



ORIGINAL ARTICLE

Distribution of Antibodies Specific to the 19-kDa and 33-kDa Fragments of *Plasmodium vivax* Merozoite Surface Protein 1 in Two Pathogenic Strains Infecting Korean Vivax Malaria Patients

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Abstract

Objectives: *Plasmodium vivax* merozoite surface protein 1 (PvMSP1) is the most intensively studied malaria vaccine candidate. Although high antibody response-inducing two C-terminal fragments of PvMSP1 (PvMSP1-19 and PvMSP1-42) are currently being developed as candidate malaria vaccine antigens, their high genetic diversity in various isolates is a major hurdle. The sequence polymorphism of PvMSP1 has been investigated; however, the humoral immune responses induced by different portions of this protein have not been evaluated in Korea.

Methods: Two fragments of PvMSP1 were selected for this study: (1) PvMSP1-19, which is genetically conserved; and (2) PvMSP1-33, which corresponds to a variable portion. For the latter, two representative strains, Sal 1 and Belem, were included. Thus, three recombinant proteins, PvMSP1-19, PvMSP1-33 Sal 1, and PvMSP1-33 Belem, were produced in *Escherichia coli* and then tested by enzyme-linked immunosorbent assays using sera from 221 patients with vivax malaria.

Results: Of the 221 samples, 198, 142, and 106 samples were seropositive for PvMSP1-19, PvMSP1-33 Sal 1, and PvMSP1-33 Belem, respectively. Although 100 samples were simultaneously seropositive for antibodies specific to all the recombinant proteins, 39 and six samples were respectively seropositive for antibodies specific to MSP1-33 Sal 1 and MSP1-33 Belem. Antibodies specific to PvMSP1-19 were the most prevalent.

Conclusion: Monitoring seroprevalence is essential for the selection of promising vaccine candidates as most of the antigenic proteins in *P. vivax* are highly polymorphic.

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1. Introduction

Plasmodium vivax is the most prevalent species that causes malaria in humans [1]. It is endemic in the tropical and subtropical countries of Africa, the Middle East, the South Pacific, Central and South America, and in Asia, including the Republic of Korea (ROK) [2,3]. In recent years, several reports throughout the world have linked *P. vivax* to severe disease and death [4–6]. These findings associated with the emergence of drug-resistant strains have increased concerns regarding this species [7]. Since an effective malaria vaccine capable of inducing robust and long-lasting protection in naturally exposed individuals would be an important tool for malaria control, studies evaluating immune responses against different *P. vivax* vaccine candidates are urgently required.

Proteins expressed on the surface of *P. vivax* merozoites are important candidates for malaria vaccine development. Among these proteins, merozoite surface protein 1 (MSP1) is the most intensively studied. MSP1 is synthesized as a high molecular weight precursor (approximately 200 kDa), which is then processed into several smaller MSPs [8]. During invasion, the C-terminal 42-kDa fragment (MSP1-42) is further processed into 33-kDa (MSP1-33) and 19-kDa (MSP1-19) fragments. Only the MSP1-19 fragment remains on the merozoite surface and is transported into the invaded erythrocytes [9,10]. The C-terminus of MSP1 reportedly induces high antibody responses in hosts, and specific antibodies against this region are known to inhibit merozoite invasion [11,12]. Although both MSP1-19 and MSP1-42 are being considered as potential vaccine candidates, the processing and presentation of these fragments may be problematic due to the large number of disulfide linkages in the two epidermal growth factor-like regions of MSP1-19 [13,14]. In addition, the MSP1-33 fragment, which is the fragment of MSP1-42 without MSP1-19, shows an extensive polymorphism in malaria patient populations [15]. Three representative MSP1 variants of *P. vivax* (PvMSP1)—Belem, Sal-1, and recombinant types—have been observed in the ROK [16,17]. In addition, single-nucleotide polymorphisms have frequently been observed in *P. vivax* isolates from vivax malaria patients [15]. Studies on the MSP1 polymorphism have been performed in the ROK; however, the distribution of strain-specific antibodies has not yet been evaluated [18,19]. In this study, we generated three recombinant proteins of which two correspond to the polymorphic variants of PvMSP1-33 (PvMSP1-33 Sal 1 and PvMSP1-33 Belem) and the other corresponds to the conserved PvMSP1-19. We also evaluated antibody responses to these proteins in individuals infected with *P. vivax* in ROK to determine the frequency and the magnitude of the humoral response against different *P. vivax* vaccine candidate antigens.

