



PCR-RFLP for Rapid Subtyping of *Plasmodium vivax* Korean Isolates

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Abstract: *Vivax* malaria reemerged in Korea in 1993 and the outbreak has been continued with fluctuating numbers of annual indigenous cases. Understanding the nature of the genetic population of *Plasmodium vivax* circulating in Korea is beneficial for the knowledge of the nationwide parasite heterogeneity and in the implementation of malaria control programs in the country. Previously, we analyzed polymorphic nature of merozoite surface protein-1 (MSP-1) and MSP-3 α in Korean *P. vivax* population and identified the Korean *P. vivax* population has been diversifying rapidly, with the appearance of parasites with new genetic subtypes, despite the recent reduction of the disease incidence. In the present study, we developed simple PCR-RFLP methods for rapid subtyping of MSP-1 and MSP-3 α of Korean *P. vivax* isolates. These PCR-RFLP methods were able to easily distinguish each subtype of Korean *P. vivax* MSP-1 and MSP-3 α with high accuracy. The PCR-RFLP subtyping methods developed here would be easily applied to massive epidemiological studies for molecular surveillance to understand genetic population of *P. vivax* and to supervise the genetic variation of the parasite circulating in Korea.

Key words: *Plasmodium vivax*, PCR-RFLP, MSP-1, MSP-3 α , subtyping

INTRODUCTION

Plasmodium vivax is the most widely distributed human malaria parasite worldwide and has reemerged in many regions of the world where malaria has been eliminated [1,2]. *Vivax* malaria was eradicated in Korea in the late 1970's as the result of a national malaria eradication program supported by the World Health Organization [3]. However, malaria reemerged in the country in 1993 near the demilitarized zone (DMZ) bordering North Korea, and the outbreak has spread into cities and counties adjacent to the DMZ and has continued until now with fluctuating numbers of annual indigenous cases [4].

Understanding the nature of genetic structure of *P. vivax* population circulating in Korea is beneficial to figure out the

nationwide parasite heterogeneity and to implement malaria control programs. Therefore, many studies have attempted to understand the genetic characteristics of *P. vivax* Korean isolates [5-13]. In our previous study, we performed combinational analysis of genetic structures of 2 major polymorphic molecular markers of *P. vivax*, i.e., merozoite surface protein-1 (MSP-1) and MSP-3 α , in Korean *P. vivax* isolates collected over an extended period from 1998 to 2013 [14]. Both MSP-1 and MSP-3 α are differentiated into 7 distinct subtypes (subtypes A to G for MSP-1 and subtype 1 to 7 for MSP-3 α) among the analyzed Korean *P. vivax* isolates. Combinational genetic analysis of polymorphic patterns of MSP-1 and MSP-3 α in the isolates suggests that the *P. vivax* population in Korea has been diversifying rapidly, with the appearance of parasites with new haplotypes, despite the recent reduction of the disease incidence [14]. Several reports also supported the notion that Korean *P. vivax* population is rapidly disseminated in recent years [6,8,9,15]. These results highlight the importance of molecular epidemiological surveillances to supervise the genetic variations of the parasite in Korea [14]. However, routine amplification by PCR followed by sequencing analysis for polymorphic

•Received 19 December 2016, revised 8 February 2017, accepted 19 February 2017.

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marker genes of *P. vivax* is costly and time- and labor-consuming and does not provide large amounts of information quickly for epidemiological purposes. Therefore, an accurate and powerful tool that can replace the existing cumbersome and time-consuming sequencing method is needed.

In this study, we developed reliable PCR-restriction fragment length polymorphism (PCR-RFLP) methods for rapid subtyping of MSP-1 and MSP-3 α of *P. vivax* Korean isolates. These methods can easily distinguish different subtypes of MSP-1 and MSP-3 α of *P. vivax* Korean isolates in a simple and single step.

