Development and Evaluation of a Rapid Diagnostic Test for *Plasmodium falcifarum*, *P. vivax*, and Mixed-Species Malaria Antigens

Gyu-Cheol Lee, Eun-Sung Jeon, Dung Tien Le, Tong-Soo Kim, Jong-Ha Yoo, Hak Yong Kim, and Chom-Kyu Chong*

*Water Analysis and Research Center, K-Water, Daejeon, Republic of Korea; Institute of BioMedicals, Bioland Ltd.,
Cheongwon, Chungbuk, Republic of Korea; Research Team for Vector-borne Diseases, National Agriculture Research Center, Ibaraki, Japan;
Department of Parasitology, School of Medicine, Inha University, Incheon, Republic of Korea;
Department of Laboratory Medicine, National Health Insurance Corporation Ilsan Hospital, Goyang, Gyounggi, Republic of Korea;
Department of Biochemistry, College of Natural Sciences, Chungbuk National University, Cheongju, Chungbuk, Republic of Korea*

Abstract. *Plasmodium falciparum* and *P. vivax* malaria are endemic to many parts of the world and humans can be co-infected with both species. Because each *Plasmodium* species has different biological and clinical characteristics, accurate differentiation of the infecting species is essential for effective treatment. Therefore, we produced three mono-clonal antibodies that recognize the lactate dehydrogenase of *P. falciparum*, *P. vivax*, or both to develop the first *P. falci-parum*, *P. vivax*, and mixed-species infections malaria antigen detection kit. The detection limits of this kit were 150 and 250 parasites/mL for *P. falciparum* and *P. vivax*, respectively, and the kit was able to detect mixed-species infections. The sensitivity and specificity of this kit was assessed with 722 clinical specimens. Our results showed that its sensitivities for *P. falciparum*, *P. vivax*, and mixed-species infection were 96.5%, 95.3%, and 85.7%, respectively. In addition, its specificity was high (99.4%).

INTRODUCTION

Malaria is a severe human health problem in many parts of the world. It is caused by one or more infections with *Plasmodium falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*. Among these parasites, *P. falciparum* and *P. vivax* are the most widespread and common causes of mixed-species malaria, which is defined as co-infection with more than one species or genotype of *Plasmodium*.[1] Clinical diagnosis of mixed-species malaria is difficult because the ability of existing assays to identify and differentiate the mixed-species infections is limited. As a result, mixed-species malaria infections are often not properly diagnosed and their prevalence is underestimated.[2,3] For example, in Asia, surveys report that less than 2% of malaria infections are caused by mixed species. However, therapeutic studies have demonstrated a high prevalence (up to 30%) of infection with other malaria species during convalescence, which suggests co-infection.[1,4,5]

The diagnosis of mixed-species malaria is critical for therapeutic decisions, including the selection, dose, and timing of anti-malarial drugs. Mistreatment of a single species, rather than multiple species, can have serious clinical consequences. For example, Boyd and Kitchen reported that *P. falciparum* can suppress *P. vivax* parasitemia in patients,[6,7] which is known as interspecies inhibition.[8,9] Likewise, Mason and McKenzie predicted that when mixed-species malaria is misdiagnosed as a single-species *P. vivax* infection, treatment of *P. vivax* may increase *P. falciparum* parasitemia.[10] Many clinical studies have found high rates of *P. vivax* infection after treatment of single-species *P. falciparum* infection.[11,12] In addition, mixed-species infections are related to the growing problem of anti-malarial drug resistance, which can enable emergence of a drug-resistant population of *Plasmodium* parasites and relapse infection.

Microscopy and polymerase chain reaction have been the diagnostic methods of choice for malaria, including identification of species and detection of mixed-species infections.[13–16] However, these methods have several limitations, including expertise and time, which make them unfavorable for point-of-care testing. As a result, several rapid diagnostic tests (RDTs) have been developed to simplify point-of-care testing for malaria. The target antigens in these tests are usually histidine-rich protein-II (HRP-II), *Plasmodium* lactate dehydrogenase (pLDH), and *Plasmodium* aldolase.[17–20] Although commercially available RDTs are able to differentiate *P. falciparum* from non-*P. falciparum* infections, they are not able to differentiate non-*P. falciparum* species or identify mixed-species infections. We report the development and evaluation of the first RDT, known as the *P. falciparum*, *P. vivax*, and mixed-species infections malaria antigen (FVM Ag) detection kit, which can identify both single-species *P. falciparum* or *P. vivax* infections and mixed infections with these two species.

