

## Evaluation of Diagnostic Methods of Re-emerging Malaria in Korean Patients

Hwan Sub Lim<sup>1</sup>, and Hyon-Suk Kim<sup>2</sup>

<sup>1</sup>Department of Clinical Pathology, Kwandong University College of Medicine, Kangnung, Korea

<sup>2</sup>Department of Clinical Pathology, Yonsei University College of Medicine, Seoul, Korea

Malaria is one of the most important parasitic diseases especially in tropical areas. Over 300 million people are affected and the condition causes 1-3 million deaths each year. It is transmitted by the bite of infected *Anopheles* mosquitoes. Although Korea was declared to be free of Malaria by the WHO in 1979, malaria re-emergence has been apparent since 1993 amongst soldiers located near the De-Militarized Zone (DMZ) in the northern part of the country.

Conventional microscopic examination of thin and thick blood films demonstrates the presence of the parasite and thus this method has been used to confirm the diagnosis of malaria, but it is a labor-intensive procedure and relies upon subjective interpretation. To overcome these limitations, fast and reliable methods for malaria detection have been recently introduced. In this study, we compared three kinds of antibody detection kits and one biochemical test kit that determines the presence of *Plasmodium* lactate dehydrogenase (pLDH) with conventional peripheral blood smears. The antibody detection methods examined were, two rapid test pack format methods and a single microplate format enzyme-linked immunosorbent assay (ELISA) kit, as manufactured by Korean companies.

The sensitivities of the three commercial antibody detection kits in the early stage of malaria were 70.8%, 77.4%, and 63.6%, their corresponding specificities 90.5%, 91.8%, and 80.9%, and their accuracies 87.6%, 87.0%, and 76.7%. The sensitivity and specificity of the pLDH assay were 100% apiece and the results were in 100% concordance with the microscopy of thick blood films.

Thus, the pLDH assay may be used as an alternative for conventional microscopic blood film examination, especially in emergency situations when prompt treatment is necessary.

**Key Words:** Malaria, *plasmodium vivax*, pLDH, enzyme-linked immunosorbent assay (ELISA)

## INTRODUCTION

Malaria is a parasitic disease transmitted by the *Anopheles* mosquito, which is common in tropical areas and presents a danger to travelers.<sup>1</sup>

The re-emergence of *Plasmodium vivax* malaria has been reported in several countries, such as Peru, India, China and Korea, and has become a serious public problem in these countries.<sup>2,3</sup> In Korea, malaria was declared to have been eradicated in 1979 by the WHO<sup>4</sup>, but in 1993, there was a report of malaria in a young soldier working near the De-Militarized Zone (DMZ) in northernmost South Korea.<sup>5</sup> Imported cases of malaria have been reported frequently in Korea,<sup>6-10</sup> but in these cases the mosquito species were mainly *P. falciparum* and *P. vivax*, though one case of *P. ovale* infection was observed in a Malaysian.<sup>9</sup>

The diagnosis of *P. vivax* malaria is usually made by microscopic examination of peripheral blood films, but accurate diagnosis needs a skilled microscopist and the interpretation is too subjective for routine use in clinical laboratories, particularly now that laboratories are under pressure to shorten the turn around time produce the correct results. The microscopy necessary requires the reading of more than 100 fields of Wright-Giemsa stained peripheral blood smears. And, to increase the sensitivity of the microscopy, both thin and thick blood smears should be examined, by experienced technicians or doctors.

The development of rapid and specific tests to identify malaria is being undertaken in an effort

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to control this disease especially in transfusion services and travel medicine. The polymerase chain reaction (PCR) has been applied to the identification of human malarial infection<sup>11</sup> and it showed high sensitivity and specificity.<sup>12</sup> However, the PCR method may not be the correct clinical diagnostic tool because it does not provide a response after successful treatment,<sup>13</sup> and can only be used to complement some other method. In this study, we evaluated the results obtained using recently introduced diagnostic methods for malaria, including, traditional microscopic examination of thin and thick blood films, Plasmodium lactate dehydrogenase (pLDH) enzyme produced both in its sexual and asexual forms for the assay of parasites and three antibody detection kits manufactured by Korean companies.

