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Rapid genotyping of *Plasmodium vivax Pvs25* and *Pv38* genes by using mismatch specific endonuclease

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Abstract. Mismatch specific endonuclease (MSE) method was used to detect natural polymorphisms in *Pvs25* and *Pv38* genes of *Plasmodium vivax*. Eighty seven patients with *P. vivax* were recruited in the Republic of Korea (ROK). *Pvs25* and *Pv38* genes were amplified by polymerase chain reaction (PCR), and the PCR amplicons were mixed with reference DNA sequences. Following the denaturation and gradual annealing, the product mixtures were cleaved by the MSE. Heteroduplex types were readily detected by gel electrophoresis, where extra bands with shorter sizes would appear from the cleavage. After MSE cleavage of 657-bp product from *Pvs25* mixtures, three genotypes were detected, while *Pv38* mixtures with 1220-bp products presented two genotypes in ROK isolates. After the MSE cleavage, the mismatched samples of *Pvs25* and *Pv38* were completely sequenced, and the results were in complete agreement with the MSE analyses. In conclusion, genotyping of *Pvs25* and *Pv38* with MSE cleavage could be a potential method for the high-throughput screening of the large field samples.

INTRODUCTION

Plasmodium vivax is the most widespread malaria parasite in human, affecting almost 40% of population worldwide (Guerra *et al.*, 2006; WHO, 2010). Although *P. vivax* malaria is rarely lethal, it has large impact on overall morbidity of humans outside of Africa, and it also can lead to severe malaria symptoms (Kochar *et al.*, 2005; Ozen *et al.*, 2006).

Based on the non-human malaria models, the protective immunity could be formed by targeting the sexual stages of parasites (Carter & Chen, 1976; Gwadz, 1976). Transmission-blocking immunity is effective with antibodies against the surface proteins of the parasite, which are isolated from the midgut of a blood-fed mosquito (Tsuboi *et al.*, 2003). In *Plasmodium falciparum*, the surface proteins of the gamete, zygote, and ookinete stages are candidate proteins for the transmission-blocking vaccine (TBV) (Kaslow *et al.*, 1988; Kocken *et al.*, 1993; Williamson *et al.*, 1993). The ookinete surface proteins of *P. falciparum*, *Pfs25* and *Pfs28*, were chosen and further developed as targeted antigens for TBV (Duffy & Kaslow, 1997; Gozar *et al.*, 1998). These proteins contain four epidermal growth factor (EGF)-like cysteine-rich domains, which contains an affinity to the surface of the parasite with glycosyl phosphatidylinositol anchor. EGF-like domains are predominantly found in extracellular proteins of eukaryotes, where they usually participate in adhesion and signaling (Appella *et al.*, 1988). Majority of amino acid substitutions are found within the EGF-like domains. The analogous proteins

of *Pfs25* and *Pfs28* in *P. vivax* are *Pvs25* and *Pv28*. *Pvs25* has been selected for the development of multitarget/multispecies TBV (Tsuboi *et al.*, 1998).

Since *Pv38* contains high degree of homology with *Pf38*, it was recently chosen as a new antigen candidate for TBV (Mongui *et al.*, 2008). *Pv38* is encoded by a single exon gene, and is composed of 355 amino acids. *Pv38*, as surface protein, contains the s48/45 domain, which is found on gametocytes. The association of *Pv38* with detergent-resistant membranes and its expression in the mature blood stages of the parasite (mainly schizonts) has been reported (Mongui *et al.*, 2008).

Genetic polymorphism of the surface proteins of *P. vivax* ookinete stage has been reported from the field samples (Tsuboi *et al.*, 1998, 2004). To develop better TBV against *P. vivax*, an efficient high throughput screening method for detecting genetic polymorphism should be developed for handling large numbers of samples. Many advanced technologies have been introduced for mutation analyses, such as direct sequencing, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), PCR-single stranded conformational polymorphism (SSCP), real-time single-nucleotide polymorphism (SNP) analysis, high resolution melting curve analysis, and whole genome analysis. However, these methods are expensive or need special instruments, where large quantities of unnecessary data could be produced. Presently, no reliable and efficient method is available to detect mutations and polymorphism by utilizing their electrophoretic properties.

