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▣ ORIGINAL ARTICLE

Evaluation of the Accuracy of the EasyTest™ Malaria Pf/Pan Ag, a Rapid Diagnostic Test, in Uganda

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Abstract: In recent years, rapid diagnostic tests (RDTs) have been widely used for malaria detection, primarily because of their simple operation, fast results, and straightforward interpretation. The Asan EasyTest™ Malaria Pf/Pan Ag is one of the most commonly used malaria RDTs in several countries, including Korea and India. In this study, we tested the diagnostic performance of this RDT in Uganda to evaluate its usefulness for field diagnosis of malaria in this country. Microscopic and PCR analyses, and the Asan EasyTest™ Malaria Pf/Pan Ag rapid diagnostic test, were performed on blood samples from 185 individuals with suspected malaria in several villages in Uganda. Compared to the microscopic analysis, the sensitivity of the RDT to detect malaria infection was 95.8% and 83.3% for *Plasmodium falciparum* and non-*P. falciparum*, respectively. Although the diagnostic sensitivity of the RDT decreased when parasitemia was ≤500 parasites/μl, it showed 96.8% sensitivity (98.4% for *P. falciparum* and 93.8% for non-*P. falciparum*) in blood samples with parasitemia ≥100 parasites/μl. The specificity of the RDT was 97.3% for *P. falciparum* and 97.3% for non-*P. falciparum*. These results collectively suggest that the accuracy of the Asan EasyTest™ Malaria Pf/Pan Ag makes it an effective point-of-care diagnostic tool for malaria in Uganda.

Key words: *Plasmodium falciparum*, rapid diagnostic test, malaria, point-of-care testing, field test, Uganda

INTRODUCTION

Malaria is a major infectious disease that is a significant global health concern because of its high morbidity and mortality worldwide [1]. The Securing Ugandans' Right to Essential Medicines (SURE) program of the Uganda Ministry of Health reported in its The Value Chain Newsletter that approximately 31% of adults and 36% of children aged less than 5 years in Uganda had been diagnosed with malaria in 2010 [2]. Although many medical service organizations and health centers are working to prevent and control malaria, they do not have sufficient medical or human resources to accomplish these goals [2]. Therefore, malaria-related mortality in Uganda remains high and without significant reduction in the last decade [1,2]. Because the clinical symptoms of malaria are very similar to those of other febrile illnesses, diagnosis of malaria based on clinical manifestations alone is unreliable [3]. The microscopic examination of thick blood films is the gold-standard method for malaria diagnosis [4,5], but requires expensive and fragile microscopes as well as well-trained microscopists. Furthermore, the diagnostic reliability of microscopy can be questionable, especially in samples with low parasitemia or with mixed species infections [6-8]. To overcome these diagnostic problems, detection methods such as rapid diagnostic tests (RDTs) and nucleic acid tests (NATs) have been developed for malaria diagnosis [9-12]. Because RDTs are easy to use, fast to assay, and require no special instruments, these tests have been widely used in recent years to detect malaria infections. Several antigens, such as *Plasmodium* histidine-rich protein 2 (HRP2) [13], lactate dehydrogenase (pLDH) [14], aldolase [15], and glutamate dehydrogenase [16], have been identified and used in RDTs to detect malaria infections.

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Among them, HRP2 and pLDH are targets used in most currently deployed RDTs [17-20].

The Asan EasyTest™ Malaria Pf/Pan Ag is an RDT that detects parasite HRP2 and pLDHs and has been approved by the Korean Food and Drug Administration (KFDA). This RDT is widely used in many clinic centers and hospitals in Korea and India, but further evaluation of its diagnostic accuracy is required, especially in endemic countries on other continents. In this study, we assessed the diagnostic accuracy of the Asan EasyTest™ Malaria Pf/Pan Ag by comparing the sensitivity, specificity, and positive and negative predictive values of this RDT with those of microscopic examinations in symptomatic malaria patients in Uganda.

