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Evaluation of Rapid Diagnostics for *Plasmodium falciparum* and *P. vivax* in Mae Sot Malaria Endemic Area, Thailand

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Abstract: Prompt and accurate diagnosis of malaria is the key to prevent disease morbidity and mortality. This study was carried out to evaluate diagnostic performance of 3 commercial rapid detection tests (RDTs), i.e., Malaria Antigen Pf/Pan™, Malaria Ag-Pf™, and Malaria Ag-Pv™ tests, in comparison with the microscopic and PCR methods. A total of 460 blood samples microscopically positive for *Plasmodium falciparum* (211 samples), *P. vivax* (218), mixed with *P. falciparum* and *P. vivax* (30), or *P. ovale* (1), and 124 samples of healthy subjects or patients with other fever-related infections, were collected. The sensitivities of Malaria Ag-Pf™ and Malaria Antigen Pf/Pan™ compared with the microscopic method for *P. falciparum* or *P. vivax* detection were 97.6% and 99.0%, or 98.6% and 99.0%, respectively. The specificities of Malaria Ag-Pf™, Malaria Ag-Pv™, and Malaria Antigen Pf/Pan™ were 93.3%, 98.8%, and 94.4%, respectively. The sensitivities of Malaria Ag-Pf™, Malaria Antigen Pf/Pan™, and microscopic method, when PCR was used as a reference method for *P. falciparum* or *P. vivax* detection were 91.8%, 100%, and 96.7%, or 91.9%, 92.6%, and 97.3%, respectively. The specificities of Malaria Ag-Pf™, Malaria Ag-Pv™, Malaria Antigen Pf/Pan™, and microscopic method were 66.2%, 92.7%, 73.9%, and 78.2%, respectively. Results indicated that the diagnostic performances of all the commercial RDTs are satisfactory for application to malaria diagnosis.

Key words: *Plasmodium falciparum*, *Plasmodium vivax*, malaria diagnosis, rapid detection test (RDT), Thailand

INTRODUCTION

Malaria is the major causes of morbidity and mortality in the world. The disease affects the populations of tropical and subtropical areas worldwide. Of the 5 species of *Plasmodium* that cause malaria in humans, *Plasmodium falciparum* is the most dangerous and responsible for most morbidity and mortality [1]. Malaria control relies principally on prompt and accurate diagnosis and chemotherapy with effective antimalarial drugs [2]. Prompt and accurate diagnosis is the key to prevent morbidity and mortality while it is avoiding unnecessary use of antimalarial agents. The traditional malaria diagnosis is based on the examination of stained blood smears under light microscope. The method remains the gold standard for malaria diagnosis as it is inexpensive and sensitive (5-10 parasites/μl blood) [3]. However, it is labor-intensive, time-consuming, and more importantly, requires skill and experienced microscopists.

Recently, alternative methods, such as immunochromatographic assay, molecular amplification method, fluorescence microscopy, mass spectrometry, and flow cytometry have been developed for malaria diagnosis [3-10]. These methods have some advantages and also some limitations. PCR is considered the most sensitive and specific method, but is expensive, requiring PCR machine, relatively sophisticated and time-consuming procedure, which may not be applicable for malaria diagnosis in remote areas. Malaria rapid detection tests (RDTs) which are based on capture of the parasite antigen by monoclonal antibodies incorporated into a test strip provide a possibility to replace microscopic diagnosis. Although there have been a number of RDTs commercially available, their sensitivity and specificity remain uncertain. RDTs can be divided into 2 major types. The first type detects histidine-rich protein 2 (HRP2), a protein uniquely synthesized by *P. falciparum* and present in the blood stream of an infected individual [11]. Some HRP2 tests are designed to also detect aldolase enzyme, a protein synthesized by all 4 human-infecting *Plasmodium* species

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[12]. The second type detects parasite lactate dehydrogenase (pLDH), an enzyme produced by all 4 human malaria species [13,14]. HRP2 test kits have generally shown higher sensitivity for *P. falciparum* detection and can be less costly than the pLDH [15-18]. Nevertheless, several studies have demonstrated that HRP2 remains in the blood stream for an extended time following successful eradication of the parasite with effective antimalarial treatment, contributing to false positive results and limited specificity [19,20]. In areas along the Thai-Myanmar border with a high annual malaria incidence, malaria diagnosis is an important tool in controlling disease morbidity and mortality [21]. RDTs would be an effective alternative diagnostic tool or used as an adjunct to microscopy for successful malaria control.