2. Materials and methods

2.1. Ethics statement

This study was approved by the research ethics committee of Kyungpook National University (Daegu, Korea). All the participants signed written informed consent forms and agreed to provide 5-mL blood samples.

2.2. Sample collection

The samples were collected at hospitals and health centers throughout the northern region of the ROK, where vivax malaria is endemic in the summer season (June to August). In 2015, 90.4% (619/685) of vivax malaria cases reported in ROK had occurred in this area. Venous blood samples with EDTA were obtained from 221 individuals showing classic symptoms of malaria, who sought treatment at the health facilities mentioned below. The samples were first diagnosed as vivax malaria using a rapid diagnostic test kit (NanoSign Malaria P.f/P.v; Bioland, Seoul, Korea) at a hospital or health center. After blood collection and diagnosis, all the patients were treated with chloroquine. First of all, 600 mg chloroquine was administered, and then three more doses of 300 mg chloroquine were administered at 6 hours, 24 hours, and 48 hours after the first dose.

The blood samples were centrifuged at 1,500g for 15 minutes to obtain plasma for further studies. The plasma samples were transported on ice to a laboratory in the Department of Parasitology and Tropical Medicine at Kyungpook National University and were stored at -70°C until required. The 221 blood samples from vivax malaria patients consisted of 140 and 81 samples collected in 2012 and 2013, respectively. All the samples were collected from symptomatic vivax malaria patients who had not been previously infected with *P. vivax*. The mean age of the patients at sampling in 2012 and 2013 was 43.2 years and 36.4 years, respectively. Of all patients from whom samples were collected from in 2012 and 2013, 40.0% (56/140) and 30.9% (25/81) were from men, respectively. In most samples, the *P. vivax* parasite was not observed using microscopy due to low parasitemia, even though the rapid diagnostic test and nested-polymerase chain reaction (PCR) were positive. Samples were also obtained from 30 healthy volunteers who resided in nonendemic areas (southern parts) of the ROK and had not traveled to vivax malaria endemic areas.

2.3. Microscopic examination and nested-PCR for vivax malaria diagnosis

All the blood samples were examined using microscopic analysis of Giemsa-stained thick and thin blood films and using nested-PCR, targeting the 18S ribosomal RNA as described previously [20,21]. Briefly, 5 μL of extracted DNA was used as the template in a primary

PCR, and 1 μ L of the primary PCR product was used as a template in the second amplification. The cycling conditions for the primary PCR were as follows: initial denaturation at 94°C for 5 minutes, followed by 25 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute. The cycling conditions for the second PCR were: initial denaturation at 92°C for 5 minutes, followed by 25 cycles of denaturation at 92°C for 20 seconds, annealing at 52°C for 20 seconds, and extension at 78°C for 20 seconds. The final PCR products were detected by staining with ethidium bromide.

2.4. PvMSP1 cloning

Genomic DNA was extracted from a Korean *P. vivax* isolate using the QIAamp DNA blood kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The extracted DNA was used to amplify the *MSP1-33* Sal 1, *MSP1-33* Belem, and *MSP1-19* genes. Oligonucleotide primer sets containing *Bam*HI and *Eco*RI restriction enzyme sites were designed based on the following reference genes: PvMSP1 Sal 1 strain XM001614792 for *PvMSP1-33* Sal 1 and *PvMSP1-19*, and PvMSP1 Belem strain AF435594 for *PvMSP1-33* Belem. The following oligonucleotide primers were used: for PvMSP1-19 (450 bp), forward primer 5'-CGTGGATCCAATGAAGTGAAGTCTTCTG-3' and reverse primer 5'-TTCGAATTCAAGCTCCATGCA-CAGGAG-3'; for PvMSP1-33 Sal 1 (854 bp), forward primer 5'-GCGGGATCCGTTAAGGAAGCTTTGCA-AG-3' and reverse primer 5'-CGCGAATTTCGCAATAAATTCGTTCTTTTAC-3'; and for PvMSP1-33 Belem (993 bp), forward primer 5'-CGTGGATCCA-TAAGCTACCTGTCCAGTG-3' and reverse primer 5'-CGCGAATTCCATCGATTGGGTCTTTTCAGTAG-3'. Amplification was performed in 50 μ L 1 \times PCR buffer (Takara, Tokyo, Japan) containing 2.5 U Taq polymerase, 2 μ L DNA template, 10 pmol of each primer, and 2mM of each deoxynucleoside triphosphate. The cycling conditions were as follows: initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, and a final extension step at 72°C for 5 minutes. The PCR product was cloned into the *Bam*HI and *Eco*RI restriction enzyme sites of the pGEM-4T-1 vector. The resulting plasmid construct was confirmed with restriction enzyme digestion and nucleotide sequencing. PvMSP1-33 Sal 1 and PvMSP1-33 Belem constructs were selected based on sequencing and Basic Local Alignment Search Tool search results.

