptiMAL test, 44% performed suboptimally), but they had good sensitivity above this cutoff (ICT Malaria Pf/Pv test, 77% sensitivity; OptiMAL test, 96% sensitivity). However, it was of particular concern that both the tests failed to detect at least five patients

TABLE 1. Parasite species detected in 750 clinically suspected malaria patients

Species	No. of patients infected		
	Microscopy	ICT malaria Pf/Pv test	OptiMAL test
P. falciparum	54	48	50
P. vivax	187	118	162
Mixed (*P. falciparum* and *P. vivax*)	24	12	18
P. malariae	2	0	0
P. ovale	4	0	0
Total positives	271	178	230
Total negatives	479	572	619

TABLE 2. Parasite densities of specimens from patients with microscopy-confirmed malaria infections

Parasite density (parasites/μl)	No. of specimens with indicated density as determined by:		
	Microscopy	ICT Malaria Pf/Pv test	OptiMAL test
<500	48	11	21
500–5,000	192	144	188
>5,000	25	23	21
Total	265	178	230

(two by ICT Malaria test and three by OptiMAL test) that had parasitemias of >5,000/μl (Table 2). In addition, both the ICT Malaria Pf/Pv and OptiMAL tests yielded positive tests for *P. falciparum* and *P. vivax* infection with samples which were negative by microscopy (ICT Malaria test, four cases of falciparum infection and 9 cases of vivax infection; OptiMAL test, three cases of falciparum infection and 14 cases of vivax infection).

DISCUSSION

The development of easy, rapid, and accurate tests for the detection of plasmodial infection is highly desirable. In this study we investigated the performance of the ICT Malaria Pf/Pv test and OptiMAL test, relative to microscopy, with samples from symptomatically diagnosed malaria patients. Previous studies of OptiMAL and ICT tests have shown variable results (2, 5, 12–14, 18–20, 26; Garcia et al., letter).

Compared with microscopy, the ICT Malaria Pf/Pv test performed well with *P. falciparum* (sensitivity 81%); however, it failed to detect *P. vivax* infection in 69 (42%) patients.

The results of this study show that both ICT Malaria and OptiMAL tests detect falciparum malaria with high sensitivities: 81 and 87%, respectively. Detection of falciparum antigen by both the tests was very specific (99%). Similar sensitivities and specificities have been recorded for other test kits detecting the HRP-2 antigen of *P. falciparum* (5, 12–14, 18–20, 26; Garcia et al., letter). However, data regarding the detection of *P. vivax* using the ICT Malaria Pf/Pv test are scarce and variable. The sensitivity of the ICT Malaria Pf/Pv test for vivax malaria conducted at the village level in Myanmar was 66.7 to 79% (3), and that within the Network TropNet Europ was 76% (15). Compared with microscopy the performance of this test to detect vivax infection was less satisfactory (sensitivity, 58%; specificity. 98%) in our study, and the sensitivity fell to only 23% in patients with parasitemias of <500/μl. Further studies with a large set of patients with *P. vivax* infection from various areas of endemicity need to be investigated before definitive recommendations for the use of the ICT Malaria Pf/Pv test kit in diagnosing vivax malaria are possible.

Both the ICT Malaria Pf/Pv and OptiMAL tests detected *P. falciparum* and *P. vivax* infections in samples which were negative by microscopic examination (ICT Malaria Pf/Pv test, four patients with falciparum infection and nine patients with vivax infection; OptiMAL test, three patietns with falciparum infection and 14 patients with vivax infection). False-positive tests have been reported in earlier investigations (3, 12, 13), but they appear to play a minor role in evaluating the usefulness of malaria tests for clinical settings. False-positive reactions may occur in individuals who have been recently treated for malaria as reported earlier (2, 24–26) or if patients have circulating rheumatoid factors (3, 15; M. P. Grobusch, U. Alpermann, S. Schwenkl, T. Jelinek, and D. C. Warhurst, Letter, Lancet **353:** 297, 1999). The preliminary data show that PfHRP-2 antigen, which is detected by the ICT Malaria Pf/Pv test, may persist for up to 7 to 10 days after asexual parasite clearance (7, 24; Garcia et al., letter), whereas circulating pLDH activity, which is detected by the OptiMAL test, drops profoundly immediately after the parasites are cleared from the peripheral blood (21). Thus, the OptiMAL test may provide the potential to monitor the effectiveness of antimalarial therapy and thus assist in the detection of drug-resistant infections.

In addition, false-negative results by microscopy can occur if patients have undertaken self-medication prior to presentation. It is likely that some of our patients with false-positive results may have performed self-medication with antimalarial drugs during an attack of fever. However, it is unlikely that these factors account for the entire set of false-positive cases. It is more probable that most of the false-positive cases were true positives which were not detected by microscopy, due to sequestration limiting the number of circulating parasites at the time of blood collection or due to the parasitemia being below the detection limit of approximately 50/μl by microscopy.

The performance of both the ICT Malaria Pf/Pv and OptiMAL tests was greatly influenced by the level of parasitemia in peripheral blood. The sensitivity of the OptiMAL test was 96% and that of the ICT Malaria test was 77% at parasitemias of >500/μl; however, the sensitivity dropped to 44 and 23%, respectively, at parasitemias of <500/μl. Our findings are consistent with earlier findings (8, 12, 17, 19, 22, 23, 27, 32). This can potentially be dangerous, as to miss the diagnosis of malaria in an ambulant, febrile patient may mean that complications develop because appropriate treatment was not instituted in time. The assessment of a negative result in this situation will be clearly influenced by the clinical features. In this study both the antigen test kits missed at least five cases of malaria in samples with high-level parasitemia (>5,000 parasites/μl). False-negative dipstick test results in samples with higher parasitemias have been observed in earlier studies, but the underlying reason is not known (1, 4, 9, 16, 20, 28).

In conclusion, our results add to the evidence that these nonmicroscopical rapid tests for the detection of plasmodial antigens may develop into important diagnostic tools and can prove to be a valuable adjunct to clinical assessment of the patient and blood film microscopy under certain circumstances. These tests are rapid and simpler to perform and to interpret. However, their sensitivity indicates that they should not yet be regarded as first-line diagnostic tests. In our study the performance of the OptiMAL test to detect *P. falciparum* and *P. vivax* infection in symptomatically diagnosed patients was better than that of the ICT Malaria Pf/Pv test. Our results raise particular concern over the sensitivity of the ICT Malaria Pf/PV test for nonfalciparum (*P. vivax*) infection, which was only 58%. Thick blood film examination is still the standard method for diagnosing malaria because it detects all *Plasmo-*

dium species and offers the clear distinctions between parasite growth stages, which are essential for therapeutic decisions.

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