Mismatch specific endonuclease (MSE) belongs to the CEL family of plant endonucleases, which can cleave mismatched base substituted or distorted sites within DNA with high specificity (Oleykowski *et al.*, 1998; Yang *et al.*, 2000). When mutant and wild-type alleles are mixed, heated, and then annealed to form heteroduplexes, MSE can cleave the heteroduplex fragments. Here, MSE method was performed to detect natural polymorphism in *Pvs25* and *Pv38* genes of *P. vivax*.

MATERIAL AND METHODS

Subjects and sample collection

A total of 87 patients with *P. vivax* were recruited between 2006 and 2011 at the Korea University Guro Hospital (Seoul, Republic of Korea). Patients had fever or recent history of fever at the time of blood smear preparations. All patients gave written informed consent under the approved protocol, which was reviewed and approved by the Korea University Guro Hospital. All samples were frozen at -80°C until further use.

Extraction of the genomic DNA

After thawing samples from -80°C, the genomic DNA was extracted from whole blood or packed cells using AxyPrep™ Blood Genomic DNA Miniprep kit (Axygen Bioscience, Union City, CA, USA).

PCR of the *Pvs25* and *Pv38* genes

Pvs25 and *Pv38* genes of *P. vivax* were amplified by PCR using oligonucleotide primers as listed (Fig. 1), *Pvs25* F2: 5'-CACCGACCACAAAAACTTAT-3'(158-178, AF083502), *Pvs25* R2: 5'-AACGTAAAGCC TTCCATACA-3' (785-814, AF083502), *Pv38*F2:5'-GTAGAGGTACCCCCGCGTAG-3' (129184-129203, CM000444), *Pv38*R2: 5'ATTAACGCAGCTTCGCAACG-3' (130384-130404, CM000444). Primers were used with a final concentration of 0.1 µM in a 100 µL reaction mixture (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP), containing 10 µL of DNA and 2.5 units of ExTaq polymerase (Takara Shuzo, Shiga, Japan). Reaction mixtures underwent 30 cycles of amplifications: denaturation for 30 seconds at 95°C, annealing for 30 seconds at 58°C, and extension for 1 minute at 72°C in a thermocycler (Mastercycler gradient, Eppendorf, Hamburg, Germany). Amplified PCR products were resolved by electrophoresis on 2% agarose gels, containing ethidium bromide at 0.5 mg/mL.

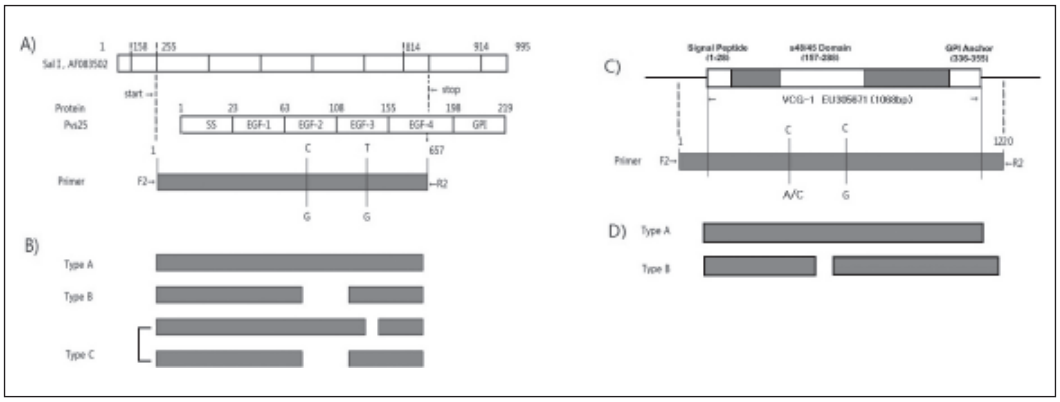

Figure 1. Schematic representations of the genes and protein structures of *Pvs25* and *Pv38*. Locations of the start and stop codons, mismatch sites, oligonucleotide primers for PCR, and the nucleotide substitutions were indicated. EGF, epidermal growth factor; GPI, glycosyl phosphatidylinositol; SS, signal sequence.