MATERIALS AND METHODS

Ethics statement

All participants and patients were informed of this study, and the signed informed consent was obtained according to ethical standards. Written confirmation with full information, including the procedures and the potential risks and benefits of this study, was provided before blood collection. Blood collection was conducted with approval from the Ministry of Health, Uganda. Blood samples from children were obtained after consent from their parents. All personal identifiers and patient information were anonymized. Pregnant women and patients with signs of severe and complicated *Plasmodium falciparum* malaria as defined by the World Health Organization (WHO) [21] were excluded from this study. This study was conducted according to the principles of the Declaration of Helsinki.

Study area and blood samples

Field studies were carried out in primary health centers in 6 villages of Kiyuni Parish, Kyankwanzi District, Uganda in April and September 2011. Malaria transmission in this area has not been described in detail, but the primary mosquito vector *Anopheles funestus* has been frequently observed in association with *P. falciparum* infection. Patients with suspected malaria who sought treatment at health centers for febrile symptoms (axillary or internal ear temperature more than 37.5℃ or history of fever in the previous 24 hr) were selected, and approximately 3 ml of venous blood was drained into an EDTA anticoagulant tube. In children aged less than 5 years, only fingertip blood samples were collected. A total of 184 blood samples were analyzed in this study.

Microscopic examinations

Thick and thin blood smears were prepared immediately after blood collection and stained with 4% Giemsa for 20 min. Three experienced Ugandan technicians examined the blood films following the standard protocols [22]. Parasites in thick blood films were counted against 299-500 white blood cells. The parasite density was estimated assuming 8,000 white blood cells/μl of blood [6,22]. Each examination was performed in duplicate by independent microscopists to minimize the error or potential bias. Patients diagnosed with malaria by microscopy received WHO-recommended anti-malarial therapy. The microscopic analysis was confirmed by the Department of Parasitology, Inha University School of Medicine, Korea to minimize diagnostic errors.

PCR analysis

To confirm the results of microscopic examinations, we also performed 18s ribosomal RNA (18s rRNA)-based nested PCR analysis as described previously [23]. Genomic DNA was extracted from 100 μl of whole blood sample using a QIAamp Blood Kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. Amplicons from the nested PCR were separated by electrophoresis on a 2% agarose gel and stained with ethidium bromide for visualization using ultraviolet trans-illumination.

Rapid diagnostic test (RDT)

The RDT used in this study was the Asan EasyTest Malaria Pf/Pan Ag (lot. no.: D3036, exp. date: 2012/06/25) manufactured by Asan Pharmaceutical Co. Ltd. (Hwaseong, Gyeonggi-do, Korea) under the technical assistance from GenBody Inc. (Cheonan, Chungcheongnam-do, Korea), which is designed to detect *P. falciparum*-specific HRP2 and pan-specific pLDH. The RDT contains a membrane strip, which is pre-coated with 2 monoclonal antibodies as 2 separate lines (line 1, a monoclonal antibody specific to *P. falciparum* HRP2; line 2, a monoclonal antibody specific to pLDH) and a control line (line 3). This RDT is therefore able to differentiate between *P. falciparum* and non-*P. falciparum* malaria. The RDT was used according to the manufacturer's instructions. In brief, 20 μl of whole blood was loaded into the injection well of the device. Four drops of assay diluent were added to the buffer well. The test result was interpreted within 20 min.

Data analysis

RDT results were compared with those of microscopic examinations. A value of $P < 0.05$ was considered statistically significant. The sensitivity, specificity, and predictive values of the RDT were calculated using the formula generally used. To determine sensitivity and specificity, RDT results were compared with the microscopy results. The sensitivity was defined as the percentage of positive results among samples confirmed to be malaria positive by microscopic examinations. The specificity was the percentage of negative results among samples whose thick blood films were negative for malaria. The positive predictive value (PPV) and the negative predictive value (NPV) were the proportion of true-positive results among all positive samples and the proportion of true negative results among all negative samples, respectively.

