

SENSITIVITY AND SPECIFICITY OF AN ANTIGEN DETECTION ELISA FOR MALARIA DIAGNOSIS

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Abstract. Enzyme-linked immunosorbent assays (ELISAs) allow for the testing of large numbers of samples within a short time frame. We tested the sensitivity and specificity of a histidine-rich protein 2 (HRP2)-based, commercially available ELISA antigen detection assay for *Plasmodium falciparum* (Malaria Antigen CELISA; Cellabs, Sydney, Australia). A total of 700 whole blood samples obtained from symptomatic outpatients of malaria clinics along the Thai–Myanmar border were tested relative to blinded duplicate expert microscopy adjusted with species-specific polymerase chain reaction (PCR). PCR-adjusted microscopy showed that 79 (11.3%) were infected with *P. falciparum*, 118 (16.9%) with *P. vivax*, 1 (0.1%) with *P. malariae*, 7 (1.0%) with mixed infections (*P. falciparum* and *P. vivax*), and 495 (70.7%) were negative. The geometric mean parasite density for *P. falciparum* was 7547/μL (range: 12–363,810/μL). The overall sensitivity of the HRP2 ELISA for *P. falciparum* malaria was 98.8% (95% CI, 93.6–100%) and the specificity was 100% (95% CI, 99.5–100%). The positive and negative predictive values for the ELISA were 100% (95% CI, 96.5–100%) and 99.8% (95% CI, 99.1–100%), respectively. The results for *P. falciparum* were clearly superior to expert microscopy alone, particularly in mixed infections. Microscopy combined with ELISA reaches a sensitivity and specificity similar to PCR-adjusted microscopy for the diagnosis of *P. falciparum* while being considerably less expensive and faster. We conclude that ELISA serves as an excellent tool to augment microscopy as the gold standard for *P. falciparum* diagnosis in research settings and should be further evaluated for screening in blood banks.

INTRODUCTION

In recent years, devices for the diagnosis of *Plasmodium falciparum* malaria based on histidine-rich protein 2 (HRP2) have significantly gained importance.^{1,2} The abundance of the antigen and the resulting high sensitivity of the diagnostic devices combined with the simplicity of their application makes them an obvious alternative in settings where microscopy is not available or not of sufficiently high quality standard.^{3,4}

Nonetheless, the detection of asexual parasites by light microscopy of Giemsa-stained thick and thin films still remains the gold standard for malaria diagnosis. The detection of low parasite densities and mixed infections, however, poses a significant challenge, even to highly experienced microscopists.⁵ However, the highest achievable levels of sensitivity and specificity are of enormous importance, not just for the early diagnosis of this potentially life-threatening disease, but also for malaria research, such as the validation of new diagnostic devices or for epidemiologic studies. In these cases, advanced techniques, such as polymerase chain reaction (PCR) or enzyme-linked immunosorbent assay (ELISA) may provide the required additional sensitivity to augment the gold standard for the diagnosis of falciparum malaria.^{6,7}

In this study, we validated the sensitivity and specificity of a commercial ELISA test kit based on HRP2 (malaria antigen CELISA; Cellabs, Sydney, Australia) for the diagnosis of *P. falciparum* malaria against Giemsa-stained light microscopy adjusted with species-specific PCR in a population with symptoms consistent with malaria.

MATERIALS AND METHODS

Study site. The study was performed along the Thai–Myanmar border, at the malaria clinics of Chedi Koh and So Oh, Tak Province, under the responsibility of the Mae Sot Vector Borne Disease Control Unit in Tak Province, Thailand. The study was approved by the Ethical Review Committee for Research in Human Subjects, Ministry of Public Health, Nonthaburi, Thailand, and the Human Use Review Committee (HURC) of the Walter Reed Army Institute of Research, Silver Spring, MD.

Subjects and sample collection. A total of 700 adults seeking diagnosis and treatment at the two malaria clinics were enrolled into the study. Eligible subjects were those ≥ 20 years old with symptoms of an oral temperature of $\geq 38^{\circ}\text{C}$, headache, or history of fever within the past 72 hours. Written informed consent was obtained from all study participants, and signs and symptoms of malaria, travel, and medication history were assessed. Whole blood was collected in sterile EDTA tubes, and thick and thin blood films were prepared in duplicate immediately after blood draw from venous blood. Immediately after completion of the study procedures, the patients were released to standard medical care by the staff of the malaria clinics. A sample from the blood specimen was processed for a white blood cell (WBC) count using an automated blood cell counter (Coulter T-890; Beckman-Coulter, Fullerton, CA).