In the present study, 3 commercial RDTs (SD BIOLINE: Standard Diagnostics, Seoul, Republic of Korea), i.e., Malaria Antigen Pf/Pan™, Malaria Antigen Ag-Pf™, and Malaria Antigen Ag-Pv™, were assessed for their diagnostic performance for *P. falciparum* and *P. vivax* malaria. Ag-Pf™ detects HRP2 specific to *P. falciparum*, whereas Pg-Pv™ detects pLDH specific to *P. vivax*, and Pf/Pan™ detects both HRP2 and pLDH specific to *Plasmodium* species in human blood. The assessment of their diagnostic performance was performed in comparison with the gold-standard microscopic and reference PCR methods.

MATERIALS AND METHODS

Sample collection

The study was cross-sectional and was conducted during May 2008- April 2009 at Mae Sot General Hospital, Mae Sot District, Tak Province, Thailand, an area along the Thai-Myanmar border with a high annual malaria incidence (http://www.thaivbd.org/cms/index.php). The inclusion criteria were blood samples obtained from febrile patients (oral temperature > 37.5℃) with acute uncomplicated malaria and healthy subjects who had no previous sign of fever for at least weeks. The exclusion criteria were blood samples from those having previous antimalarial treatment or presence of clinical signs and symptoms of severe malaria. *P. falciparum* accounts for 50-60% of the *Plasmodium* species in this region. A total of 584 blood samples, 500 μl each for microscopic examination and 100 μl finger-prick blood for PCR and RDTs, were collected for malaria diagnosis by 3 methods, microscopic, PCR, and RDTs. Among them, 460 were collected from patients with signs and symptoms of malaria, 72 were from patients with fever related to other infections (10 typhus, 17 scrub typhus, and 45 dengue hemorrhagic fever), and 52 normal blood samples obtained from blood bank of Mae Sot General Hospital. Blood smears of 460 malaria samples were confirmed by 2 microscopists; 211, 218, 30, and 1 samples were identified as infections with *P. falciparum*, *P. vivax*, mixed infection with *P. falciparum* and *P. vivax*, and *P. ovale*, respectively.

Malaria diagnosis

Microscopic examinations

Thick blood smears were prepared for all blood samples and stained with 10% Giemsa. The malaria parasite was detected under light microscopy. The number of parasites was counted against 200 leucocytes and parasite density was estimated by assuming 8,000 leucocytes/μl blood. Samples were considered negative when no parasite was detected after examining 100 microscopic fields. The malaria microscopic examination was performed by 2 independent experienced microscopists from Mae Sot General Hospital and Pharmacology and Toxicology Laboratory, Thammasat University, Thailand. Each blood slide was blinded and the result was masked to both of the 2 microscopists. In order to check for inter-observer variability, a double blind cross reading of a random sampling of 100 blood slides was carried out by the senior microscopist.

Rapid diagnostic tests (RDTs)

Malaria diagnosis by 3 commercial RDTs, i.e., Malaria Antigen Pf/Pan™ (Catalogue No.05FK60), Malaria Ag-Pf™ (Catalogue No. 05FK50), and Ag-Pv™ (Catalogue No. 05FK70) (SD BIOLINE) were performed in all blood samples (finger-prick blood) using the method described by the manufacturer (http://standardia.com/html_e/mn03/mn03_01.asp). The presence of both the control and test lines indicated a positive result for *P. falciparum* and *P. vivax*, whereas the presence of only the control line indicated a negative result. All RDT kits were stored as directed by the manufacturer and the quality of package desiccant was checked before use.

PCR analysis

PCR analysis was performed in a total of randomly selected 129 (20%) finger-prick blood samples. The fresh blood sample (20 μl) was spotted onto a filter paper (Whatmann No. 3), and the dried blood spot paper was stored in a zipper plastic bag containing desiccant, and transported to he Pharmacology Unit, Graduate Program in Biomedical Sciences, Thammasat

University for PCR analysis. Genomic DNA was extracted using Chelex-resin (Biorad, Hercules, California, USA) according to the method of Wooden et al. [22]. The previously published nested PCR methods were employed to detect malaria species specific reactions [23]. The method is highly specific and can differentiate *P. falciparum*, *P. vivax*, and other malaria species.