MATERIALS AND METHODS

Protein expression and purification. Recombinant *P. falciparum* and *P. vivax* LDH (*Pf*LDH and *Pv*LDH, respectively) were expressed in *Escherichia coli*, and then purified by using diethylaminoethyl ion exchange chromatography, followed by Blue Sepharose column chromatography. Briefly, bacterial strains of *E. coli* BL21(DE3) cells were transformed with the pET-15b expression vector (Novagen, Merck, Darmstadt, Germany) that contained the genes for *Pf*LDH and *Pv*LDH. The transformants were grown at 37°C in Luria-Bertani medium to an optical density at 600 nm (optical density at 600 nm [OD_{600}]) of 0.7–0.8. Protein expression was induced with 0.5 mM isopropyl-β-D-thiogalatoside for 4 hours at 30°C, and cells were then harvested by centrifugation at $5,000 \times g$ for 20 minutes. The cell pellets were resuspended in a standard buffer of 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride and lysed by sonication. Subsequently, the homogenate was centrifuged at $20,000 \times g$ for 20 minutes, and the supernatant then was applied to a diethylaminoethyl ion exchange column. The unbound proteins were loaded onto a Blue Sepharose column (GE Healthcare, Piscataway, NJ) that was equilibrated with the standard buffer. After washing the unbound proteins off with the standard

————————
*Address correspondence to Chom-Kyu Chong, Department of Biochemistry, College of Natural Sciences, Chungbuk National University, 52 Naesudong-ro, Heungdeok-gu, Cheongju, Chungbuk 361-763, Republic of Korea. E-mail: ckchong01@hotmail.com

buffer, the bound proteins were eluted with the elution buffer (standard buffer plus 10 mM nicotinamide adenine dinucleotide). Proteins in the eluent were identified by using sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis,[21] and quantified by using the Bradford method.[22] The purified proteins were used as immunogens and antigens in the hybridoma screening.

Preparation of polyclonal antibodies. Goat anti-mouse IgG was produced by immunizing goats with mouse IgG, and then purified by using affinity chromatography with protein G Sepharose (Thermo Fisher Scientific, Waltham, MA). Goat anti-mouse IgG was used to capture antibodies on the control line of the RDT.

Preparation of monoclonal antibodies. Recombinant *Pf*LDH and *Pv*LDH were used to immunize seven-week-old female BALB/c mice. The recombinant proteins were mixed with complete Freund's adjuvant for the first immunization and with incomplete Freund's adjuvant for the second and third immunizations. The immunizations were conducted at two-week intervals. The titer of antibodies against pLDH was monitored by using an enzyme-linked immunosorbent assay in a multiwall plate coated with 2 mg/mL of pLDH. When the OD_{450} of a 1,000-fold dilution of blood was greater than 0.2, the next booster immunization step was performed. The booster immunization was performed by injecting 0.2 mL of a solution of pLDH into the tail vein. Three days after the final booster immunization, mouse spleen cells were fused with Sp2/0 myeloma cells. The resulting hybridomas were produced as described.[23]

Hybridomas that produced positive antibodies in the screening were selected for single-cell subcloning by using the limited dilution method. The hybridomas were then used to produce ascitic fluid in the mice. IgG was purified by using protein G Sepharose. The cloned monoclonal antibodies (mAbs) were isotyped by using the Mouse Monoclonal Antibody Isotyping Kit (Sigma-Aldrich, St. Louis, MO).

Preparation of colloidal gold–conjugated monoclonal antibodies. Colloidal gold particles (mean diameter = 40 nm) were prepared according to the method of Frens.[24] Briefly, 100 mL of 0.01% $HAuCl_4$ was boiled. A total of 1.8 mL of 1% trisodium citrate solution was then added while constantly stirring the solution. After the color of the solution changed to wine-red for approximately 45 seconds, the solution was boiled for 5 minutes and cooled and stirred for 10 minutes. The colloidal gold solution was stored in a dark bottle at 4°C and used to prepare colloidal gold-conjugated mAb.

Monoclonal antibody against pLDH was conjugated with colloidal gold particles as described.[25–27] Briefly, mAbs were dialyzed against phosphate-buffered saline for three hours at 4°C. The gold solution was adjusted to pH 9.0 with 0.2 M K_2CO_3, and then mixed with the mAb (1 mg of mAb/100 mL of gold solution; OD_{540} = 2). The resulting mixture was incubated for 30 minutes at room temperature. Subsequently, the gold conjugate was blocked with 1% bovine serum albumin for 30 minutes under the same conditions. Finally, the gold conjugate was washed three times with phosphate-buffered saline containing 1% bovine serum albumin and the absorbance was measured at 450 nm.