MATERIALS AND METHODS

Blood samples

The blood samples used in this study were obtained from Korean patients infected with *P. vivax* in Korea between 1998-2013 [14]. All the patients inhabited in malaria endemic areas, i.e., Ilsan, Kimpo, Paju, Gangwha, or Yoncheon, and have not been abroad at least in recent 2 years when their blood samples were collected. All the patients were confirmed to be infected with *P. vivax* by microscopic examinations and PCR. Monoclonal *P. vivax* infections were confirmed by genotyping of *P. vivax* positive samples at the highly polymorphic loci MS16 and MSP-1F3 as described previously [16]. Blood samples were collected under protocols reviewed and approved by the Ethics Committees of Inha University School of Medicine and Gachon University of Medicine and Science. Blood samples were stored as aliquots of heparinized blood frozen at -70°C.

Genomic DNA extraction

Genomic DNA was extracted from 200 μ L of each blood sample using a QIAamp DNA Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

PCR

Polymorphic regions of MSP-1 (block 5 to 6) and MSP-3 α (block I and II) were amplified with PCR using specific primers for each gene [17-19]. ExTaq DNA polymerase (Takara, Otsu, Japan) was used in all PCR amplifications to minimize possible amplification error.

Selection of restriction enzymes and RFLP analysis

Based on nucleotide sequences of 255 MSP-1 and 255 MSP-3 α genes (GenBank accession nos. KU893351 to KU893860)

amplified from Korean *P. vivax* isolates [14], we surveyed the restriction enzyme sites in each subtype of MSP-1 and MSP-3 α using the Restriction Mapper program (<http://www.restriction-mapper.org/>). The restriction enzymes with digestion sites that were conserved within a subtype in a given sequence were selected. The selected restriction enzymes and their predicted cleavage sites in each subtype of MSP-1 and MSP-3 α were shown in Fig. 1A and Fig. 3A. PCR products of each subtype MSP-1 were digested with *PvuII*, *SpeI*, and *StyI* (each 3 U; Takara) in a total volume of 30 μ L at 37°C for 2 hr. RFLP analysis of each subtype of MSP-3 α was carried out by digesting the PCR products with the *PvuII*, *StyI*, and *EcoRI* (each 3 U; Takara) in a total volume of 30 μ L at 37°C for 2 hr. The resulting digested fragments were separated in 3% agarose gels by electrophoresis in 0.5 \times Tris-acetate-EDTA (TAE) buffer, stained with Red-Safe Nucleic Acid Staining Solution (Intron, Seongnam, Korea) and visualized under ultraviolet light. The 100-bp DNA ladder or 1-kb DNA ladder (Life Technologies, Rockville, Maryland, USA) was used as the DNA size marker.

Blind analysis of PCR-RFLP methods

To evaluate the applicability of the developed PCR-RFLP methods for subtyping of MSP-1 and MSP-3 α in Korean *P. vivax* isolates, a blind test was performed with 50 Korean *P. vivax* isolates. The PCR reactions were set to the same conditions as above, and the PCR products were subsequently digested with the corresponding restriction enzymes for MSP-1 and MSP-3 α . The reactants were analyzed by 3% agarose gel electrophoresis as described above and observed the profiles of restriction fragments. Sequencing analysis of amplified MSP-1 and MSP-3 α was also performed for each *P. vivax* isolate by automatic nucleotide sequencing to evaluate the accuracy of the PCR-RFLP methods.