Polymorphism detection using mismatch specific endonuclease (MSE)

Mismatch specific endonuclease (MSE) in SURVEYOR Mutation Detection Kit (Transgenomic) was used to detect polymorphisms in the *Pvs25* and *Pv38* genes. Before the nuclease digestion, PCR products were both self-hybridized and cross-hybridized with the amplified reference DNA. If the DNA sample was heterozygous with mutation(s), self-hybridization would produce DNA heteroduplexes. Next, if the DNA sample contained homozygous mutation(s), cross-hybridization with the amplified reference DNA would also produce heteroduplexes. Hence, the resulting heteroduplexes with sequence deviations between the wild type samples and the reference sequence would contain mismatches, then these mismatches would be cleaved by MSE.

Equal amount of PCR-amplified reference DNA (KPV10-11-1 for *Pvs25* and *Pv38* each) and unknown PCR products were mixed and hybridized directly. The hybridization was performed in a PCR thermocycler using the following program: 95°C for 2 minutes; 95°C to 85°C with a cooling rate of 2°C/sec; 85 to 25°C with a cooling rate of 0.2°C/sec; and held at 4°C. Hybridized DNA (5 µL) was digested by adding 1 µL of MSE directly into the PCR reaction buffer at 42°C for 20 minutes. Digestion controls were used, according to the manufacturer's instructions. The incubation was terminated with an addition of 1/10 volume of ethylenediaminetetraacetic acid (0.5 M, pH 8.0). Digestion products were analyzed with agarose gel (2%) electrophoresis. Nucleotide changes or mutations would reveal digested fragments from heteroduplexes. Mutations were confirmed from the DNA sequencing analysis.

Sequencing

The targeted PCR products were purified using the Qiagen PCR Cleanup kit (Qiagen, Valencia, CA, USA), and sequenced using the BigDye Terminator DNA sequencing kit (Applied Biosystems, Foster City, CA, USA). Sequencing was performed in an ABI Prism 3100 genetic analyzer (Applied Biosystems). Sequencing data of the *Pvs25* and *Pv38* genes were compared with MSE cleavage patterns for confirmation. The sequences were submitted to GenBank with accession numbers JF427569, JF427570 and GU971416 to GU971513.

RESULTS

***Pvs25* and *Pv38* gene mutation using mismatch specific endonuclease**

Amplified *Pvs25* gene fragments of 657 bp were generated from 87 *P. vivax* isolates.

After the MSE cleavage, different amplicon patterns were analyzed in parallel with 2% agarose gel electrophoresis. Three allelic variants were observed from the enzyme digestion in comparison to the reference DNA sequences. Twenty-seven Korean isolates revealed no cleavage products. Forty-nine isolates had cleavage products with 271 bp and 386 bp long. Eleven isolates had four cleavage products; 151 bp, 271 bp, 386 bp, 506 bp (Fig. 2).

From the PCR amplification of *Pv38*, a 1,220 bp product was identified, and after MSE cleavage, two allelic variants were identified in comparison to the reference DNA sequences. Fifty-three Korean isolates had no cleavage products. The other thirty-four isolates produced cleavage products of 558 bp and 662 bp long (Fig. 2).