Preparation of the RDT for malaria. The FVM Ag RDT was prepared as follows. First, 1.0 mg/mL of goat anti-mouse IgG, 1.0 mg/mL of mAb against *Pf*LDH (clone 2E11), and 1.2 mg/mL of mAb against *Pv*LDH (clone 1H3) were immobilized on the control line (C), test line 1 (T1), and test line 2 (T2),

respectively, of the nitrocellulose membrane. The membrane was then dried for one day at room temperature at a relative humidity of less than 20%. The colloidal gold–conjugated mAb (clone 8C10) was dried on a glass fiber and used as the conjugate pad. The sample pad was prepared by treating cellulose paper with 0.1 M Tris-Cl, pH 8.0, containing 0.5% polyvinyl alcohol and 0.5% Tween-20. The absorbance pad consisted of untreated cellulose paper. All pads overlapped partially to ensure continuous migration of the sample solution along the test strip.

Assay procedure and interpretation of FVM Ag rapid diagnostic test result for malaria. The assay procedure of the FVM Ag RDT is shown in Figure 1A. Briefly, the test is started by loading 5 μL of a blood sample onto the hemolysis pad, and then dispensing 4 drops (approximately 100 μL) of 0.1 M Tris-Cl buffer, pH 8.0, containing 0.1% casein and 1% Tween-20 onto the sample pad. The results can be interpreted approximately 15–20 minutes later. The control line should appear in all tests. If a red band appears at T1, the sample is *P. falciparum*-positive infection. If a band appears at T2, the sample is *P. vivax*-positive infection. If there are bands at both T1 and T2, then the sample is a mixed-species (*Pf* + *Pv*) infection (Figure 1B).

Analytical sensitivity of FVM Ag rapid diagnostic test for malaria. Specimens for which parasite density had been verified by microscopic analysis were serially diluted with uninfected donor blood (10,000–150 parasites/μL for *P. falciparum* and 2,000–60 parasites/μL for *P. vivax*). The analytical sensitivity of
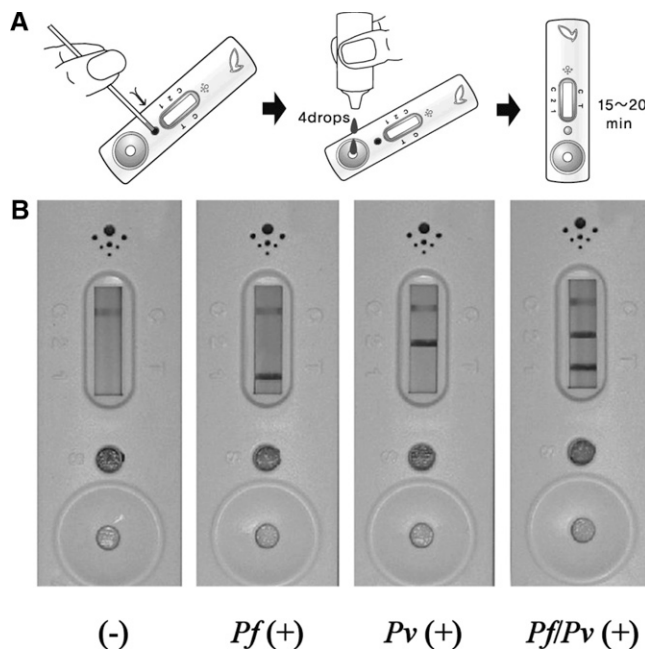

FIGURE 1. Assay procedure and interpretation of the mixed-species infections malaria antigen (FVM Ag) rapid diagnostic test kit to diagnose mixed-species malaria. **A**, Assay procedure. First, the blood sample is loaded onto the hemolysis pad, and four drops of buffer (see Materials and Methods section for composition) is dispensed onto the sample pad. Results are visible after 15–20 minutes. **B**, Interpretation of the results. Absence of any bands (–) indicates an uninfected sample. Presence of a red band at T1 or T2 indicates a single *Plasmodium falciparum*–positive (*Pf*(+)) or *P. vivax*-positive (*Pv*(+)) sample, respectively. If there are bands at T1 and T2, the sample is a mixed-species infection (*Pf*/*Pv*(+)).

the FVM Ag RDT was compared with those of two common commercial kits in Korea, namely, NanoSign™ Malaria Pf/Pan Ag (Bioland Ltd., Ochang, Republic of Korea) and BioLine™ Malaria Pf/Pan Ag (Standard Diagnostics Inc., Suwon, Republic of Korea). These kits detect HRP-II (T1) and pLDH (T2) to diagnose *P. falciparum* and pan malaria infections, respectively.