RESULTS

MSP-1 subtyping PCR-RFLP

On the basis of computational restriction fragment analysis of currently known 7 MSP-1 subtypes in Korean *P. vivax* isolates, restriction enzymes *PvuII*, *SpeI*, and *StyI* were chosen, as they were expected to produce fragments of different sizes and subtype-specific restriction profiles (Fig. 1A). PCR amplifications of polymorphic regions (block 5 to 6) of 7 distinct Korean *P. vivax* MSP-1 subtypes (subtypes A to G) revealed that the amplified products showed only slightly different length poly-

morphisms between the subtypes (subtype A, 588 bp; subtype B, 591 bp; subtype C, 591 bp; subtype D, 495 bp; subtype E, 495 bp; subtype F, 498 bp; subtype G, 507 bp), which did not clearly discriminate each other on a gel (Fig. 1B upper panel). Digestion of the amplified PCR products with *Pvu*II, *Spe*I, and *Sty*I resulted in different sets of digestion profiles between the subtypes as expected (Fig. 1B, lower panel). Subtypes A and B generated 2 fragments of approximate sizes of 450 bp and 140 bp, while subtype C yielded 3 fragments of 360, 140, and 90

bp in their approximate sizes. Subtype D produced 3 different sized fragments with approximate 260, 140, and 100 bp. Subtype E generated 4 digested fragments with approximate sizes of 170, 140, 100, and 90 bp. In the cases of subtypes F and G, 3 fragments with approximate lengths of 250, 170, and 90 bp were produced. These results showed that the 7 MSP-1 subtypes in Korean *P. vivax* isolates produced different RFLP profiles that can be distinguished each other, even though *Sal* I type subtypes (subtypes A and B) and Belem type subtypes