Data analysis

The performances of all RDTs were evaluated based on the following criteria: sensitivity, specificity, positive prediction value (PPV), negative prediction value (NPV), false positive rate, and false negative rate, in 2 separate analyses; (i) diagnostic performance of the 3 RDTs in comparison with the microscopic method (gold standard), and (ii) comparative diagnostic performances of the 3 RDTs in comparison with the microscopic method and PCR (reference). The sensitivity of the test was calculated as (number of true positives/[number of true positives + number of false negatives]) x 100, and the specificity of the test was calculated as (number of true negatives/[number of true negatives + number of false positives]) x 100, PPV and NPV were determined from (number of true positive/[number of true positive + number of false positive]) x 100, and (number of true negative/[number of true negative + number of false negative]) x 100, respectively. The false positive and the false negative rates were determined from 1-specificity, and 1- sensitivity, respectively. The detection limit was calculated from the sample with the lowest parasitemia with the true positive result, and was confirmed by the laboratory clone *P. falciparum* culture with different dilutions of parasitemia.

Statistical analysis was performed by the chi-square test at a statistical significance level of $P=0.05$, using the SPSS version 12.0.

RESULTS

Diagnostic performance of RDTs in comparison with microscopy

The parasite density of *P. falciparum* and *P. vivax* were 2-70,000 and 2-27,200 parasites/μl, respectively. A double blind cross reading of a random sample of 100 slides showed 1% inter-observer variability.

The diagnostic performances of all the 3 commercial RDTs were evaluated in 584 blood samples, in comparison with the microscopy method. Table 1 summarizes the number of blood samples with positive and negative detection of *P. falciparum* and *P. vivax*. A total of 229 and 211 samples, respectively, showed positive for *P. falciparum* by Ag-Pf™ and the microscopic method. *P. vivax* was detected in 219 and 218 samples by Ag-Pv™ and the microscopic method, respectively. *P. falciparum* and *P. vivax* , respectively, were detected in 232 and 200 samples by Pf/pan™. The sensitivity, specificity, PPV, NPV, false positive rate and false negative rates of the 3 RDTs compared with the microscopic method are shown in Table 2. The sensitivity of Ag-Pf™ and Pf/Pan™ compared with the microscopic method for detection of *P. falciparum* was 97.6% and 99.0%, respectively. The sensitivity of Ag-Pv™ and Pf/Pan™ for detec-

Table 1. Detection of *P. falciparum* and *P. vivax* by Malaria Ag-Pf, Ag-Pv, and Malaria Antigen Pf/Pan tests[a] in comparison with the microscopic method

Species (microscopy positive)	Ag-Pf kit	Ag-Pv kit	P.f/Pan
P. falciparum (211)	229	0	232
P. vivax (218)	0	219	200
P. falciparum and *P. vivax* mixed (30)	29	30	28
Negative or *P. ovale* (125)	326	335	124
Total (584)	584	584	584

[a]Data are presented as the number of positive samples by the 3 RDTs and microscopic method (in parenthesis).

Table 2. The test performance[a] of Malaria Ag-Pf, Ag-Pv, and Malaria Antigen Pf/Pan for detection of *P. falciparum* and *P. vivax* in comparison with the microscopic method

	Ag-Pf	Ag-Pv	Pf/Pan
Sensitivity for *P. falciparum*	97.6 (94.7-98.9)	-	99.0 (96.9-99.7)
P. vivax	-	98.6 (96.5-99.6)	99.0 (95.6-99.5)
Specificity	93.3 (90.1-95.5)	98.8 (97.0-99.5)	94.4 (88.8-97.0)
Positive predictive value (PPV)	90.0 (86.8-92.2)	98.2 (96.4-99.4)	98.2 (95.6-99.5)
Negative predictive value (NPV)	98.5 (95.3-99.7)	99.1 (97.3-99.8)	80.3 (77.7-85.5)
False positive rate	6.7 (4.5-9.9)	1.2 (0.5-3.0)	5.6 (3.0-11.2)
False negative rate	2.4 (1.1-5.3)	1.4 (0.4-35)	6.8 (4.5-9.6)
Detection limit (parasites/μl)	>2-5	>2	Pf> 2-5, Pv>2