2.5. Recombinant PvMSP1 expression and purification

PvMSP1-33 Sal 1, PvMSP1-33 Belem, and PvMSP1-19 were expressed individually as glutathione S-transferase (GST) fusion proteins in the *Escherichia coli* BL21 (DE3) strain (GE Healthcare, Chicago, IL, USA)

according to the manufacturer's instructions. *E. coli* cells expressing these proteins were washed three times with phosphate-buffered saline (PBS), lysed in 1% Triton X-100-PBS, sonicated, and then centrifuged at 10,000g for 10 minutes at 4°C. Soluble GST-fused rPvMSP1-33 Sal1, rPvMSP1-33 Belem, or rPvMSP1-19 proteins were purified from the cell lysates with glutathione-Sepharose 4B beads (GE Healthcare) according to the manufacturer's instructions. After purification, the GST tag was subsequently removed from the GST-fusion proteins using a Thrombin Kit (Novagen, Darmstadt, Germany).

2.6. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blotting

The expression of recombinant proteins was verified using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining, and the antigenicity of the expressed recombinant proteins was confirmed using Western blot analysis. To identify the antigenicity of the recombinant proteins, we applied pooled serum from 30 patients infected with *P. vivax* to the Western blot. In addition, 10 patients each infected with *P. vivax* classified as MSP1 Sal 1 and MSP1 Belem in a previous study [18] were used for another Western blot analysis in order to determine whether the two antigens, PvMSP1-32 Sal 1 and Belem, could distinguish the respective sera. The recombinant proteins were separated by 14% SDS-PAGE and then electroblotted onto a nitrocellulose membrane [22]. The membranes were blocked with 5% skimmed milk in PBS plus 0.05% Tween 20 and then probed with pooled serum (1:100) as the primary antibody. After the membrane was washed with PBS plus Tween 20, horseradish peroxidase-conjugated antihuman immunoglobulin G (1:2,000; Bethyl Laboratories, Montgomery, TX, USA) was applied as the secondary antibody. Finally, the reacted proteins were detected via chemiluminescence using enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ, USA).

2.7. Enzyme-linked immunosorbent assay

The optimal concentrations or dilutions of the antigen, serum, enzyme-antibody conjugate, and substrate solutions were determined through "checkerboard" titrations of each reagent against all other reagents. Individual wells of a microtiter plate (Nunc, Roskilde, Denmark) were coated overnight at 4°C with the purified rPvMSP1-19, rPvMSP1-33 Sal1, and rPvMSP1-33 Belem proteins (1 mg/mL) dissolved in 0.05M carbonate-bicarbonate buffer (pH 9.6). The plates were then blocked with 3% skim milk solution for 1 hour at 37°C. After washing with 0.05% Tween 20 in PBS, the plates were incubated with sera at a dilution of 1:100. The binding of the antibody was detected via incubation with horseradish peroxidase-conjugated antihuman

immunoglobulin G (IgG, 1:5,000; Bethyl Laboratories) or antihuman IgG1, IgG2, IgG3 (1:5,000; MyBioSource, San Diego, CA, USA) and 2,20-aminobis (3-ethylbenzthiazolone sulfonic acid; Sigma-Aldrich, St. Louis, MO, USA). The optical density (OD) was measured at 415 nm using an MTP-500 microplate reader (Corona Electric, Ibaraki, Japan). The mean OD values of triplicate enzyme-linked immunosorbent assay (ELISA) results were then calculated. The ELISA titer was considered as positive when the OD was ≥ 0.08 , ≥ 0.1 , and ≥ 0.17 for rPvMSP1-19, rPvMSP1-33 Sal 1, and rPvMSP1-33 Belem, respectively. The OD cutoff values (0.08, 0.1, and 0.17) were calculated by determining the mean OD for normal sera (sera from 30 healthy people) plus threefold standard deviation.

2.8. Genetic polymorphisms of the PvMSP1 gene

The PvMSP1 sequences in the study samples were obtained from a previous study wherein the same samples were classified by the PvMSP1 genotype as PvMSP1 Sal 1, Belem, or recombinant type [18].