## MATERIALS AND METHODS

### Patients

Blood samples were collected from subjects showing either symptoms of malaria or from those with a confirmed diagnosis using blood films. Study samples were limited to the early stage of malaria. One hundred eighty two venous blood samples of patients suspected as having malaria were obtained from Myongji Hospital in Ilsan City, Kyunggi Do, Korea and samples of patients confirmed with malaria were obtained from the Severance Hospital, Yonsei University College of Medicine in Seoul, Korea between June and August, 2000.

### Diagnostic test methods

*Detection of human IgG & IgM to Plasmodium vivax using a one-step test pack from Green Cross Inc. (Youngin, Korea)*

Genedia<sup>®</sup> Malaria (*P. vivax*) Ab Rapid: human IgG and IgM antibodies to *Plasmodium vivax* (*P. vivax*) were obtained from Green Cross Inc. (Youngin, Korea).

This test kit detects the antibodies to the merozoite surface protein (MSP), Duffy-binding protein (DBP), and circumsporozoite protein (CSP) antigens of *P. vivax*, and is composed of a

small cassette with a nitrocellulose membrane and an absorbent pad. On the cassette, the test and control lines are immobilized with 3 kinds of *P. vivax* specific recombinant antigens and anti-human IgG and IgM. All reagents should be brought up to room temperature, 15~30 minutes before use, to prevent moisture formation inside the bag. Two drops of the first buffer solution (phosphate casein buffer with normal goat serum, Tween 20) were added to the well of this device. 40  $\mu$ L of the patients serum/plasma was then added and allowed for complete absorption. Two drops of the first washing solution were then added and allowed for complete absorption. After a second washing with distilled/deionized water, lyophilized gold conjugate, which contains anti-human IgG and IgM conjugated with colloidal gold, were reconstituted, and 40  $\mu$ L of this mixture was then added with a micropipette. Finally, three drops of the second washing solution were added and the result read and recorded within 10 minutes.

*Detection of IgG, IgA and IgM Antibodies to Plasmodium vivax using a one-step test pack from Standard Diagnostics Inc. (SD<sup>®</sup>, Suwon, Korea)*

SD Malaria (*P. vivax*) IgG, IgA and IgM antibodies to *P. vivax* were obtained from Standard Diagnostics Inc. (Suwon, Korea).

This test uses an immunochromatographic method for the qualitative detection of antibodies. The total procedure took 20~25 minutes. Ten microliters of serum was added to the well of the device and three drops of assay diluent provided by the manufacturer added. At the beginning of the test, a purple color moves across the result window in the center of the test device. After incubation at room temperature for 10 minutes, the result was read and recorded within 20 minutes.

*Detection of antibody to Plasmodium vivax by ELISA (LG<sup>®</sup> Chemical Inc. Iksan, Korea)*

LG Malaria Anti-PV: antibody to *P. vivax* by ELISA from LG Chemicals Inc. (Iksan, Korea) kit was also examined.

Cloned merozoite surface protein is used in the kit in the form of a purified merozoite surface protein-coated microplate. Before the test, all kit

reagents should be brought up to room temperature and gently mixed. Serum samples from patients and horse-radish peroxidase (HRP)-conjugated antigen solution were added to the wells of the microplate for the reaction with the malarial antibody in sandwich method. After incubation, excess serum and conjugate solution were removed by washing. To perform the test at least six control wells should be used (1 well as a blank, 3 wells as negative controls, and 2 wells as positive control) in addition to patients sera. Each 100  $\mu$ L of negative and positive control, patients serum sample was added to each well respectively. HRP-conjugated malarial antigen was added to each well except for the blank control. After gently shaking the microplate for 5~10 seconds, it was incubated at  $37 \pm 1^\circ\text{C}$  for 90 minutes. Then, after washing each well with washing solution containing polysorbate 20 six times, each well was completely emptied. Substrate solution containing hydrogen peroxide (25~33%) was then added to each empty well including the blank control. After another incubation at  $15\sim30^\circ\text{C}$  for  $30 \pm 1$  minutes, stopping solution (Sulfuric acid, 49 mg/mL, 100  $\mu$ L) was added, absorbance was read at 450 nm and the result determined.