***Pvs25* and *Pv38* sequence confirmations**

Of the 87 samples, 3 allelic types were found for *Pvs25*, based on two nucleotide substitutions. The locations of the mutations were at nucleotide position number 386 with G/C and 488 with C/A substitutions, resulting in amino acid changes at position 97 from Glu to Gln (E97Q), and at position 131 from Gln to Lys (Q131K), respectively, in comparison to the reference DNA (AF083502). For locating polymorphism in *Pv38*, two patterns emerged from the 87 samples, resulting from nucleotide substitutions at C558A in comparison with

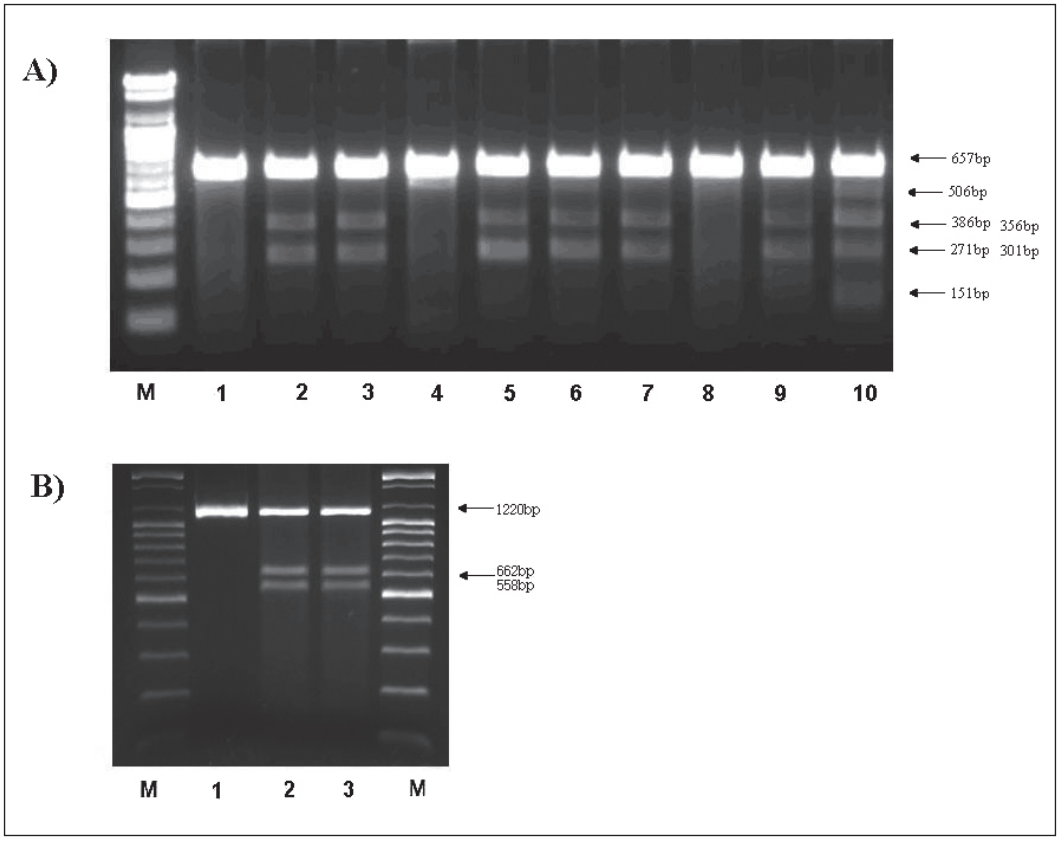

Figure 2. Identification of polymorphism by mismatch specific endonuclease (MSE) method in *Pvs25* (A), using test sample and control DNA. M: 100 bp DNA ladder, Lane 1, 4, 8: Type A without cleavage (657 bp), Lane 2, 3, 5, 6, 7, 9: Type B with a cleavage site (271 bp + 386 bp), Lane 10 Type C with two cleavage sites (151 bp + 271 bp + 386 bp + 506 bp). *Pv38* gene revealed two cleavage patterns after MSE reaction (B): Lane 1, Type A without cleavage (1,220 bp); Lane 2, 3 Type B with a cleavage site (558 bp + 662 bp).