Application of FVM Ag rapid diagnostic test for malaria to clinical samples and determination of test properties. Two hundred nineteen malaria-positive blood samples (129 for *P. vivax*, 86 for *P. falciparum*, and 7 for *P. vivax* and *P. falciparum*) were obtained from the School of Medicine, Sungkyunkwan University (Suwon, Republic of Korea); School of Medicine, Inje University (Busan, Republic of Korea); Department of Laboratory Medicine, Ilsan Hospital (Goyang, Republic of Korea); and National Institute of Malariology, Parasitology and Entomology (Hanoi, Vietnam). Five hundred malaria-negative blood samples were kindly provided by the Cheongju Medical Center, Korean Hospital Association (Cheongju, Republic of Korea). All of these samples were examined and confirmed by using microscopic analysis and polymerase chain reaction, which amplified the *Plasmodium* circumsporozoite gene.[28]

To examine the parasite density, thick and thin blood smears were made immediately after blood collection and stained with 4% Giemsa for 20 minutes, and then analyzed by light microscopy in each laboratory. Parasites in thick blood films were counted against 200–500 leukocytes. Parasite density was estimated assuming 8,000 leukocytes/μL of blood. The sensitivity, specificity, and predictive values were calculated by using the formulas sensitivity = a/(a + c), specificity = d/(b + d), positive predictive value = a/(a + b), and negative predictive value = d/(c + d), where a is the number of true positive samples, b is the number of false-positives samples, c is the number of false-negative samples, and d is the number of true negative samples.

Sequence alignment. The ClustalW program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used to generate a pairwise sequence alignment of *Pf*LDH (GenBank accession no. ABA46355) and *Pv*LDH (AAY59419).

RESULTS

Properties of mAbs against *Plasmodium* LDH. Although most of the hybridoma clones that we screened secreted antibodies that were cross-specific for *Pf*LDH and *Pv*LDH, we were able to identify 11 clones that produced antibodies with high affinities against pLDH (Table 1). Among them, four hybridoma clones (8C10, 5H11, 6G5, and 1G1) showed strong cross-reactivity with *Pv*LDH and *Pf*LDH. In addition, clones 8C10 and 5H11 had the highest mean (SD) affinities: K_d = 24.21 (2.07) nM and 12.03 (3.10) nM, respectively (Table 1). In addition, clone 8C10 had higher immunoreactivity with *Pf*LDH and *Pv*LDH. However, it did not exhibit any nonspecific reactions with malaria-negative blood samples (Figure 2). Conversely, clone 5H11 showed some nonspecific reactions with malaria-negative blood samples. As a result, we used clone 8C10 to produce the colloidal gold–conjugated mAb for our FVM Ag RDT.

Antibodies produced by hybridoma clones 2E11, 6E4, and 2H9 were *Pf*LDH specific. Because antibody produced by clone 2E11 had the highest affinity and excellent specificity for *Pf*LDH (Table 1 and Figure 2A), we used it as the *Pf*LDH-

TABLE 1
Characteristics of monoclonal antibodies against *Plasmodium* lactate dehydrogenase*

Clone	Isotype	Immunogen	K_d (nM)†	Immunogenicity
8C10	IgG1	*Pv*LDH	24.21 (2.07)	*Pv*LDH/*Pf*LDH specific
5H11	IgG1	*Pv*LDH	12.03 (3.10)	*Pv*LDH/*Pf*LDH specific
1H3	IgG1	*Pv*LDH	36.52 (1.12)	*Pv*LDH specific
3F3	IgG2a	*Pv*LDH	18.30 (1.03)	*Pv*LDH specific
1E4	IgG2a	*Pv*LDH	541.1 (30.7)	*Pv*LDH specific
6E5	IgG2a	*Pv*LDH	762.2 (50.1)	*Pv*LDH specific
6G5	IgG1	*Pf*LDH	236.4 (11.0)	*Pv*LDH/*Pf*LDH specific
1G1	IgG2b	*Pf*LDH	142.4 (14.3)	*Pv*LDH/*Pf*LDH specific
2E11	IgG1	*Pf*LDH	25.24 (0.51)	*Pf*LDH specific
6E4	IgG1	*Pf*LDH	28.33 (7.05)	*Pf*LDH specific
2H9	IgG1	*Pf*LDH	421.1 (32.1)	*Pf*LDH specific

* *Pv*LDH, *Plasmodium vivax* lactate dehydrogenase; *Pf*LDH, *P. falciparum* lactate dehydrogenase.
† The dissociation constant (K_d) was determined by using a Klotz plot, as described by Friquet and others.[29] Values are mean (SD).