RESULTS

Twice field tests were carried out in 6 villages of Kiyuni in Uganda at April and at September of 2011. Among 185 individuals with suspected malaria whose age ranged from 1 to 53 years, 110 individuals (59.5%) were positive for malaria by microscopic examinations and PCR analysis (Table 1). The mean ± SD age of the malaria patients was 10.4 ± 11.0 years; 27.2% were ≤ 3 years, 37.3% were 4-10 years, 18.2% were 11-20 years, 13.6% were 21-40 years, and 3.6% were ≥ 40 years. The ratio of male:female patients was 0.55:1. The parasitemia of the 110 positive samples ranged from 39 to 40,888 parasites/μl. Microscopic examinations of blood smears from 110 malaria-positive patients revealed that *P. falciparum* infection was most prevalent: *P. falciparum* infection (n = 71; 64.6%), non-falciparum malaria infection (n = 36; 32.7%), and mixed infection of *P. falciparum* and non-falciparum malaria (n = 3; 2.7%) (Table 1).

To evaluate the diagnostic performance of EasyTest™ Pf/Pan Ag, the 185 blood samples were also examined using this RDT. Compared to the results of microscopic examinations, 4 of 71 negative samples were positive for malaria, 2 for *P. falciparum* and 2 for non-falciparum malaria (Table 1), although the positive response in each sample was very weak. However, 8 of 110 positive samples in microscopic examinations were negative in the Asan EasyTest™ Pf/Pan Ag (Table 1). To confirm the results obtained from microscopic examinations, we also performed genus-specific PCR analysis, which resulted in the same pattern of positivity with microscopic examinations (Table 1). All non-*P. falciparum* malaria were confirmed to be *P. vivax* in the PCR analysis.

Based on these results, we analyzed the diagnostic performance of the Asan EasyTest™ Pf/Pan Ag using 95% confidence intervals (CIs). The sensitivity of the RDT was 95.8% for *P. falciparum* and 83.3% for non-falciparum malaria. The specificity was 97.3% for *P. falciparum* and 94.4% for non-falciparum malaria; the overall PPV and NPV were 96.3% and 94.9%, respectively. As expected, the sensitivity of the EasyTest™ Pf/Pan Ag test was low for blood samples with low parasitemia. For *P. falciparum*, the RDT had 100% sensitivity for samples with parasitemia > 500 parasites/μl, but only 66.6% when there were < 100 parasites/μl in the sample. Similarly, the RDT showed 100% sensitivity for non-falciparum malaria in samples with > 500 parasitemia, but it decreased to 50% in samples with < 100 parasitemia (Table 2). To determine the detection limit of the RDT, we also examined the analytical sensitivities of the RDT against experimentally diluted blood samples with different parasitemia of *P. falciparum* or *P. vivax*. The minimum detection level of the RDT was determined to be 79 parasites/μl for *P. falciparum* and 125 parasites/μl for *P. vivax*. The RDT showed similar analytical sensitivity against blood samples with mixed species of *P. falciparum* and *P. vivax* (data not shown).

Table 1. Comparison of results between microscopic examination and the Asan EasyTest™ Pf/Pan Ag

	Microscopy	PCR	EasyTest™ Pf/Pan Ag		
	No. of samples	No. of samples	No. of samples		
			Negative	*P. falciparum*	Non-*P. falciparum*
Negative	75	75	71	2	2
Positive	110	110			
P. falciparum	71	71	3	68	0
Non-*P. falciparum*	36	36	4	2	30
Mixed	3	3	1	2	0
Total	185	185	79	74	32

Table 2. Sensitivity of the Asan EasyTest™ Pf/Pan Ag based on parasitemia

Parasites/μl	No. of samples tested	No. of positive samples	Detection sensitivity (95% CIs[a])
P. falciparum			
>5,000	23	23	100
501-5,000	19	19	100
100-500	21	20	95.0
<100	8	6	75.0
Non-*P. falciparum*			
>5,000	2	2	100
501-5,000	13	13	100
100-500	17	15	88.2
<100	4	2	50.0
Mixed			
501-5,000	1	0	0
<100	2	2	100

[a]Confidence intervals.

DISCUSSION

The Asan EasyTest™ Pf/Pan Ag is an RDT approved by KFDA for detection of malaria. In recent years, its use has been increasing in many public and private clinic centers in Korea and several other countries, but its diagnostic performance in field settings had not yet been established. In this study, we evaluated the diagnostic performance of the Asan EasyTest™ Pf/Pan Ag in 6 villages of Kiyuni Parish in Uganda.