[a]Data are presented as percentage (95% confidence interval; CI).

tion of *P. vivax* was 98.6% and 99.0%, respectively. The specificity of Ag-Pf™, Ag-Pv™, and Pf/Pan™ was 93.3%, 98.8%, and 94.4%, respectively. All the 3 RDTs showed significant correlation with the microscopic method in detecting malaria parasite species ($P<0.001$). Parasitemia of the false negative samples for Ag-Pf™ and Ag-Pv™ were 2-1,446 and 4-62 parasite/μl, respectively. Parasitemia of the false negative samples by Pf/pan™ for detection of *P. falciparum* and *P. vivax* were 41-1,466 and <20 parasites/μl, respectively. The detection limit for *P. falciparum* and *P. vivax* was >2 parasites/μl for all RDT tests. For the laboratory strain *P. falciparum* dilution, the detection limit was 5 parasites/μl.

The sensitivity of the 3 RDTs categorized by parasite density is summarized in Table 3. Results clearly showed lower sensitivity of all RDTs in detecting both *P. falciparum* and *P. vivax* with parasite densities of less than 50 parasites/μl.

Diagnostic performance of RDTs, microscopy, and PCR methods

PCR was performed in a total of 129 randomly selected blood samples. The sensitivity, specificity, PPV, NPV, false positive and false negative rates of the 3 RDTs, and the microscopic method in comparison with the PCR method are shown in Table 4. Ag-Pf™ showed the highest false positive rate. The sensitivity of Ag-Pf™, Pf/Pan™, and the microscopic method for *P. falciparum* detection were 91.8%, 100%, and 96.7%, respectively. The sensitivity of Ag-Pv™, Pf/Pan™, and the microscopic method for *P. vivax* detection were 91.9%, 92.6%, and 97.3%, respectively. The specificity of Ag-Pf™, Ag-Pv™, Pf/Pan™, and the microscopic method were 66.2%, 92.7%, 73.9%, and 78.2%, respectively. All the RDTs and the microscopic method showed significant correlation with the PCR method in detecting certain species of malaria ($P<0.001$). Based on PCR results, the microscopic method provided the best diagnostic performance compared with the RDTs for detection of both *P. falciparum* and *P. vivax*.

DISCUSSION

The present study demonstrated that the 3 RDTs, Malaria

Table 3. The sensitivity[a] of Malaria Ag-Pf, Ag-Pv, and Malaria Antigen Pf/Pan tests for detection of *P. falciparum* and *P. vivax* in comparison with the microscopic method, categorized according to parasite density

Parasite density (parasites/μl)	Ag-Pf	Ag-Pv	Pf/Pan
Plasmodium falciparum			
<50 (n=29)	84.2 (62.4-94.5)	-	89.7 (73.6-96.4)
50-499 (n=74)	98.7 (92.7-99.8)	-	97.3 (90.7-99.3)
500-999 (n=35)	97.1 (85.1-99.5)	-	97.1 (85.5-99.5)
1,000-4,999 (n=64)	98.4 (91.3-99.7)	-	98.4 (91.7-99.7)
>5,000 (n=38)	100 (90.8-100)	-	100 (90.8-100)
Plasmodium vivax			
<50 (n=47)	-	76.6 (62.8-86.4)	78.7 (65.1-88.0)
50-499 (n=111)	-	95.5 (89.9-98.1)	93.7 (87.6-96.9)
500-999 (n=35)	-	97.1 (85.5-99.5)	94.3 (81.4-98.4)
1,000-4,999 (n=44)	-	90.9 (78.8-96.4)	84.1 (70.6-92.1)
>5,000 (n=11)	-	100 (74.1-100)	100 (74.1-100)

[a]Data are presented as percentage (95% CI).