2.9. Statistical analysis

The means and standard deviations were calculated, and the statistical significance of the differences was determined by Student *t* test using JMP Version 8 (SAS Institute Inc., Cary, NC, USA). Differences in the means were considered as statistically significant when the *p* values were < 0.05 .

3. Results

3.1. Production of PvMSP1-19, PvMSP1-33 Sal 1, and PvMSP1-33 Belem

Three PvMSP1 proteins, PvMSP1-19, PvMSP1-33 Sal 1, and PvMSP1-33 Belem, were selected for this study (Figure 1A), with the lengths of the nucleotide sequences of the respective coding regions being 450 bp, 854 bp, and 993 bp (data not shown). PvMSP1-19, PvMSP1-33 Sal 1, and PvMSP1-33 Belem were expressed as soluble GST fusion proteins in *E. coli*, with molecular masses of approximately 42 kDa, 57 kDa, and 61 kDa, respectively. Subsequently, 16-kDa, 31-kDa, and 35-kDa proteins of PvMSP1-19, PvMSP1-33 Sal 1, and PvMSP1-33 Belem, respectively, were produced upon GST cleavage by thrombin (Figure 1B).

3.2. Reactivity of PvMSP1-19, PvMSP1-33 Sal 1, and PvMSP1-33 Belem

To evaluate the antigenicity of PvMSP1-19, PvMSP1-33 Sal 1, and PvMSP1-33 Belem, we performed Western blotting with the three recombinant proteins and sera from patients with vivax malaria and healthy individuals. The recombinant proteins reacted with the sera from the infected patients but not with

those from healthy individuals, indicating that the recombinant proteins could be used to detect *P. vivax* infection in a serum sample (Figure 1C).

Subsequently, another Western blot analysis was performed to confirm whether rPvMSP1-33 Sal 1 and rPvMSP1-33 Belem could differentiate the sera from patients infected with *P. vivax* Sal 1 and Belem strains. Infection with *P. vivax* Sal 1 and Belem was determined based on data from our previous study where infection with these strains was detected by sequencing the *PvMSP1* gene [18]. The results showed that the serum from patients infected with *P. vivax* MSP Sal 1 reacted to rPvMSP1-33 Sal 1 but not to rPvMSP1-33 Belem. In contrast, rPvMSP1-33 Belem reacted to the serum collected from a patient infected *P. vivax* Belem, but not to rPvMSP1-33 Sal 1 (Figure 1C).

3.3. Prevalence of antibodies specific to PvMSP1-19, PvMSP1-33 Sal 1, and PvMSP1-33 Belem

Among the 221 samples from patients with confirmed vivax malaria, 198 (89.6%), 142 (64.3%), and 106 (48.0%) samples were seropositive for PvMSP1-19, PvMSP1-33 Sal 1, and PvMSP1-33 Belem, respectively (Table 1). In addition, the levels of PvMSP1-19 antibodies were significantly higher than the levels of PvMSP1-33 Sal 1 and Belem antibodies (Figure 2). However, the percentage of samples that were seronegative for PvMSP1-33 Sal 1 (35.7%) and PvMSP1-33 Belem (52.0%) was higher than that for PvMSP1-19 (10.4%). Moreover, 100 (45.3%) samples were seropositive for antibodies to all three recombinant proteins (Table 2).

4. Discussion

Surveillance studies for the prevalence of antibodies specific to vaccine candidates must be performed before selecting a candidate for the development of serodiagnostic tools and vaccines, especially since most of the promising vaccine and serodiagnostic candidates are highly polymorphic. Therefore, this study investigated the prevalence of antibodies specific to PvMSP1-19, PvMSP1-33 Sal 1, and PvMSP1-33 Belem, which are promising vaccine candidates, in Korean *P. vivax* isolates.