According to the WHO recommendations for RDT performance, only 2 RDTs have been approved; these RDTs have a reported sensitivity greater than 95% for samples with parasitemias ≥ 100 parasites/μl: 96.8% for the CareStart™ Malaria test and 95.2% for the OptiMAL-IT™ test [24]. However, the present study shows that the sensitivity of the Asan EasyTest™ Malaria Pf/Pan Ag is 96.8% (95.8% for *P. falciparum* and 83.3% for non-*P. falciparum*) for samples having the same parasite density, similar to the sensitivity reported for the CareStart™ Malaria test. Although we found 4 false-positive results with the RDT, we could not fully exclude the possibility that false-positive results were actually true-positives, which could be missed by microscopic examinations because of sequestration that limited the number of circulating parasites at the time of blood collection [25] or that the blood samples were from individuals with circulating rheumatoid factors [19,26,27]. As shown in Table 1, 3 cases (1.6%) were identified as mixed infections. The rate of malaria co-infection was similar to that reported in previous surveys [28,29]. However, the RDT was unable to detect mixed infections in the presence of *P. falciparum* because of cross-reactivity with the pan-specific pLDH band.

In conclusion, we evaluated the clinical performance of the Asan EasyTest™ Malaria Pf/Pan Ag using blood samples collected in Uganda. The accuracy of the RDT was similar to that of RDTs recommended by WHO. Therefore, the Asan EasyTest™ Malaria Pf/Pan Ag may be a reliable diagnostic tool to detect *P. falciparum* and non-*P. falciparum* malaria infections in Uganda, and can contribute to malaria control efforts in the country. However, we used a limited number of blood samples and a small number of sites in this study; more extensive tests with more samples from various global populations are needed to fully evaluate the diagnostic performance of the Asan EasyTest™ Malaria Pf/Pan Ag RDT.

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CONFLICT OF INTEREST

We have no conflict of interest related to this work.

REFERENCES

1. World Health Organization. World Malaria Report 2011. Available from http://www.who.int/topics/malaria/en/.
2. USAID. The Malaria End-Use Verfication (EUV) Report 2010. Available from http://sure.ug/?Publications.
3. World Health Organization. New perspectives: malaria diagnosis. Report of a Joint WHO/USAID Informal Consultation 2010. WHO/MAL/2000.1091.
4. Moody A. Rapid diagnostic tests for malaria parasites. Clin Microbiol Rev 2002; 15: 66-78.
5. Benito A, Roche J, Molina R, Amela C, Altar J. Application and evaluation of QBC® malaria diagnosis in a holoendemic area. Appl Parasitol 1994; 35: 266-272.
6. World Health Organization. Management of uncomplicated malaria and the use of antimalarial drugs for the protection of travellers. Report of an Informal Consultation 1996. WHO/MAL/96.
7. Molyneux M, Fox R. Diagnosis and treatment of malaria in Britain. BMJ 1993; 306: 1175-1179.
8. McKenzie FE, Sirichaisinthop J, Miller RS, Gasser RA Jr, Wongs-