specific capture antibody for T1 in our RDT. Similarly, antibodies produced by hybridoma clones 1H3, 3F3, 1E4, and 6E5 were *Pv*LDH specific. Because antibody produced by clone 1H3 had strongest immunoreactivity for *Pv*LDH: mean (SD) K_d = 36.52 (1.12) nM, without cross-reacting with *Pf*LDH (Figure 2A), we used it as the *Pv*LDH-specific capture antibody for T2 in our RDT.
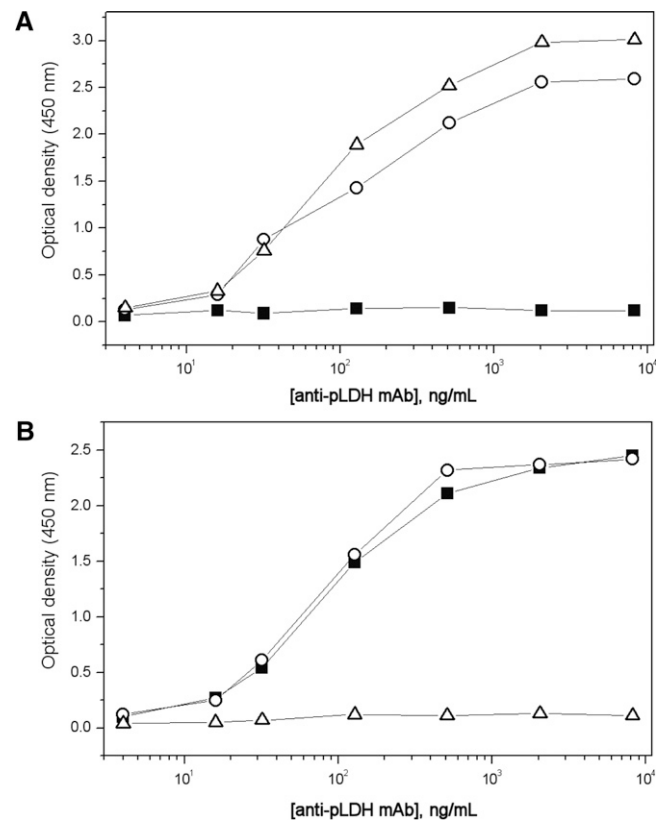

FIGURE 2. Immunoreactivity of monoclonal antibody (mAb) against *Plasmodium vivax* lactate dehydrogenase (LDH) (**A**) and *P. falciparum* LDH (**B**). Shown are hybridoma clones 2E11 (■), 8C10 (X), and 1H3 (△).

Analytical sensitivity of mixed-species infections malaria antigen (FVM Ag), NanoSign™ Malaria *Pf*/Pan Ag, and BioLine™ Malaria *Pf*/Pan Ag rapid diagnostic tests*

Parasite load (parasites/μL)		FVM Ag			NanoSign™ Malaria *Pf*/Pan Ag		BioLine™ Malaria *Pf*/Pan Ag	
Pf	*Pv*	*Pf*	*Pv*	Mixed	*Pf*	Non-*Pv*	*Pf*	Non-*Pv*
10,000	0	+	−	−	+	+	+	+
5,000	0	+	−	−	+	+	+	+
2,500	0	+	−	−	+	+	+	+
1,250	0	+	−	−	+	−	+	−
600	0	+	−	−	+	−	+	−
300	0	+	−	−	+	−	+	−
150	0	+	−	−	+	−	+	−
60	0	−	−	−	+w	−	+w	−
0	2,000	−	+	−	−	+	−	+
0	1,000	−	+	−	−	+	−	+
0	500	−	+	−	−	+	−	+
0	250	−	+	−	−	+w	−	−
0	125	−	−	−	−	−	−	−
0	60	−	−	−	−	−	−	−
2,500	1,000	+	+	M	+	+	+	+
1,250	500	+	+	M	+	+	+	+
600	250	+	+	M	+	+w	+	−
300	125	+	−	−	+	−	+	−
150	60	+	−	−	+	−	+	−

*All results were confirmed by two independent technicians. *Pf* = *Plasmodium falciparum*; *Pv* = *P. vivax*; + = positive; − = negative; +w = weakly positive; M = mixed infection.

Analytical sensitivity of the FVM Ag rapid diagnostic test for malaria. As shown in Table 2, the detection limit of our FVM Ag RDT for *P. falciparum*-positive blood samples (150 parasites/μL) was higher than that of the two commercial RDTs (< 60 parasites/μL^{-1}). The detection limit of our RDT for *P. vivax*-positive specimens was 250 parasites/μL, which was the same as that of the NanoSign™ Malaria Pf/Pan Ag kit and lower than that of the BioLine Malaria Pf/Pan Ag kit (500 parasites/μL). In addition, our FVM Ag RDT was able to detect and differentiate specimens with mixed-species infections.

Properties of the FVM Ag rapid diagnostic test for malaria. As shown in Table 3, the sensitivity of our FVM Ag RDT for *P. falciparum* was 96.5% (83 of 86) and that for *P. vivax* was 95.3% (123 of 129). In addition, our RDT correctly diagnosed 6 (85.7%) of 7 specimens with known mixed-species infections; one case was misdiagnosed as *P. falciparum* positive. The specificity of our RDT for malaria-negative blood samples was 99.4% (497 of 500). The positive predictive value of our RDT for *P. falciparum* was 97.6% (83 of 86) and that for *P. vivax* was 98.4% (123 of 125), and the negative predictive value was 98.2% (497 of 506).