Malaria antigen CELISA. The ELISA kit (Malaria Ag CELISA; Cellabs) evaluated in this study is a commercial ELISA test kit designed as a confirmatory test for *P. falciparum* malaria and is similarly used in recently developed drug sensitivity assays.⁸ It is based on the detection of HRP2, a highly sensitive marker of falciparum malaria, in blood samples. The EDTA blood samples were frozen-thawed twice to obtain full hemolysis before being tested in the ELISA.⁹ One hundred microliters of the samples was transferred into the ELISA in duplicate. Positive and negative controls, as

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well as serial dilutions of positive controls, were tested on every plate. Forty samples were tested on each 96-well plate. The plates pre-coated with monoclonal antibodies against PfHRP2 (capture antibody of IgM class; code: CPF4) were incubated at room temperature for 1 hour. Subsequently, the plates were washed five times with the provided washing solution, and 100 μ L of the diluted Ab-conjugate (indicator antibody of IgG1 isotype; code: CPF6) was added to each well. After further incubation for 1 hour, the plates were once again washed five times, and 100 μ L of the diluted TMB chromogen (1:20) was added to each well. The plates were incubated for another 15 minutes in the dark, and 50 μ L of the stopping solution was added. Spectrophotometric analysis was performed with an ELISA plate reader (SpectraMAX 340 Microplate Spectrophotometer; Molecular Devices, Sunnyvale, CA) at an absorbance maximum of 450 nm. The complete ELISA takes ~3 hours to perform.

Reference microscopy. Thick and thin blood films were prepared immediately by drawing aliquots from the venous blood. Using a micropipette, 6 μ L of blood was placed on a pre-cleaned slide for the thick smear and 4 μ L of blood from the same sample was placed on the slide for the thin smear. Two such slides were made from each sample. The slides were thoroughly dried overnight before being stained for 25 minutes in 5% Giemsa solution. One slide was stored for reference; the other was read by two expert microscopists blinded to each other's readings and the ELISA results. The thick films were screened for 200 oil-immersion fields ($\times 100$ oil immersion objective) before declaring a film to be negative. If positive, parasite species were separately counted against 200 WBC or against 500 WBC if < 10 asexual parasites were found after reaching the 200th WBC. The automated WBC count was used as the basis for the calculation of parasite density. Any disagreement between the two microscopists as to the presence of parasitemia, quantity of asexual parasitemia outside of a factor of 2, or *Plasmodium* species was considered to be non-concordance. In these cases ($N = 29$), the slides were re-examined by a reference microscopist, whose reading was accepted as final.

Polymerase chain reaction. In all samples ($N = 12$) in which there was a qualitative discrepancy between microscopy and CELISA results, molecular assays were done from the EDTA blood. Nested PCR was performed to amplify species-specific sequences of the small subunit RNA (18s SSU rRNA).¹⁰ The PCR technician was blinded to the results of microscopy and ELISA.

Data analysis. For the performance characteristics, the following values were used: true positive (TP), false positive (FP), true negative (TN), and false negative (FN). Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and pre-test probability (PTP) were calcu-

lated. Exact confidence intervals were calculated following the method of Clopper and Pearson.¹¹

RESULTS

Of the 700 individuals who were enrolled in the study, 480 (68.6%) were men and 220 (31.4%) were women. All patients were symptomatic. The age ranged from 20 to 65 years (overall median: 26 years; 25 years for men and 29 for women).

ELISA test results were considered positive when the optical density was > 0.1 over the negative control. Of 700 individual tests, 28 had to be repeated because the result was not interpretable or the duplicate tests showed non-concordant results. None of the tests remained non-concordant on repeated testing.

PCR-adjusted blinded duplicate microscopy showed that 79 (11.3%) were infected with *P. falciparum*, 118 (16.9%) with *P. vivax*, 1 (0.1%) with *P. malariae*, 7 (1.0%) with mixed infections (*P. falciparum* and *P. vivax*), and 495 (70.7%) were negative. The geometric mean parasite density was 7,547/ μ L (range: 12–363,810/ μ L) for *P. falciparum*, 1,854/ μ L (range: 10–103,982/ μ L) for *P. vivax*, and 418/ μ L for *P. malariae*. The pre-test probability for *P. falciparum* was 12.1% (95% CI: 9.9–14.8%).

The overall sensitivity of the HRP2 ELISA for *P. falciparum* malaria was 98.8% (95% CI: 93.6–100%), and the specificity was 100% (95% CI: 99.5–100%; Table 1). The PPV for the ELISA was 100% (95% CI: 96.5–100%), and the NPV was 99.8% (95% CI: 99.1–100%). One sample with a parasite density of 2,194/ μ L tested negative in the ELISA while testing positive in microscopy and PCR, as well as in a HRP2-based rapid diagnostic device (NOW ICT Malaria Pf/Pv; Binax, Portland, ME). *P. falciparum* was reliably detected in all mixed infections. None of the *P. vivax* or *P. malariae* infections or malaria negative samples tested positive in the HRP2-ELISA.

Compared with PCR-adjusted microscopy, duplicate expert microscopy alone had a sensitivity of 92.9% (95% CI: 85.3–97.4%) and a specificity of 98.8% (95% CI: 99.1–100%) for the detection of *P. falciparum*. The PPV and NPV for the microscopy were 98.9% (95% CI: 93.7–100%) and 98.8% (95% CI: 99.1–100%), respectively. Microscopy missed mixed infections with *P. falciparum* in four cases, which were diagnosed as *P. vivax*. In addition, one *P. falciparum* monoinfection and one mixed infection were read as negative by microscopy. Only two of seven mixed infections were correctly diagnosed by expert microscopy. Interestingly, four mixed infections were diagnosed as *P. vivax* only, one as negative, and none as *P. falciparum* only.