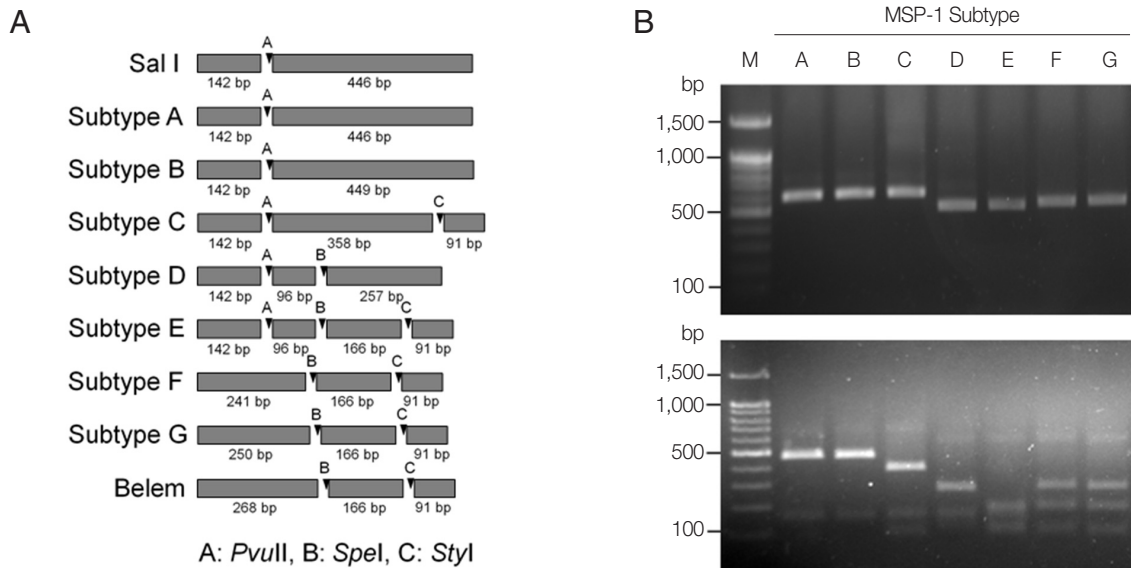


Fig. 1. PCR-RFLP subtyping method for MSP-1 of Korean *P. vivax* isolates. (A) Schematic view for predicted restriction positions and expected sizes of restriction fragments of the 7 subtypes of Korean *P. vivax* MSP-1 by 3 restriction enzymes, *Pvu*II, *Spe*I, and *Sty*I. Subtypes A-B, *Sal* I types; subtypes C-E, recombinant types; subtypes F-G, Belem types. Predicted restriction positions for MSP-1 sequences of *Sal* I (AF435593) and Belem (AF435594) were also presented. (B) Amplification and PCR-RFLP analysis of the 7 subtypes of Korean *P. vivax* MSP-1. (Upper panel) PCR amplifications of each subtype MSP-1. Polymorphic region (block 5-6) of MSP-1 was amplified from each subtype and analyzed. (Lower panel) RFLP patterns for each MSP-1 subtype amplicon generated by digestion with *Pvu*II, *Spe*I, and *Sty*I. Different patterns of digested fragments for each subtype were identified. Lane M, 100 bp DNA ladder; lanes A-G, subtypes A to G, respectively.

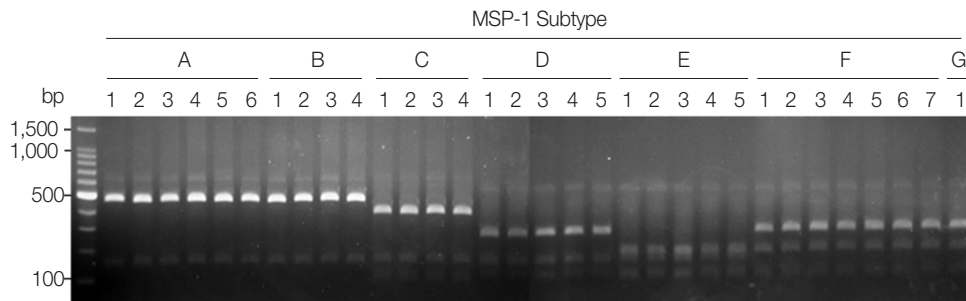


Fig. 2. Blind evaluation of MSP-1 PCR-RFLP method for Korean *P. vivax* isolates. The MSP-1 polymorphic region (block 5-6) was amplified from genomic DNA purified from 50 *P. vivax* Korean isolates by PCR, respectively. Each PCR product was digested with *Pvu*II, *Spe*I, and *Sty*I and analyzed on a 3% agarose gel. The nucleotide sequence of each amplicon was also confirmed to evaluate the accuracy of the method. Only 32 representative results were presented.

(subtypes F and G) did not clearly differentiated due to their high sequence identities. To evaluate the applicability of the PCR-RFLP, a blind analysis using 50 Korean *P. vivax* isolates was performed. As expected, distinct RFLP profiles for each subtype were successfully obtained from the analyzed samples (Fig. 2). The MSP-1 amplicons were sequenced for validation of the method and confirmed that the accuracy of this method was 100% in the analyzed 50 samples.

MSP-3α subtyping PCR-RFLP

For subtype-specific PCR-RFLP method for MSP-3α, *PvuII*, *StyI*, and *EcoRI* restriction enzymes were selected by in silico restriction fragment analysis (Fig. 3A). PCR amplifications of polymorphic regions (block I and II) of the 7 Korean *P. vivax* MSP-3α subtypes (subtypes 1 to 7) resulted in different sizes of amplicons, but they were not clearly discriminated into the corresponding subtypes on a gel (Fig. 3B, upper panel). Amplifications of MSP-3α followed by RFLP analysis using the 3