DISCUSSION

Although microscopy is still the gold standard for malaria diagnosis, it requires experienced technicians and a microscope,

Properties of the mixed-species infections malaria antigen (FVM Ag) rapid diagnostic test for malaria

FVM Ag result	Microscopy/polymerase chain reaction result			
	Plasmodium falciparum	*P. vivax*	Mixed	Negative
P. falciparum	83	0	1	1
P. vivax	0	123	0	2
Mixed	0	0	6	0
Negative	3	6	0	497
Total	86	129	7	500

which are not always available in remote areas. In addition, variations in operator technique of preparing, staining, and interpreting blood films can produce different diagnoses.[30–32] Moreover, in the case of samples with mixed-species infections, it is difficult to make a differential diagnosis by using microscopy. These shortcomings of using microscopy for malaria diagnosis motivated us to develop an improved and cost-effective RDT for on-site identification of mixed-species infections.

Unlike existing RDTs, which target two malaria antigens, our FVM Ag RDT targeted two similar antigens (*Pf*LDH and *Pv*LDH). Because the amino acid sequences of these two proteins are more than 90% identical by pairwise sequence alignment, it was extremely difficult to develop specific mAbs. To overcome this difficulty, we used tagless *Pv*LDH and *Pf*LDH as immunogens to expose species-specific epitopes near their N-termini.

The targets of the two commercial RDTs that we compared in this study were *Pf*HRP2 and pLDH. For the detection of *P. falciparum*-positive samples, the difference in the detection limit of these RDTs was caused by one of the two targets. Although HRP2 provides better sensitivity than pLDH, it has problems with false-positive results because of its persistence in the circulatory system after parasite death.[33–37] In this study, the detection threshold of our RDT for *P. vivax*-positive samples was similar to that of NanoSign™ Malaria Pf/Pan Ag but higher than that of BioLine Malaria Pf/Pan Ag. These results might be caused by the difference in immunoreactivity of the pLDH antigens. The analytical sensitivity of our RDT for *P. falciparum* and *P. vivax* was comparable with that of the commercial RDTs and is suitable for diagnostic applications, including on-site identification of mixed-species malaria.

Previous clinical studies have shown that the sensitivity of RDTs is generally 84–100% for *P. falciparum* and 75–96% for *P. vivax*, and the overall specificity is 75–96%.[20,30,36–38] For example, Ratsimbasoa and others[30] reported that the sensitivity of various RDTs for *P. falciparum* and *P. vivax* were 97.0% and 92.3% for the CareStart Malaria Ag kit (AccessBio Inc., Monmouth Junction, NJ), 89.4% and 73.3% for the BioLine Malaria Ag (Standard Diagnostics Inc., Suwon, Republic of Korea), and 92.6% and 86.7% for the OptiMAL Malaria Pf/Pv Ag (DiaMed AG; Cressier sur Morat, Switzerland), respectively. Unlike these RDTs, which exhibited a lower sensitivity for *P. vivax* than *P. falciparum*, the sensitivity of our RDT was similar for both parasites (96.5% for *P. falciparum* and 95.3% for *P. vivax*). Collectively, these results showed that our FVM Ag RDT not only has superior sensitivity and specificity compared with existing RDTs but also the ability to differentiate *P. falciparum*, *P. vivax*, and mixed-species malaria infections.

To our knowledge, our FVM Ag kit is the first RDT with these capabilities. We anticipate that our RDT will be a useful diagnostic tool that will help improve the diagnosis and treatment of malaria, particularly in remote areas of the world.

Authors' addresses: Gyu-Cheol Lee, Research Team of Microbiology, Water Analysis and Research Center, Korea Institute of Water and Environment, K-water, 560 Sintanjin-ro, Daedeok-gu, Daejeon 306-711, Republic of Korea, E-mail: gclee@kwater.or.kr. Eun-Sung Jeon, Institute of BioMedicals, Bioland Ltd., 644-6 Gak-ri, Ochang,

Cheongwon, Chungbuk 363-883, Republic of Korea, E-mail: espluto@hanmail.net. Dung Tien Le, Research Team for Vectorborne Diseases, National Agriculture Research Center, Kannondai 3-1-1, Tsukuba, Ibaraki 305-8517, Japan, E-mail: letiendzung@yahoo.com. Tong-Soo Kim, Department of Parasitology, School of Medicine, Inha University, Third Street, Sinheung-dong, Choong-gu, Inchon 400-712, Republic of Korea, E-mail: tongsookim@inha.ac.kr. Jong-Ha Yoo, Department of Laboratory Medicine, National Health Insurance Corporation Ilsan Hospital, 1232 Baeksok-dong, Ilsandong-gu, Goyang, Gyounggi 410-719, Republic of Korea, E-mail: jhyoo92@nhimc.or.kr. Hak Yong Kim and Chom-Kyu Chong, Department of Biochemistry, College of Natural Sciences, Chungbuk National University, 52 Naesudong-ro, Heungdeok-gu, Cheongju, Chungbuk 361-763, Republic of Korea, E-mails: hykim@chungbuk.ac.kr and ckchong01@hotmail.com.