*Dipstick pLDH assay (OptiMAL<sup>®</sup>) from Flow Inc. (Portland, OR, U.S.A.).*

This immunochromatographic test is commercially available under the name OptiMAL and was provided by Flow Inc. (Portland, OR, U.S.A.). This assay detects the presence of pLDH enzyme produced in both sexual and asexual forms of parasites. In addition to a control antibody reaction zone at the top of the test strip, the assay contains 2 test lines/reaction zones. The first encountered by the sample is comprised of an antibody that is specific to *P. falciparum* pLDH. The second test line is comprised of a pan-specific pLDH monoclonal antibody that recognizes *P. vivax*, *P. malariae*, and *P. ovale*. Before the test, one drop of buffer was dispensed to conjugate well and 4 drops into washing well. After 1 minute, ten microliter ( $\mu$ L) of blood sample was added to the conjugate well and mixed gently and incubated for 1 minute. The test dipstick was then placed vertically into the conjugate well and allowed to

stand for 10~15 minutes. The entire sample in the conjugate well was allowed to wick up the test dipstick towards the filter pad and then control band appeared progressively. The dipstick was moved from the conjugate well to washing well. After clearing the reaction field of the dipstick, the control band became clearly visible within 5~10 minutes. Results were interpreted by a trained technician or doctor.

#### *Microscopy of Peripheral Blood Films*

Peripheral blood films for malaria testing were made by the traditional method with both thin and thick smears. Detection of the parasite was performed by a skilled medical technologist and confirmed by a clinical pathologist after examining at least in 100 oil-immersion high power fields at 1,000 x magnification.

#### **Data analysis**

For statistical analysis, only the results on the initial blood sample were included, although several samples were available from some patients. Sensitivities, specificities and overall accuracies were analyzed on the basis of the result of the thick blood film microscopy, because this remains the method used as a confirmatory diagnostic test for malaria.<sup>12,13</sup>

## **RESULTS**

Blood samples from 104 male and 78 female patients were included in this study. The mean age of the patients was 34.4 years (Table 1). Thirty-three patients (27 male and 6 female) were diagnosed with malaria by blood film microscopy.

The concordance rate between microscopy and (1) Genedia<sup>®</sup> Malaria (*P. vivax*) Ab Rapid: human

**Table 1.** Sex Distribution and Mean Age of Patients Included

	Mean Age	No. of Patients
Male	30.5 $\pm$ 21.1*	104
Female	39.6 $\pm$ 24.6	78
Total	34.4 $\pm$ 23.0	182

\*Mean  $\pm$  SD

IgG and IgM antibody was 56.7% (17 out of 30), (2) SD<sup>®</sup> Malaria (*P. vivax*): IgG, IgA and IgM antibodies to *P. vivax*, 82.8% (24 out of 29), and (3) LG<sup>®</sup> Malaria Anti-PV: antibody by ELISA for malaria infection, 51.9% (14 out of 27), respectively. The results are shown in Tables 2 and 3. Concordance rate between microscopy and the pLDH assay (OptiMAL<sup>®</sup>) was 100% (21 out of 21) as in Table 2.

Sensitivities of the 4 diagnostic methods (1) Genedia<sup>®</sup> Malaria (*P. vivax*) Ab Rapid: human IgG and Ig M antibodies to *P. vivax*, (2) SD<sup>®</sup> Malaria (*P. vivax*): IgG, IgA and IgM antibodies to *P. vivax*, (3) LG<sup>®</sup> Malaria Anti-PV: antibody to *P. vivax* by ELISA, and (4) pLDH assay (OptiMAL<sup>®</sup>) - were 70.8%, 77.4%, 63.6%, and 100.0%, respectively (Table 3). Specificities of the methods (1) human IgG and Ig M antibodies to *P. vivax*, (2) IgG, IgA and IgM antibodies to *P. vivax*, (3) antibody to *P. vivax* by ELISA, (4) pLDH assay - were 90.5%, 91.8%, 80.9%, and 100.0%, respectively (Table 3).