richanalai C. Dependence of malaria detection and species diagnosis by microscopy on parasite density. Am J Trop Med Hyg 2003; 69: 372-376.
9. Lee GC, Jeon ES, Le DT, Kim TS, Yoo JH, Kim HY, Chong CK. Development and evaluation of a rapid diagnostic test for *Plasmodium falciparum*, *P. vivax*, and mixed-species malaria antigens. Am J Trop Med Hyg 2011; 85: 989-993.
10. Chilton D, Malik ANJ, Armstrong M, Kettelhut M, Parker-Williams J, Chiodini PL. Use of rapid diagnostic tests for diagnosis of malaria in the UK. J Clin Pathol 2006; 59: 862-866.
11. Kain KC, Brown AE, Mirabelli L, Webster HK. Detection of *Plasmodium vivax* by polymerase chain reaction in a field study. J Infect Dis 1993; 168: 1323-1326.
12. Kain KC, Kyle DE, Wongrichanalai C, Brown AE. Qualitative and semi-quantitative polymerase chain reaction to predict *Plasmodium falciparum* treatment failure. J Infect Dis 1994; 170: 1626-1630.
13. Rock EP, Marsh K, Saul SJ, Wellems TE, Taylor DW, Maloy WL, Howard RJ. Comparative analysis of the *Plasmodium falciparum* histidine-rich proteins HRP1, HRP2 and HRP3 in malaria diagnosis of diverse origin. Parasitology 1987; 95: 209-227.
14. Makler MT, Piper RC, Milhous WK. Lactate dehydrogenase and the diagnosis of malaria. Parasitol Today 1998; 14: 376-377.
15. Meier B, Döbeli H, Certa U. Stage-specific expression of aldolase isoenzymes in the rodent malaria parasite *Plasmodium berghei*. Mol Biochem Parasitol 1992; 52: 15-27.
16. De Dominguez N, Rodriguez-Acosta A. Glutamate dehydrogenase antigen detection in *Plasmodium falciparum* infections. Korean J Parasitol 1996; 34: 239-246.
17. Piper R, Lebras J, Wentworth L, Hunt-Cooke A, Houzé S, Chiodini P, Makler M. Immunocapture diagnostic assays for malaria using *Plasmodium* lactate dehydrogenase (pLDH). Am J Trop Med Hyg 1999; 60: 109-118.
18. Quintana M, Piper R, Boling HL, Makler M, Sherman C, Gill E, Fernandez E, Martin S. Malaria diagnosis by dipstick assay in a Honduran population with coendemic *Plasmodium falciparum* and *Plasmodium vivax*. Am J Trop Med Hyg 1998; 59: 868-871.
19. Shiff CJ, Premji Z, Minjas JN. The rapid manual ParaSight-F® test. A new diagnostic tool for *Plasmodium falciparum* infection. Trans R Soc Trop Med Hyg 1993; 87: 646-648.
20. Tjitra E, Suprianto S, Dyer M, Currie BJ, Anstey NM. Field evaluation of the ICT malaria P.f/P.v immunochromatographic test for detection of *Plasmodium falciparum* and *Plasmodium vivax* in patients with a presumptive clinical diagnosis of malaria in eastern Indonesia. J Clin Microbiol 1999; 37: 2412-2417.
21. World Health Organization. Monitoring antimalarial drug resistance. Report of WHO Consultation 2001. WHO/CDS/CSR/EPH/2002.7.
22. Warhurst DC, Williams JE. Laboratory diagnosis of malaria. J Clin Pathol 1996; 49: 533-538.
23. Brown AE, Kain KC, Pipithkul J, Webster HK. Demonstration by the polymerase chain reaction of mixed *Plasmodium falciparum* and *P. vivax* infections undetected by conventional microscopy. Trans R Soc Trop Med Hyg 1992; 86: 609-612.
24. World Health Organization. Malaria rapid diagnosis: making it work. Meeting Report January 20-23. Manila, 2003.
25. Bell D, Wongsrichanalai C, Barnwell JW. Ensuring quality and access for malaria diagnosis: how can it be achieved? Nat Rev Microbiol 2006; 4: 682-695.
26. Grobusch MP, Alpermann U, Schwenke S, Jelinek T, Warhurst DC. False-positive rapid tests for malaria in patients with rheumatoid factor. Lancet 1999; 353: 297.
27. Humar A, Ohrt C, Harrington MA, Pillai D, Kain KC. ParaSight® F test compared with the polymerase chain reaction and microscopy for the diagnosis of *Plasmodium falciparum* malaria in travelers. Am J Trop Med Hyg 1997; 56: 44-48.
28. Mayxay M, Pukrittayakamee S, Newton PN, White NJ. Mixed-species malaria infections in humans. Trends Parasitol 2004; 20: 233- 240.
29. Co EMA, Dennull RA, Reinbold DD, Waters NC, Johnson JD. Assessment of malaria in vitro drug combination screening and mixed-strain infections using the malaria Sybr green I-based fluorescence assay. Antimicrob Agents Chemother 2009; 53: 2557-2563.