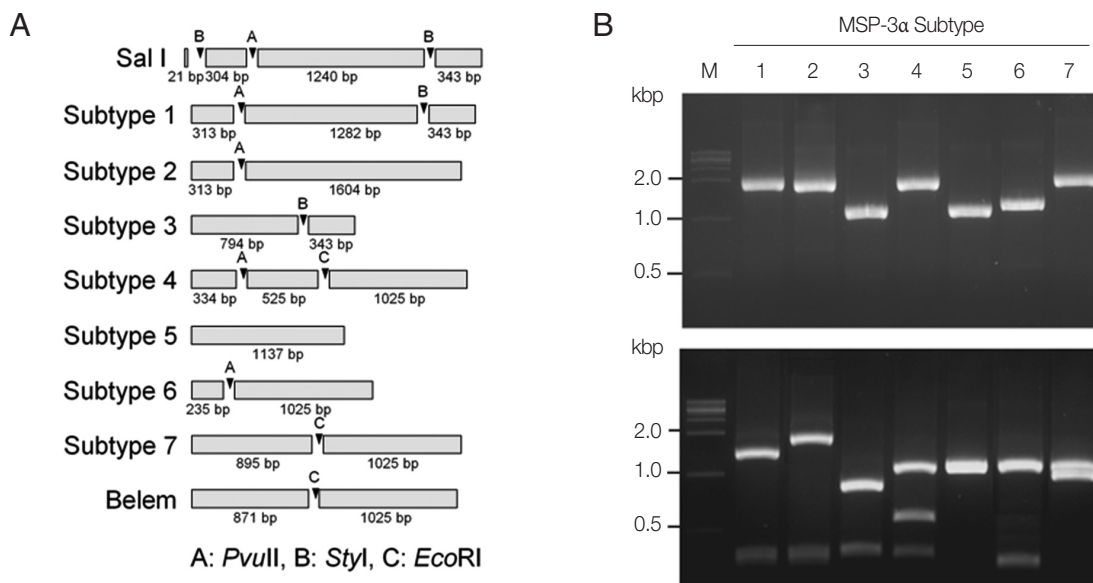


Fig. 3. PCR-RFLP subtyping method for MSP-3α of Korean *P. vivax* isolates. (A) Schematic view for predicted restriction positions and expected sizes of restriction fragments of the 7 subtypes of Korean *P. vivax* MSP-3α by 3 restriction enzymes, *PvuII*, *StyI*, and *EcoRI*. Predicted restriction positions for MSP-3α sequences of Sal I (XM_001613154) and Belem (AF093584) were also presented. (B) Amplification and PCR-RFLP analysis of the 7 subtypes of Korean *P. vivax* MSP-3α. (Upper panel) PCR amplifications of each subtype MSP-3α. Polymorphic region (block I-II) of MSP-3α was amplified from each subtype and analyzed. (Lower panel) RFLP patterns for each MSP-3α subtype amplicon by digestion with *PvuII*, *StyI*, and *EcoRI*. Different patterns of digested fragments for each subtype were identified. Lane M, 1 kbp DNA ladder; lanes 1-7, subtypes 1 to 7, respectively.

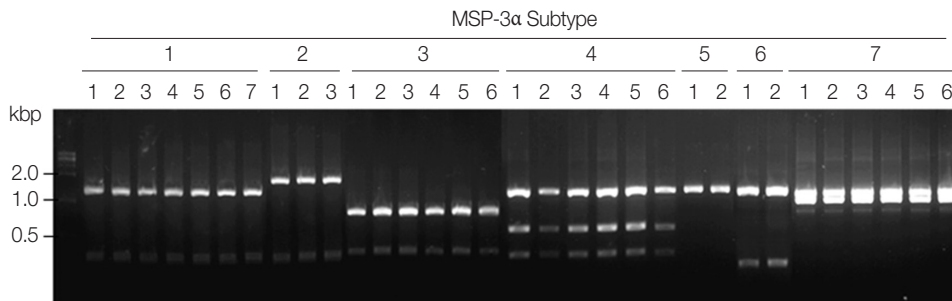


Fig. 4. Blind evaluation of MSP-3α PCR-RFLP method for Korean *P. vivax* isolates. The MSP-3α polymorphic region (block I-II) was amplified from genomic DNA purified from 50 *P. vivax* isolates by PCR. Each PCR product was digested with *PvuII*, *StyI*, and *EcoRI* and analyzed on a 3% agarose gel. The nucleotide sequence of each amplicon was also confirmed to evaluate the accuracy of the method. Only 32 representative results were presented.