Overall accuracies of the methods (1) human IgG and Ig M antibodies to *P. vivax*, (2) IgG, IgA and IgM antibodies to *P. vivax*, (3) antibody to *P. vivax* by ELISA, and (4) pLDH assay were 87.6%, 87.0%, 76.7%, and 100.0%, respectively (Table 3).

One patient was diagnosed as *P. vivax* by thick blood film microscopy, but the pLDH assay showed *P. falciparum* instead of *P. vivax*. In another case, a patient was admitted with a diagnosis of malaria, but the presence of the malaria parasite could not be demonstrated by microscopy or pLDH assay. Nevertheless, he had been diagnosed with malaria 2 days before his admission and had received medication before being transferred. We retrieved his initial blood sample from the hospital where he was diagnosed. In that initial blood sample, pLDH assay and thick blood film microscopy were both positive for *P. vivax*.

In other case, a 27 year-old man was diagnosed with malaria in his initial visit to hospital and

**Table 2.** Results of Each Method Compared to Thick Blood Film Microscopy

Method		Thick blood film microscopy		Total
		Positive	Negative	
(1) Human IgG and IgM to <i>P. vivax</i>	Positive	17	7	24
	Negative	13	124	137
(2) IgG, IgA and IgM Abs to <i>P. vivax</i>	Positive	24	7	31
	Negative	5	56	61
(3) Ab to <i>P. vivax</i> by ELISA	Positive	14	8	22
	Negative	13	55	68
(4) pLDH assay	Positive	21	0	21
	Negative	0	70	70
Total		33	149	182

**Table 3.** Sensitivity, Specificity, False Positive Rate, False Negative Rates and Overall Accuracy of the Methods

	Sensitivity (%)	Specificity (%)	False positive rate (%)	False Negative rate (%)	Overall accuracy (%)
(1) Human IgG, IgM antibody (Genedia <sup>®</sup> )	70.8	90.5	9.5	29.2	87.6
(2) IgG, IgA and IgM antibody (SD <sup>®</sup> )	77.4	91.8	8.2	22.6	87.0
(3) Antibody by ELISA (LG <sup>®</sup> )	63.6	80.9	19.1	36.4	76.7
(4) pLDH assay (OptiMAL <sup>®</sup> )	100	100	0	0	100

received medication. He showed a positive result on thick blood film microscopy, pLDH assay and IgG, IgA and IgM antibodies to *P. vivax*. After treatment, results of the pLDH assay and thick blood film microscopy became negative, but IgG, IgA and IgM antibodies to *P. vivax* showed still a positive result. Antibody detected by ELISA was negative in the initial sample, but became a borderline positive after 2 weeks.

## DISCUSSION

Malaria has been an important worldwide communicable parasitic disease for many years, and prevailed throughout Korea until the 1950s. Recently malaria has re-emerged in South Korea and has become a threat to public health and in particular to blood donation.<sup>4</sup>

A prolonged incubation period has been observed in many strains of *P. vivax*, such as Dutch, Madagascan, Russian and Korean, mainly in temperate climates. The first report of a long-term incubation period for Korean *P. vivax* was made by Hasegawa.<sup>14</sup> Brunetti et al. reported an outbreak of *P. vivax* malaria in California, U.S.A., which resulted from veterans returning from the Korean War.<sup>15</sup> Among them, 26 persons became ill after an incubation period of 217~316 days.<sup>15</sup> Tiburskaja and Vrublevskaia experimentally proved cases involving infection with a North Korean strain of *P. vivax*.<sup>16</sup> They found that 18 out of 77 patients showed malaria symptoms after 11 months incubation period.<sup>16</sup> In 1998, 3,982 cases were reported in Korea. Most of these cases (87.6%) were reported between June and August, and only 0.2% in January, February, March, and December.<sup>17</sup> The seasonal occurrence of the re-emerging malaria is similar to that of the pattern shown in the 1960s in Korea.<sup>18</sup> Clinically vivax malaria in Korea reported as an unstable type that could be influenced by changes of climatic, socio-ecological, political (such as war), entomological and parasitological factors.<sup>16</sup>

Relapse of vivax malaria may be due to a mixture of sporozoites with different abilities to undergo hepatic schizogony, some are capable of making schizonts quickly, but others need longer time to complete schizogony. The latter can lead

to prolonged incubation periods as well as repeated relapses.<sup>19</sup> Since the re-emergence of *P. vivax* malaria, very few relapsed cases have been reported.<sup>20</sup> This may be due to the successful treatment of almost all patients with the standard chloroquine and primaquine combination therapy, if diagnosed accurately as *P. vivax*.<sup>20</sup> The courses of illnesses were relatively mild and prognosis was good in most of the patients. Drug-resistance of the re-emerging *P. vivax* has not been reported and no mortality has been reported to date.

