

Diagnostic Performance of Three Rapid Diagnostic Test Kits for Malaria Parasite *Plasmodium falciparum*

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Abstract: Malaria is a potent burden on public healthcare worldwide due to requiring rapid diagnosis and treatment. Nowadays, prompt diagnosis with rapid diagnostic tests (RDTs) has been widely accepted as an effective diagnostic technique in malaria-endemic countries, primarily due to their easy operation, fast output, and straightforward interpretation. The global availability and use of RDTs have gradually grown over recent decades as field-applicable diagnostic tests for the reliable confirmation of malaria infection and proper case management. This study was conducted to evaluate diagnostic performance of 3 commercially available malaria RDT kits : BIOCREDIT™ Malaria Ag Pf(pLDH), Malaria Ag Pf(pLDH/pHRPII), and Malaria Ag Pf/Pv(pLDH/pLDH) (where pLDH and pHRPII stand for plasmodium lactate dehydrogenase and histidine-rich protein 2, respectively) for the specific detection of *Plasmodium falciparum*. A total of 1,129 blood samples including 95 blood samples, confirmed as vivax malaria infection by microscopic examinations and a nested-PCR method, were tested for falciparum malaria infection. The overall sensitivity and specificity of Malaria Ag Pf(pLDH/pHRPII), Malaria Ag Pf/Pv(pLDH/pLDH), and Pf(pLDH) for *P. falciparum* were 99.0% and 100%, 95.8% and 100%, and 100% and 100%, respectively. It is proposed that the 3 RDT kits perform reliable level of diagnostic accuracy of detection for *P. falciparum* parasites.

Key words: *Plasmodium falciparum*, malaria, rapid diagnostic test, diagnostic performance

INTRODUCTION

Malaria is one of the oldest known and most prevalent parasitic diseases with an estimated 3.2 billion people being at risk of infection [1]. Despite significant control efforts, morbidity and mortality induced by malaria remain high in many developing countries, especially in areas characterized by tropical and subtropical ecosystems [2-5]. In practice, the accurate diagnosis of malaria is the primary tool for effecting rational therapy. Still, access to adequate diagnosis and treatment is insufficient, resulting in a large treatment gap where many cases

are managed sub-optimally or even go untreated. Thus, it is pivotal to have sensitive and specific malaria diagnostic tools to prevent the injudicious use of anti-malaria drugs and over-treatment. The microscopic examination of Giemsa-, Wright-, or Wright-Giemsa-stained blood smears as a routine reference test has been used as the gold standard for malaria diagnosis in many malaria-endemic areas despite the infrastructural and technical requirements that are not always available in resource-limited settings [6-8]. Thus, many medical resources cannot offer round-the-clock dependable smear-based diagnosis. Inconsistency due to intense inter-observer variability, particularly for samples with low parasitemia or mixed *Plasmodium* species, has been regarded as a major shortcoming of microscopic examination [8]. These limitations have fostered the development of non-microscopic alternatives for the diagnosis of malaria, especially in field diagnosis [9-12].

Although the World Health Organization (WHO) launched a comparative study of some rapid diagnostic kits (RDTs) on

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selected samples containing *P. falciparum* and *P. vivax* in 2008 [13], studies using clinical samples have been highly informative regarding test performance in routine usage. In practice, malaria RDTs from different companies can show wide variation, especially in terms of performance characteristics, and can be affected by many factors that potentially cause false-negative results [14]. This study was performed to determine functionality of 3 commercially available RDTs Malaria Ag Pf(pLDH), Malaria Ag Pf(pLDH/pHRPII), and Malaria Ag Pf/Pv(pLDH/pLDH) for specific detection of *P. falciparum*.

MATERIALS AND METHODS

Ethics statements

The anonymous samples used in this study were donated from the Global Resource Bank of Parasitic Protozoa Pathogens at the Inha University School of Medicine (NRF-2017M3A9B8069530), some of which were exempted from Institutional Review Board (IRB) review and the rest were frozen resources used after the IRB had approved the research (Inha 1712081A).