REFERENCES

1. Mayxay M, Pukrittayakamee S, Newton PN, White NJ, 2004. Mixed-species malaria infections in humans. *Trends Parasitol 20:* 233–240.

2. Chilton D, Malik AN, Armstrong M, Kettelhut M, Parker-Williams J, Chiodini PL, 2006. Use of rapid diagnostic tests for diagnosis of malaria in the UK. *J Clin Pathol 59:* 862–866.

3. McKenzie FE, Bossert WH, 1997. Mixed-species *Plasmodium* infections of humans. *J Parasitol 83:* 593–600.

4. Co EM, Dennull RA, Reinbold DD, Waters NC, Johnson JD, 2009. Assessment of malaria *in vitro* drug combination screening and mixed-strain infections using the malaria Sybr green I-based fluorescence assay. *Antimicrob Agents Chemother 53:* 2557–2563.

5. Siribal S, Nakasiri S, Looareesuwan S, Chavalitshewinkoon-Petmitr P, 2004. Identification of human malaria parasites and detection of mixed infection in Thai patients by nested PCR. *Southeast Asian J Trop Med Public Health 35:* 5–9.

6. Boyd MF, Kitchen SF, 1937. Simultaneous inoculation with *Plasmodium vivax* and *Plasmodium falciparum. Am J Trop Med 17:* 855–861.

7. Boyd MF, Kitchen SF, 1938. Vernal vivax activity in persons simultaneously inoculated with *Plasmodium vivax* and *Plasmodium falciparum. Am J Trop Med 18:* 505–514.

8. James SP, 1931. Some general results of a study of induced malaria in England. *Trans R Soc Trop Med Hyg 24:* 477–525.

9. Mayne B, Young MD, 1938. Antagonism between species of malaria parasites in induced mixed infections. *Public Health Rep 53:* 1289–1291.

10. Mason DP, McKenzie FE, 1999. Blood-stage dynamics and clinical implications of mixed *Plasmodium vivax-Plasmodium falciparum* infections. *Am J Trop Med Hyg 61:* 367–374.

11. Looareesuwan S, White NJ, Chittamas S, Bunnag D, Harinasuta T, 1987. High rate of *Plasmodium vivax* relapse following treatment of falciparum malaria in Thailand. *Lancet 2:* 1052–1055.

12. Meek SR, Doberstyn EB, Gauzere BA, Thanapanich C, Nordlander E, Phuphaisan S, 1986. Treatment of falciparum malaria with quinne and tetracycline or combined mefloquine/sulfadoxine/pyrimethamine on the Thai-Kampuchean border. *Am J Trop Med Hyg 35:* 246–250.

13. Barker RH Jr, Banchongaksorn T, Courval JM, Suwonkerd W, Rimwungtragoon K, Wirth DF, 1992. A simple method to detect *Plasmodium falciparum* directly from blood samples using the polymerase chain reaction. *Am J Trop Med Hyg 46:* 416–426.

14. Franzen L, Westin G, Shabo R, Aslund L, Perlmann H, Persson T, Wigzell H, Pettersson U, 1984. Analysis of clinical specimens by hybridisation with probe containing repetitive DNA from *Plasmodium falciparum.* A novel approach to malaria diagnosis. *Lancet 1:* 525–527.

15. Mehlotra RK, Lorry K, Kastens W, Miller SM, Alpers MP, Bockarie M, Kazura JW, Zimmerman PA, 2000. Random distribution of mixed species malaria infections in Papua New Guinea. *Am J Trop Med Hyg 62:* 225–231.

16. Warhurst DC, Williams JE, 1996. ACP Broadsheet no 148. July 1996. Laboratory diagnosis of malaria. *J Clin Pathol 49:* 533–538.

17. Piper R, Lebras J, Wentworth L, Hunt-Cooke A, Houze S, Chiodini P, Makler M, 1999. Immunocapture diagnostic assays for malaria using *Plasmodium* lactate dehydrogenase (pLDH). *Am J Trop Med Hyg 60:* 109–118.