Some methods, such as the selective detection of pLDH activities<sup>21</sup> and the Parasight-F kit detection of histidine rich protein for *P. falciparum* have been introduced as replacements for microscopy.<sup>22</sup> Various other reports have been issued regarding the replacement of traditional microscopic blood film examination with rapid diagnostic methods, such as the pLDH assay. The pLDH assay (OptiMAL<sup>®</sup> test) is reported to have sensitivities of 88~94%, and specificities of 99~100%.<sup>23</sup> In another report, irrespective of stage in the diagnosis of *P. falciparum*, the OptiMAL test had a sensitivity of 91.3%, a specificity of 94.7%, a positive predictive value of 87.2%, and a negative predictive value of 94.7% at initial diagnosis.<sup>24</sup> This diagnostic kit could be used where microscopy is not available and for urgent diagnosis of malaria in the emergency room at nights and weekends, when laboratory support is unavailable, or when relatively inexperienced microscopists may be on duty. An outstanding feature of pLDH enzyme activity based tests is that when parasites are cleared from the peripheral blood, its activity is reduced immediately.<sup>25</sup> pLDH activity could be useful for managing drug-resistant malaria and for patient management as well as consistent relationship of infection and activities in whole blood and packed cells. Activity of the enzyme declines rapidly after treatment with anti-malarial drugs and when patients are cured.<sup>26</sup> The test was improved by using 3-acetyl pyridine adenine dinucleotide (APAD) instead of nicotinamide adenine dinucleotide (NAD) as a substrate. In our study, we also observed the conversion of the pLDH assay from positive to negative in a patient that had been treated successfully. Activities detected in whole blood and packed red cells were similar and followed an identical

pattern with parasite detection by thick blood film microscopy, but activity of pLDH in plasma did not correlate with infection and showed lower activity than in whole blood and packed cells.<sup>25</sup>

The ELISA based methods for detecting antibodies to *P. vivax* have an inherent limitation because the antibodies are induced by a sequential immune response after initial exposure. Sensitivity and specificity of the antibody-based detection kits ranged from 63.6 to 77.4%, and from 80.9 to 91.8%, in our study. Overall accuracy of the antibody detection methods was 76.7~87.6% in our study subjects. Specificities of the antibody detection kits were high enough for primary diagnosis and for previous history of malaria, but due to its low sensitivity and low concordance rate with blood film microscopy in the early stage, antibody detection methods could not be offered as replacement methods of blood film microscopy. pLDH assay was believed to offer an alternative to traditional blood film microscopy, but the pLDH assay presents a problem as it is unable to differentiate mixed infection between *P. falciparum* and *P. vivax*.<sup>26</sup> In our study, one patient found to have *P. falciparum* by pLDH assay was proved to be *P. vivax* positive by thick blood film microscopy.

Although conventional microscopy of thick blood films is still regarded as the gold standard for the diagnosis of malaria, the pLDH assay kit could be used instead of thick blood film microscopy when results are urgently needed for the differential diagnosis of patients, for example, in the absence of expert microscopists or clinical pathologists. Because the pLDH assay does not need any blood preparation or careful examination prior to diagnosis, it only takes about 20 minutes and the procedure is very simple. Antibody detection kits cannot replace thick blood film microscopic examination, and could only be used to complement microscope based diagnostic methods.

Hyon Suk Kim, M.D.

Department of Clinical Pathology, Yongdong Severance Hospital, Yonsei University College of Medicine, Yongdong P.O. Box 1217, Seoul 135-720, Korea,

Tel: 82-2-3497-3531, Fax: 82-2-3462-9493

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