The optical density values measured in the ELISA corre-

TABLE 1

Cross-tabulation of CELISA and unadjusted duplicate expert microscopy by PCR-adjusted microscopy for *P. falciparum* asexual parasites only

PCR-adjusted microscopy	CELISA		Microscopy alone (<i>P. falciparum</i> asexual parasites only)	
	Negative	Positive	Negative	Positive
Negative	615	0	614	1
<i>P. falciparum</i> asexual	78	77	1	77
Mixed <i>P. falciparum</i> and <i>P. vivax</i>	7	7	5	2
Total	700	84	620	80

lated with asexual *P. falciparum* parasite densities ($R = 0.367$; $P = 0.001$ on log-transformed values). There was no obvious relation between parasite density and the sensitivity of the ELISA (Table 2).

DISCUSSION

Few situations are conceivable where ELISA would be used in everyday clinical diagnosis of malaria. However, in research settings and similarly for blood bank screening, where large numbers of samples have to be screened, ELISA can provide a fast, relatively inexpensive, and reliable way to detect *P. falciparum*.¹² In these settings, ELISA may serve as a suitable adjunct to microscopy. The fact that HRP2-based ELISA test kits and monoclonal antibodies are available on the market greatly improves the applicability of these assays for the diagnosis of *P. falciparum*.

Unlike many malaria rapid diagnostic tests, the sensitivity of the ELISA did not decrease with parasite density. Even low parasite densities were reliably detected in this HRP2 ELISA. One sample that was diagnosed in the ELISA had a parasite density of only 12 asexual parasites/ μL , considerably below the detection limit for most microscopists.⁵ Validation of the ELISA in a population of asymptomatic persons with *P. falciparum* infection, who may carry lower parasite burdens, should be performed to better define the use for blood bank screening.¹³

Mixed infections are a particular challenge for microscopy. The reason obviously lies in the morphologic similarities between early developmental stages of malaria parasites of different species. In Thailand, ~8% of patients treated for *P. vivax* malaria are subsequently found to be co-infected with *P. falciparum*.¹⁴ The biggest challenge seems to be the detection of *P. falciparum* parasitemia when it is superimposed by *P. vivax*. In these cases, additional tests, such as PCR or ELISA, are essential for an accurate diagnosis.

Despite all its advantages, ELISA will not be able to replace microscopy. Microscopy is still more flexible and offers the immense advantage of providing species diagnosis and exact parasite densities. Compared with PCR, the detection of HRP2 in an ELISA is considerably faster and cheaper. However, currently, the availability of ELISA test kits only for *P. falciparum* limits the diagnostic options. For the additional time and money, PCR provides data for species differentiation, which an ELISA based on antibodies directed against a single antigen cannot provide. Some rapid diagnostic tests (RDTs) use a second, pan-genus antibody (such as

against aldolase) for detecting malaria species other than *P. falciparum*. The addition of such an antibody, or even better, of species-specific antibodies, to the ELISA procedure could greatly benefit the application of these tests for other *Plasmodium* species. This would also overcome the problem found with many RDTs of not being able to distinguish between *P. falciparum* mono-infections and mixed infections.

The sensitivity of microscopy generally varies with the skill and experience of the slide reader, quality of smear preparation and staining, microscope quality, magnitude of parasitemia, and number of fields read. The potential role of an FDA-approved RDT particularly in settings where malaria microscopy is either not available or of poor quality should therefore not be underestimated.

The relatively weak correlation between optical density readings and parasite densities can be explained by the fact that not all ELISA plates were processed at the same time and that some plates therefore had considerably higher values for positive controls than others. Moreover, samples with higher parasite densities quickly exceed the dynamic range of the ELISA and therefore require serial dilutions of the samples to get accurate HRP2 measurements.^{15,16}

The high sensitivity rates found in this study confirm that HRP2 is a highly sensitive marker of *P. falciparum* infection. The fact that one sample tested negative in the ELISA is surprising considering the fact that the sample has a relatively high parasite density. However, this case does not confirm the previously published theory that some *P. falciparum* parasites do not produce HRP2, because this sample tested positive in a rapid diagnostic test also based on the detection of HRP2. However, it may be an indication that there may be structural variations in the HRP2 molecule that are not recognized by all anti-HRP2 antibodies.

We conclude that, despite the high sensitivity, specificity, and convenience of the ELISA procedure, particularly for handling of large numbers of samples, the application will remain limited to research settings and blood bank screening as long as there are no commercial species-specific ELISA tests kits for all species. Further validation in populations with asymptomatic *P. falciparum* infections should be performed.

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TABLE 2

Cross-tabulation of unadjusted duplicate expert microscopy for *P. falciparum* ($n = 80$) by CELISA results broken down into parasite densities

<i>P. falciparum</i> density of asexual parasites per microliter of blood in microscopy	CELISA	
	Negative	Positive
≤ 100	0	2
101–500	0	6
501–1,000	0	3
1001–5,000	1	17
> 5,000	0	51
Total	1	79

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