Samples

The blood samples used in this study were retrieved from the -80°C stock in Department of Tropical Medicine, Inha University School of Medicine. The samples used in this study were confirmed using 18S ribosomal RNA-based genus-specific nested-PCR analyses for *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* [15]. The *P. falciparum*-positive samples were of 191 Ugandan patients [16]. The *P. vivax*-positive samples were collected from 95 Korean patients by the Global Resource Bank of Parasitic Protozoa Pathogens in the Inha University School of Medicine. The *Plasmodium*-negative blood samples were of 843 healthy people. Positive samples of *P. malariae* and *P. ovale* were excluded from this study.

Analysis on functionality of RDTs

All blood samples were assayed with each of the 3 BIO-CREDIT™ Malaria RDTs: Malaria Ag Pf/Pv(pLDH/pLDH) (Lot. No. H016005), Malaria Ag Pf(pLDH) (Lot. No. H019005), and Malaria Ag Pf(pLDH/pHRPII) (Lot. No. H052005) manufactured by RapiGEN Inc. (Gunpo, Korea). These kits were designed to detect *P. falciparum*-specific pLDH and *P. vivax*-specific pLDH, *P. falciparum*-specific pLDH, and *P. falciparum*-specific pLDH and pHRPII, respectively. Each RDT comprised a membrane strip pre-coated with antibodies specific to each target

protein and was tested according to the manufacturer's instructions. In brief, 5 µl of whole blood was loaded into the sample well of the device and 3 drops of assay diluent were added to the buffer well. The test results, interpreted within 35 min, were recorded as negative if only the control line appeared, which indicates that the test had been performed well. Positive or negative results for the respective malaria parasites are based on the color code shown on the strip regardless of test line intensity. Each sample was blinded against the results obtained from the other diagnostic tests. All kits were tested, and the results validated to ensure strict adherence to the manufacturer's statements.

Limit of detection (LOD)

Samples with known parasite counts per µl of whole blood determined by an expert research microscopist were used to test the LOD of the 3 Malaria RDTs. Three samples of each relevant *Plasmodium* spp. were chosen: for *P. falciparum*, 5,440 (*Pf*1), 3,104 (*Pf*2) and 995 (*Pf*3) parasites/µl, and for *P. vivax*, 2,228 (*Pv*1), 1,108 (*Pv*2), and 6,507 (*Pv*3) parasites/µl. Frozen whole blood samples were thawed and then diluted in fresh malaria-negative, non-endemic country whole blood to produce parasitemia with the diluting blood serving as a negative control by serial dilution.

Data analysis

The 3 sets of RDT results were compared with the microscopic results as the gold standard. The sensitivity, specificity, positive-predictive value (PPV), and negative-predictive value (NPV) of each RDT were calculated. Variable measures were number of true positives (TP), true negatives (TN), false positives (FP), and false negatives (FN). Sensitivity was calculated as TP/(TP+FN), specificity as TN/(TN+FP), PPV as TP/(TP+FP), and NPV as TN/(TN+FN) [17].

RESULTS

Ten out of 191 *P. falciparum*-positive samples were negative for the Pf(pLDH) antigen in Malaria Ag Pf(pLDH) (Table 1), 10/191 positive samples were negative for the Pf(pLDH) antigen and 2/191 positive ones were negative for the Pf(pHRPII) antigen in Malaria Ag Pf(pLDH/pHRPII) (Table 1), and 8/191 positive samples were negative for the Pf(pLDH) antigen in Malaria Ag Pf/Pv(pLDH/pLDH) (Table 2). Sensitivity and specificity of Malaria Ag Pf(pLDH) were 94.8% and 100%, respec-

Table 1. Comparison of *P. falciparum* detection by Malaria Ag Pf(pLDH/pHRPII) and microscopic examination (n = 1,034)

Microscopic examination	No. sample	<i>Pf</i> (pLDH)		<i>Pf</i> (pHRPII)	
		No. positive	No. negative	No. positive	No. negative
Positive	191	181	10	189	2
Negative	843	0	843	0	843
Total	1,034	181	853	189	845