In Western blotting analyses, the three recombinant proteins successfully demonstrated antigenicity. The antibody response to a *Plasmodium* antigen depends on the correct folding of conformational epitopes in the antigen. Therefore, in order to investigate the prevalence of antibodies to PvMSP1-19, PvMSP1-33 Sal 1, and PvMSP1-33 Belem, the recombinant proteins should be structurally similar to the native proteins. Previously, recombinant PvMSP1 fragments were used to determine

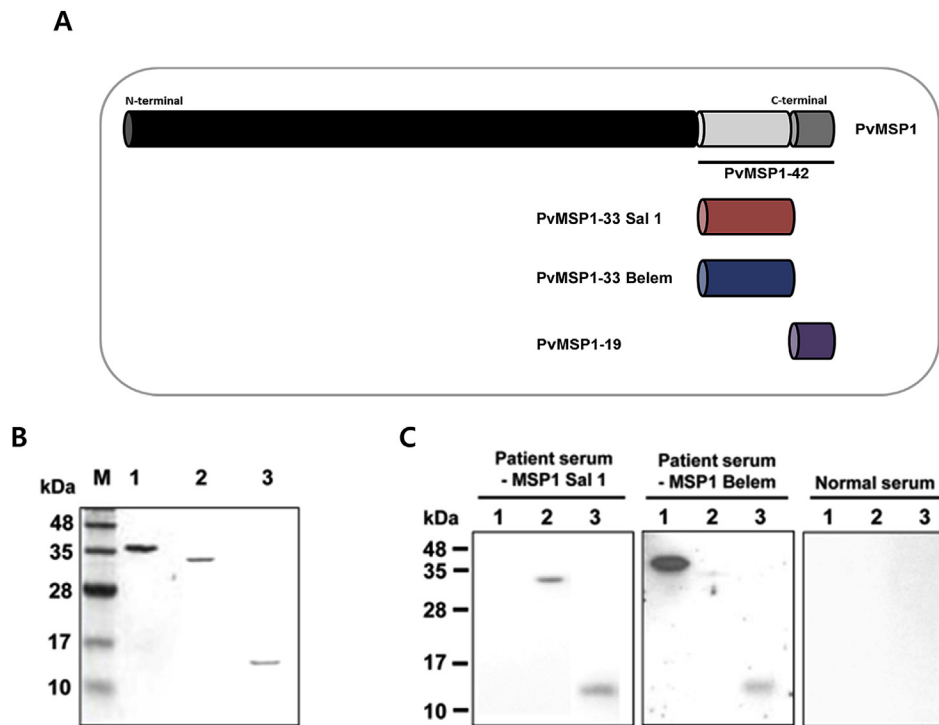


Figure 1. Diagram and antigenicity evaluation of three recombinant *Plasmodium vivax* merozoite surface protein 1 (PvMSP1) proteins: rPvMSP1-19, rPvMSP1-33 Sal 1, and rPvMSP1-33 Belem. (A) A diagram of the three recombinant proteins produced. (B) SDS-PAGE of rPvMSP1-19, rPvMSP1-33 Sal 1, and rPvMSP1-33 Belem. Successful expression of these recombinant proteins in *Escherichia coli* was confirmed using SDS-PAGE and Coomassie blue staining. (C) Western blot analyses of rPvMSP1-19, rPvMSP1-33 Sal 1, and rPvMSP1-33 Belem. Antigenicity and the ability to differentiate specific antibodies against the three recombinant proteins were analyzed via Western blotting with sera obtained from patients infected with PvMSP1-Sal 1 (patient serum-MSP1 Sal 1) and PvMSP1-Belem (patient serum-MSP1 Belem), and sera from healthy individuals (normal serum). Lanes: M, molecular mass marker; 1, rPvMSP1-Belem; 2, rPvMSP1-Sal 1; 3, rPvMSP1-19. SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

the tertiary structure of native PvMSP1 [23]. Moreover, several studies on the prevalence of specific antibodies to these PvMSP1 fragments have been conducted [12,24,25]. Therefore, the recombinant proteins generated in this study, were used to evaluate the antibody prevalence in patients with vivax malaria from the ROK.

Our ELISA results showed that the levels of PvMSP1-19 antibodies were significantly higher than the levels of PvMSP1-33 Sal 1 and Belem antibodies. This may be caused by the presence of two cysteine-rich and immunogenic epidermal growth factor-like domains in PvMSP1-19. Nonetheless, more samples were seronegative for PvMSP1-33 Sal 1 and PvMSP1-33 Belem compared with that for PvMSP1-19, possibly due to a

hypervariable region in the PvMSP1-33 fragments. In addition, approximately 100 patients possessed more than two types of PvMSP1-specific antibodies to different strains of PvMSP1, namely PvMSP1 Sal 1 and Belem. These results are consistent with previous studies that showed high multiplicities of infection for *P. vivax* in Korea [26,27]. However, these studies did not evaluate whether the high multiplicity of infection, as determined with nucleotide sequencing, results in the production of antibodies specific to each strain. In addition, 39 (17.6%) and six (2.7%) PvMSP1-19-seropositive serum samples were also seropositive for PvMSP1-33 Sal 1 and PvMSP1-33 Belem, respectively (Table 2). In a previous study by Kang et al [15], Korean

Table 1. Seroprevalence of antibodies specific to *Plasmodium vivax* merozoite surface protein 1 (PvMSP1)-19, PvMSP1-23 Sal 1, and PvMSP1-23 Belem in 221 samples from a Korean population.