Table 1. Comparison of *Pvs25* and *Pv38* gene sequences from Korean *Plasmodium vivax* isolates, which were in full agreement with the polymorphism analyses by mismatch specific endonuclease (MSE) method

Pvs25					**Pv38**					
Polymorphism location			MSE pattern		Polymorphism location				MSE pattern	
AF083502	386	488	Fragment size	N	JF427569	558	559	1,003	Fragment size	N
	C	C				C	T	G		
Type A	C	C	No cleavage	27	Type A	C	T	G	No cleavage	53
Type B	G	C	271,386	49	Type B	A	T	G	558,662	34
Type C	G	A	151,271,386,506	11						

reference strain (JF427569) (Table 1). Polymorphism in *Pvs25* and *Pv38* genes from the isolates are summarized in Table 1. Sequencing analyses provided complete agreements with the results from the MSE digestion.

DISCUSSION

To achieve prevention or elimination of malaria infections, chemotherapies or vector control would not be sufficient to control the disease, because of emerging drug resistant parasites and insecticides resistant mosquitoes. In this regard, TBV appears to be a promising strategy for disrupting the transmission of *P. vivax* and *P. falciparum* (Stowers & Carter, 2001; Tsuboi *et al.*, 2003). If the TBV is successful, it could improve global public health by reducing the mortality and prevalence of malaria.

Pvs25, *Pvs28*, *Pv38*, and *Pv41* have been recognized as leading candidates of TBV programs (Sattabongkot *et al.*, 2003; ; Malkin *et al.*, 2005; Mongui *et al.*, 2008; Angel *et al.*, 2008). These proteins contain conserved structures such as four EGF-like domains (Tsuboi *et al.*, 1998). For the development of an effective TBV against diverse *P. vivax* isolates in endemic regions, an efficient screening method to monitor the polymorphism in *Pvs25* and *Pv38* is required.

Among many advance molecular techniques to detect SNPs and mutations in large number of samples, MSE digestion would offers the efficiency and high throughput capability in determining malaria polymorphisms. MSE cleaves the 3' side of any mismatch site in both DNA strands, including all base substitutions and insertion/deletions. In comparison to PCR-RFLP or PCR-SSCP, the MSE method would have relatively better sensitivity, which could detect 1 in 32 copies of SNP.

Here, the MSE method determined the genotype variation of *Pvs25* and *Pv38* genes in 87 Korean *P. vivax* isolates. Three and two different sequence variations in *Pvs25* and *Pv38* genes were identified, respectively. These results are in full agreement with nucleotide sequencing analyses (Table 1). Moreover, the MSE procedure was rapid and relatively easy in producing reproducible and specific patterns. The method can be applied on longer fragments (Mitani *et al.*, 2006). However, the MSE method requires sufficient amount of clean PCR amplicons to yield precisely cleaved DNA fragments. It has high sensitivity in resolving samples with low parasitemia levels. Another advantage of MSE method in comparison to PCR-RFLP is its ability to detect polymorphisms that are close to each other in the gene, which are not able to be distinguished by agarose gel. Furthermore, while PCR-RFLP uses different restriction enzymes for each specific gene analysis, MSE method utilizes only one endonuclease system. The weakness of the MSE method is the requirement of known reference samples, but this can be overcome by various available reference DNA sequences. In addition, MSE method does not need expensive instruments. Therefore, its application could be extended to identify diverse malaria species, to differentiate

various infections from mixed species, or to detect unknown drug resistance genes in field samples.

In this study, MSE was used for the first time in analyzing *Pvs25* and *Pv38* genotypes, demonstrating its vast potential in high-throughput screening of large field samples. Therefore, MSE method can be used to detect genetic polymorphism for developing various TBV candidates prior to field trials with the given targeted antigens.

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Conflicts of interest

The authors have declared that no conflict of interest exists.

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