Table 2. Comparison of *P. falciparum* and *P. vivax* detection by Malaria Ag Pf/Pv(pLDH/pLDH) and microscopic examination (n = 1,129)

Microscopic examination*	No. sample	<i>Pf</i> (pLDH)		<i>Pv</i> (pLDH)	
		No. positive	No. negative	No. positive	No. negative
<i>P. falciparum</i>	191	183	8	0	0
<i>P. vivax</i>	95	0	0	95	0
Negative	843	0	843	0	843
Total	1,129	183	851	95	843

*These samples were tested and confirmed using nested-PCR.

Table 3. Performance of Malaria Ag Pf(pLDH/pHRPII) compared to microscopic examination (n = 1,034)

RDT	Sensitivity (%) (95% CI)*	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)	Accuracy (%) (95% CI)
<i>Pf</i> (pLDH)	94.8 (90.6-97.5)	100 (97.6-100)	100	93.8 (97.98-99.4)	99.2 (98.2-99.5)
<i>Pf</i> (pHRPII)	99.0 (96.3-99.9)	100 (99.6-100)	100	99.8 (99.1-99.9)	99.8 (99.3-100)
<i>Pf</i> (pLDH/pHRPII)	99.0 (96.3-99.9)	100 (99.6-100)	100	99.8 (99.1-99.9)	99.8 (99.3-100)

*Confidence interval.

Table 4. Performance comparison of Malaria Ag Pf/Pv(pLDH/pLDH) microscopic examination (n = 1,129)

	Sensitivity (%) (95% CI)*	Specificity (%) (95% CI)	PPV (%) (95% CI)	NPV (%) (95% CI)	Accuracy (%) (95% CI)
<i>Pf</i> (pLDH)	95.8 (91.9-98.2)	100 (97.6-100)	100	99.1 (98.2-99.5)	99.2 (98.5-99.7)
<i>Pv</i> (pLDH)	100 (96.2-100)	100 (99.6-100)	100	100 (99.6-100)	100 (99.6-100)

tively (Table 3). Malaria Ag Pf(pLDH/pHRPII) showed sensitivity 94.8% and specificity 100% for Pf(pLDH), and 99.0% and 100% for Pf(pHRPII). Overall sensitivity and specificity were 99.0% and 100%, respectively (Table 3). Those of Malaria Ag Pf/Pv(pLDH/pLDH) were 95.8% and 100% for Pf(pLDH), and 100% and 100% for Pv(pLDH), respectively (Table 4).

PPV and NPV of Malaria Ag Pf(pLDH) were 100% and 93.8% (Table 3), while those of Malaria Ag Pf(pLDH/pHRPII) were 100% and 98.8% for Pf(pLDH), and 100% and 99.8% for Pf(pHRPII), respectively (Table 3). Those of Malaria Ag Pf/Pv(pLDH/pLDH) were 100% and 99.1% for Pf(pLDH) and 100% and 100% for Pv(pLDH), respectively (Table 4).

LODs for *P. falciparum* with Malaria Ag Pf(pLDH) and Malaria Ag Pf(pLDH/pHRPII) RDTs were 8.4 and 10.1 parasites/ μ l on average, respectively. LODs for *P. falciparum* and *P. vivax* with Malaria Ag Pf/Pv(pLDH/pLDH) RDT were 7.6 and 38.2 parasites/ μ l on average, respectively (Table 5).