	PvMSP1-19*	PvMSP1-23 Sal 1	PvMSP1-23 Belem
Positive, <i>n</i> (%)	198 (89.6)	142 (64.3)	106 (48.0)
Negative, <i>n</i> (%)	23 (10.4)	79 (35.7)	115 (52.0)

* $p < 0.05$ in antibody levels between PvMSP1-19 and PvMSP1-23 Sal 1 or PvMSP1-23 Belem.

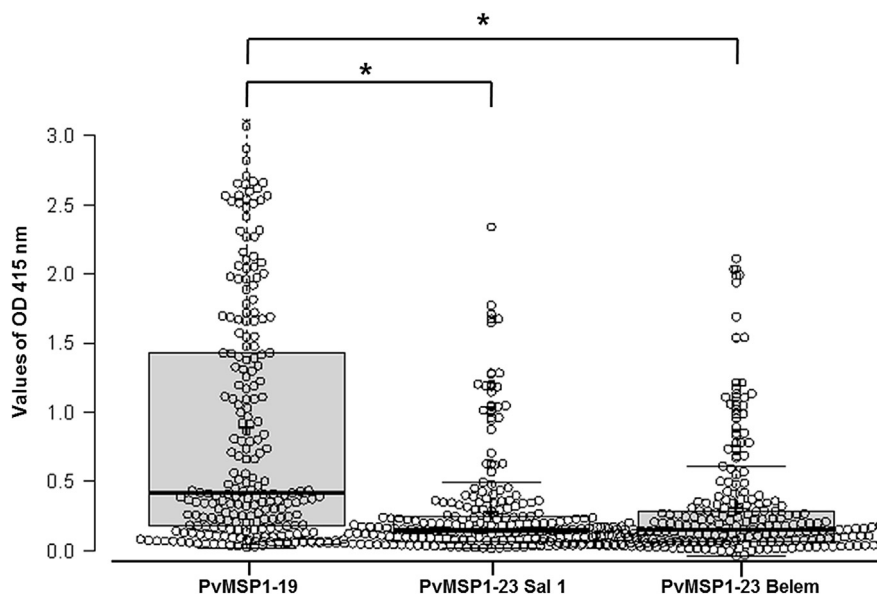


Figure 2. Levels of antibodies specific to *Plasmodium vivax* merozoite surface protein 1 (PvMSP1)-19, PvMSP1-23 Sal 1, and PvMSP1-23 Belem among malaria patients in vivax endemic regions of Korea. * $p < 0.05$. OD = optical density.

Table 2. Details of the patterns of seropositivity to *Plasmodium vivax* merozoite surface protein 1 (PvMSP1)-19, PvMSP1-23 Sal 1, and PvMSP1-23 Belem in 221 *P. vivax*-infected Korean individuals.

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Total
PvMSP1-19	+	+	+	+	-	-	-	-	
PvMSP1-23 Sal 1	+	-	+	-	+	+	-	-	
PvMSP1-23 Belem	+	-	-	+	-	+	+	-	
No. of individuals, n (%)	100 (45.3)	53 (24.0)	39 (17.6)	6 (2.7)	3 (1.4)	0 (0)	0 (0)	20 (9.0)	221 (100)

P. vivax isolates showed high genetic diversity in the PvMSP1-33 region. In addition, PvMSP1 has been classified not only as Sal 1 and Belem, but also as recombinant and mutant type [28]. This high genetic diversity might result in seropositivity for PvMSP1-19 and seronegativity for PvMSP1-33 Sal 1 and PvMSP1-33 Belem.

PvMSP1-19 is a C-terminal fragment located in a conserved region of PvMSP1. Current results showed that PvMSP1-19 is highly antigenic, and that antibodies specific to PvMSP1-19 are the most prevalent antibodies in Korean patients with *P. vivax*. In addition, seroprevalence studies for specific antibodies to the candidates of the vaccine and serodiagnostic tools are crucial for the development of these preventive and diagnostic strategies.

Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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