18. Quintana M, Piper R, Boling HL, Makler M, Sherman C, Gill E, Fernandez E, Martin S, 1998. Malaria diagnosis by dipstick assay in a Honduran population with coendemic *Plasmodium falciparum* and *Plasmodium vivax. Am J Trop Med Hyg 59:* 868–871.

19. Shiff CJ, Premji Z, Minjas JN, 1993. The rapid manual ParaSight-F test. A new diagnostic tool for *Plasmodium falciparum* infection. *Trans R Soc Trop Med Hyg 87:* 646–648.

20. Tjitra E, Suprianto S, Dyer M, Currie BJ, Anstey NM, 1999. Field evaluation of the ICT malaria *Pf/Pv* 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 37:* 2412–2417.

21. Laemmli UK, 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227:* 680–685.

22. Kruger NJ, 1994. Bradford method for protein quantitation. *Methods Mol Biol 32:* 9–15.

23. Kohler G, Milstein C, 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature 256:* 495–497.

24. Frens G, 1973. Controlled nucleation for the regulation of the particle size in monodisperse gold suspensions. *Nat Phys Sci (Lond) 241:* 20–22.

25. Cramer EM, Beesley JE, Pulford KA, Breton-Gorius J, Mason DY, 1989. Colocalization of elastase and myeloperoxidase in human blood and bone marrow neutrophils using a monoclonal antibody and immunogold. *Am J Pathol 134:* 1275–1284.

26. De Waele M, Renmans W, Segers E, De Valck V, Jochmans K, Van Camp B, 1989. An immunogold-silver staining method for detection of cell surface antigens in cell smears. *J Histochem Cytochem 37:* 1855–1862.

27. van den Brink W, van der Loos C, Volkers H, Lauwen R, van den Berg F, Houthoff HJ, Das PK, 1990. Combined beta-galactosidase and immunogold/silver staining for immunohistochemistry and DNA *in situ* hybridization. *J Histochem Cytochem 38:* 325–329.

28. Brown AE, Kain KC, Pipithkul J, Webster HK, 1992. Demonstration by the polymerase chain reaction of mixed *Plasmodium falciparum* and *P. vivax* infections undetected by conventional microscopy. *Trans R Soc Trop Med Hyg 86:* 609–612.

29. Friguet B, Chaffotte AF, Djavadi-Ohaniance L, Goldberg ME, 1985. Measurements of the true affinity constant in solution of antigen-antibody complexes by enzyme-linked immunosorbent assay. *J Immunol Methods 77:* 305–319.

30. Ratsimbasoa A, Randriamanantena A, Raherinjafy R, Rasoarilalao N, Ménard D, 2007. Which malaria rapid test for Madagascar? Field and laboratory evaluation of three tests and expert microscopy of samples from suspected malaria patients in Madagascar. *Am J Trop Med Hyg 76:* 481–485.

31. Durrheim DN, Becker PJ, Billinghurst K, 1997. Diagnostic disagreement–the lessons learnt from malaria diagnosis in Mpumalanga. *S Afr Med J 87:* 1016.

32. Kachur SP, Nicolas E, Jean-François V, Benitez A, Bloland PB, Saint Jean Y, Mount DL, Ruebush TK 2nd, Nguyen-Dinh P, 1998. Prevalence of malaria parasitemia and accuracy of microscopic diagnosis in Haiti, October 1995. *Rev Panam Salud Publica 3:* 35–39.

33. Bell DR, Wilson DW, Martin LB, 2005. False-positive results of a *Plasmodium falciparum* histidine-rich protein 2-detecting malaria rapid diagnostic test due to high sensitivity in a community with fluctuating low parasite density. *Am J Trop Med Hyg 73:* 199–203.

34. Laferi H, Kandel K, Pichler H, 1997. False positive dipstick test for malaria. *N Engl J Med 337:* 1635–1636.

35. Miller RL, Ikram S, Armelagos GJ, Walker R, Harer WB, Shiff CJ, Baggett D, Carrigan M, Maret SM, 1994. Diagnosis of *Plasmodium falciparum* infections in mummies using the rapid manual ParaSight-F test. *Trans R Soc Trop Med Hyg 88:* 31–32.

36. Moody A, 2002. Rapid diagnostic tests for malaria parasites. *Clin Microbiol Rev 15:* 66–78.

37. Playford EG, Walker J, 2002. Evaluation of the ICT malaria *Pf/Pv* and the OptiMal rapid diagnostic tests for malaria in febrile returned travelers. *J Clin Microbiol 40:* 4166–4171.

38. WHO, 1996. A rapid dipstick antigen capture assay for the diagnosis of falciparum malaria. WHO Informal Consultation on Recent Advances in Diagnostic Techniques and Vaccines for Malaria. *Bull World Health Organ 74:* 47–54.