Table 5. Detection limit of 3 Malaria Ag kits

RDT kit	Sample	Test antigen	LOD (parasites/ μ l)
Malaria Ag <i>Pf</i> (pLDH)	<i>Pf</i> 1	<i>Pf</i> LDH	13.0
	<i>Pf</i> 2	<i>Pf</i> LDH	7.5
	<i>Pf</i> 3	<i>Pf</i> LDH	4.7
Malaria Ag <i>Pf</i> (pLDH/pHRPII)	<i>Pf</i> 1	<i>Pf</i> pLDH	13.0
		<i>Pf</i> pHRPII	13.0
	<i>Pf</i> 2	<i>Pf</i> pLDH	15.0
		<i>Pf</i> pHRPII	15.0
	<i>Pf</i> 3	<i>Pf</i> pLDH	9.5
		<i>Pf</i> pHRPII	2.4
Malaria Ag <i>Pf</i> / <i>Pv</i> (pLDH/pLDH)	<i>Pf</i> 1	<i>Pf</i>	3.2
		<i>Pv</i>	Negative
		<i>Pf</i>	15.0
	<i>Pf</i> 2	<i>Pf</i>	Negative
		<i>Pv</i>	4.7
		<i>Pf</i>	Negative
	<i>Pf</i> 3	<i>Pf</i>	Negative
		<i>Pv</i>	42.2
		<i>Pf</i>	Negative
<i>Pv</i> 1	<i>Pf</i>	Negative	
	<i>Pv</i>	40.6	
	<i>Pf</i>	Negative	
<i>Pv</i> 2	<i>Pf</i>	Negative	
	<i>Pv</i>	40.6	
	<i>Pf</i>	Negative	
<i>Pv</i> 3	<i>Pf</i>	Negative	
	<i>Pv</i>	31.7	
	<i>Pf</i>	Negative	

*Three samples were chosen each from *Plasmodium* sp.: for *P. falciparum*, 5,440 (*Pf*1), 3,104 (*Pf*2) and 995 (*Pf*3) parasites/ μ l, and for *P. vivax*, 2,228 (*Pv*1), 1,108 (*Pv*2), and 6,507 (*Pv*3) parasites/ μ l.

DISCUSSION

In the present study, we examined the ability of the Malaria Ag RDTs to diagnose falciparum malaria. To determine the appropriate test for field use for *P. falciparum*, study population consisted of 2 groups of patients from Uganda for falciparum malaria and from Korea for vivax malaria. According to WHO recommendation [4], RDTs should show greater than 95% sensitivity to be useful and efficient diagnostic tools.

The WHO estimates that 219 million cases occurred during 2017 with 435,000 deaths [18]. Most malaria cases in 2017 were in Africa (approximately 200 million or 92%), followed by South-East Asia (5%) and the Eastern Mediterranean region (2%). Prompt diagnosis and treatment is the most effective means to prevent a mild case of malaria from advancing into severe disease and even death. Moreover, the clinical characteristics of malaria are nonspecific and overlap primarily with those of other febrile diseases. Thus, the WHO recommends that all patients should have a parasite-specific laboratory test performed to confirm the clinical symptoms. *P. falciparum* is the most prevalent malaria parasite in Africa, accounting for 99.7% of estimated malaria cases in 2017, as well as in South-East Asia (62.8%), and the Eastern Mediterranean (69%) and the Western Pacific regions (71.9%) [18].

In the USA, a malaria test is the only one cleared by the Food and Drug Administration for the in-vitro diagnosis of malaria. That malaria test has sensitivities of 100% and 81.6% for the detection of *P. falciparum* and *P. vivax*, respectively, in venous blood samples [19]. According to the WHO recommendations for RDT performance, only 2 RDTs with a reported sensitivity greater than 95% have been approved: 96.8% and 95.2% [20]. However, present study showed that sensitivity of the 3 Malaria Ag kits was 99.0% overall when using *Pf*(pLDH/pHRPII) RDT kit. This kit had high sensitivity and was in strong agreement with the microscopic examinations and the 18s ribosomal RNA-based nested-PCR results. HRPII-based RDTs commonly provide *P. falciparum* sensitivity higher than 90.0% in clinical cases [21,22]. However, the test results with pLDH assays have been shown to vary among studies [4,23]. In the present study, antigen *Pf*(pLDH) in the Malaria Ag *Pf*(pLDH) and *Pf*(pLDH/pHRPII) RDTs showed 94.8% sensitivity. Interestingly, sensitivity of Malaria Ag *Pf*(pLDH/pHRPII) was 99.0% and that of Malaria Ag *Pf/Pv*(pLDH) was 95.8%. It is understandable that RDTs can be influenced by several factors such as antigenic variability of the target protein, antigen persistence and parasite

density in blood samples [24,25].