Table 4. The test performance[a] of Malaria Ag-Pf, Ag-Pv, Malaria Antigen Pf/Pan, and the microscopic method for detection of *P. falciparum* and *P. vivax* compared with the PCR method

	Ag-Pf	Ag-Pv	Pf/Pan	Microscopic method
Sensitivity for *P. falciparum*	91.8 (82.2-96.5)	-	100.0 (85.7-100)	96.7 (83.3-99.4)
P. vivax	-	91.9 (83.4-96.2)	92.6 (76.6-97.9)	97.3 (86.2-99.5)
Specificity	66.2 (54.3-76.3)	92.7 (82.7-97.1)	73.9 (53.5-87.5)	78.2 (58.1-90.3)
Positive predictive value (PPV)	70.9 (59.0-81.0)	94.4 (84.4-98.8)	93.5 (86.2-99.5)	95.4 (84.3-97.6)
Negative predictive value (NPV)	90.0 (80.4-94.7)	89.5 (81.0-93.8)	77.3 (56.9-90.9)	90.0 (78.1-94.2)
False positive rate	33.8 (23.7-45.7)	7.3 (3.8-16.6)	26.1 (12.5-46.5)	21.7 (9.7-41.9)
False negative rate	8.2 (3.5-17.8)	8.1 (2.9-17.3)	4.7 (1.9-11.9)	1.9 (0.9-21.2)

[a]Data are presented as percentage (95%CI).

Antigen Pf/Pan™, Malaria Antigen Ag-Pf™, and Malaria Antigen Ag-Pv™ showed good test performances for detection of both *P. falciparum* and *P. vivax*. Pf/Pan™ is a one-step test for detection of HRP2 specific to *P. falciparum* and pLDH pan-specific to *Plasmodium* species in the human blood sample. Ag-Pv™ is a one-step detection of pLDH specific to *P. vivax*, and Ag-Pf™ is a one-step detection of HRP2 specific to *P. falciparum* in the human blood sample. Ag-Pf™ and Ag-Pv™ are specific for detection of *P. falciparum* and *P. vivax*, respectively. Both showed excellent sensitivity and specificity.

The performance of the Ag-Pf test observed in the present study was in agreement with previous studies with other HRP2-based commercial RDTs, i.e., BinaxNow Malaria™ (Binax, Inc., Inverness Medical Professional Diagnostic, Scarborough, ME, USA) [22], Paracheck Pf™ (Orchid Biomedical system) [22,23], and ParaHit PF™ (Span Diagnostic Ltd) [22,23]. The tests also showed good performance when compared with pfLDH-based commercial RDTs, such as CareStart Malaria test™ (AccessBio Inc.) [22], OptiMAL strip™ (DiaMed AG) [13], and OptiMAL-IT™ (DiaMed AG) [22-24]. Malaria Ag-Pv™ which detected pvLDH showed very good performance similarly to other commercial RDTs, such as OptiMAL strip™ (DiaMed AG) [13], and SD FK70 Malaria Antigen Pv test™ (Standard Diagnostics, Seoul, Republic of Korea) [22]. The excellent performance of Pf/Pan™ for detection of both *P. falciparum* and *P. vivax* observed in this study was also similar to that reported with OptiMAL™ (DiaMed AG) [13]. Markedly variable sensitivity and specificity have been reported for commercially available RDTs [13,22-35]. It is difficult to directly compare the diagnostic performances of these tests since results reported may be influenced by many factors, such as environmental conditions, the use of different gold-standards, as well as possible geographic variation in malaria antigens. All these factors should be taken into consideration when selecting RDTs for application to malaria diagnosis.

When PCR was the reference method, the microscopic method showed a low specificity (78.2%). The false positive was 21.7%; this was an interesting data. The explanation for this could be other species of *Plasmodium* spp. (*P. knowlesi*) that have been reported in Thailand [22,23]. The PCR method used as the reference could not differentiate this *P. knowlesi*. Therefore, the PCR result showed negative data. However, the 5 blood slides from these samples showed malaria parasites in red blood cells. False negative results of RDTs were observed and have been attributed to possible genetic heterogeneity of HRP2 or LDH expression, deletion of HRP2 or LDH gene, presence of blocking antibodies, or immune-complex formation. On the other hand, false positive tests can occur even in samples with high parasitemia, which could be due to several reasons, including viable asexual-stage parasitemia below the detection limit of microscopy (possibly due to drug resistance), persistence of antigens due to sequestration and incomplete treatment, delayed clearance of circulating antigen (free or in antigen-antibody complexes), and cross reaction with non-falciparum malaria or rheumatoid factor.

In conclusion, the test performance of these 3 RDTs as malaria diagnostic tools are promising. The tests could detect *Plasmodium* species with high accuracy, sensitivity, and specificity.

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