Most studies on the diagnostic serology of *Plasmodium* spp. include immunoassays using limited antigen, such as HRPII. Nowadays, these almost always contain the highly conserved immunodominant epitope of HRPII at a minimum. Despite the earlier detection of *Plasmodium* spp. in patients' blood samples, this first-generation RDT kit using HRPII has shown decreased sensitivity in detecting malaria infection. In general, *Pf*HRPII-based RDTs are more sensitive and heat-stable than RDTs that detect other malaria antigens, such as pan (all species) or *P. falciparum*-specific pLDH or aldolase [26,27]. However, antibodies against *Pf*HRPII can cross-react with proteins expressed by another member of the *hrp* gene family such as *pfhrp3* due to a strong similarity in the amino acid sequences. *Pf*HRPII RDTs also have limitations due to the genetic variability and polymorphism of the *pfhrp2* gene, which can affect its detection by RDTs [28-30]. The prevalence of *pfhrp2* gene deletion varies from location to location [31] and strains with partial or total *pfhrp2* deletion have been reported in South America, Africa, and India [32]. Moreover, a recent study in India reported a 2.4% prevalence of *pfhrp2* gene deletion [33]. These genetic variations in the region of the *pfhrp2* gene have caused a high rate of false-negative results when using RDTs, and the companies that make them are under pressure to develop new specific antigenic proteins as useful and essential target(s) for *P. falciparum* detection. Furthermore, RDTs used to detect malaria in pregnant women can show low sensitivity, possibly due to the sequestration of antigens in the placental circulation [34]. Therefore, it is critical to develop and improve alternative biomarkers of *P. falciparum* for the next generation RDTs for malaria parasite detection [35]. Thus, in the present study, we demonstrated that the monoclonal antibodies against pLDH in 3 commercially available 2nd generation Malaria Ag RDTs are better candidates for diagnosing falciparum malaria infection than the 1st generation HRPII-based RDT kits.

Previously we evaluated the diagnostic performances of 2 commercially available malaria RDT kits, Malaria Ag *Pf/Pv*(pLDH/pLDH) and competitor's Ag *Pf/Pv*(pHRPII/pLDH) for detectability of *Plasmodium* species in blood samples collected from Ugandan patients with malaria. The detection sensitivity of Malaria Ag *Pf/Pv*(pLDH/pLDH) and competitor for *P. falciparum* was 87.8% and 89.6%, respectively, and the specificities of the 2 RDTs were 100% for *P. falciparum* and mixed *P. falciparum/P. vivax* samples [8]. A high panel detection scores were shown with other kits, even at low parasitemia, in Round

4 of the WHO/FIND study [26]. The sensitivity and specificity of the RDTs assayed in this study were higher than estimates of those previously developed commercial RDTs. Although their diagnostic performances in a field setting have yet not been established, these Malaria Ag kits provided good diagnostic performances with *P. falciparum*/*P. vivax* -positive blood samples at a laboratory setting. Considering their performance results, we recommend these RDT kits as an appropriate option for screening for *P. falciparum* at health facilities with limited human resources and infrastructure.

In conclusion, we evaluated the clinical performance of 3 Malaria Ag kits for *P. falciparum* using whole blood samples compared to microscopic examination as the gold standard and molecular nested-PCR tests. The accuracies of the RDTs were similar to or better than those of the RDTs currently recommended by WHO [20]. Therefore, Malaria Ag kits were shown to be reliable diagnostic kits to detect falciparum malaria infections and can contribute to malaria control efforts as a possible replacement for microscopic examination in front-line diagnosis. For further studies, more extensive tests with mixed infections of *P. falciparum* and *P. vivax* and with low parasitemia values from various global populations are needed to fully evaluate the diagnostic performance of the 3 Malaria Ag kits.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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