restriction enzymes generated subtype-specific fragments of different numbers and sizes that can easily be discriminated into each subtype on an agarose gel (Fig. 3B, lower panel). Subtype 1 produced 3 fragments with approximate lengths of 1,280, 340, and 310 bp. Subtype 2 generated 2 different sized fragments of about 1,600 and 310 bp. Subtype 3 produced 2 digested fragments with approximate sizes of 790 and 340 bp. Subtype 4 yielded 3 distinct sized fragments of about 1,030, 530, and 330 bp, while subtype 5 generated only a band of approximate size with 1,140 bp since it had not any cleavage site by the 3 restriction enzymes. In the case of subtype 6, 2 different sized restriction fragments with approximate sizes with 240 and 1,030 bp were produced. Meanwhile, subtype 7 produced 2 fragments with approximate sizes of 900 and 1,020 bp. A blind analysis of 50 Korean *P. vivax* isolates for MSP-3 α showed that they are well discriminated into each subtype (Fig. 4). Further sequencing analysis of each MSP-3 α amplicon to validate the accuracy of this method confirmed that the method showed 100% concordance for the 50 tested *P. vivax* Korean isolates.

DISCUSSION

Malaria has been endemic in Korea more than 20 years after its reemergence, and there are reports suggesting various allelic variations in *P. vivax* Korean population [5-14]. Despite reduced prevalence of malaria infections in Korea in recent years, the disseminating patterns of genetic structures of Korean *P. vivax* population suggest that the population size is likely to be remained high enough to allow effective genetic recombination of the parasite and continued maintenance of genetic diversity [14]. Therefore, molecular epidemiological surveillance to supervise the genetic variation of the parasite in Korea is essential to gain an in-depth insight for the genetic change and biological nature of *P. vivax* population endemic in Korea. The MSP-1 and MSP-3 α are the most abundant proteins expressed on the surface of *P. vivax* merozoites and are potential targets for vaccine development [20-22]. Both genes are highly polymorphic among global *P. vivax* population and have been identified as reliable molecular markers to investigate genetic structures of *P. vivax* population [14,19,23,24]. Several attempts to apply PCR-RFLP methods to distinguish different types of the genes for molecular epidemiological purpose have been done [25-29]. However, those methods using *AluI* and/or *HhaI* did not clearly identify individual subtype or genotype

of MSP-1 and MSP-3 α of *P. vivax*.

In this study, we aimed to develop simple and reliable PCR-RFLP methods that are easily applicable to molecular epidemiological surveys for Korean *P. vivax* genetic heterogeneity based on MSP-1 and MSP-3 α polymorphisms. Our results suggested that the PCR-RFLP methods developed in this study could accurately identify currently identified 7 different subtypes of Korean *P. vivax* MSP-1 and MSP-3 α . Sequence differences between each subtype of MSP-1 and MSP-3 α allowed the selection of specific restriction enzymes for subtyping of Korean *P. vivax* MSP-1 and MSP-3 α . The methods are simple and feasible and can be easily applied in clinical isolates for high-throughput analysis protocol for clinical trials or epidemiological purposes at a low cost.

The limitation of RFLP analysis is that some sequence changes, such as single point mutations, substitutions, and small deletions in the target sequences could result in loss of the restriction sites, leading to an incorrect outcome. However, the cleavage sites by the restriction enzymes used in this study are well conserved in MSP-1 and MSP-3 α sequences in global *P. vivax* isolates that have been reported to date. Indeed, in silico analysis of MSP-1 and MSP-3 α in *P. vivax* isolates from other endemic areas, including India, Myanmar, and Venezuela [18,19,30,31] suggested that the PCR-RFLP methods are also applicable for subtyping of global *P. vivax* isolates. However, attention should be required to apply the methods to global *P. vivax* isolates considering that the genetic diversities of MSP-1 and MSP-3 α of *P. vivax* circulating in tropical and subtropical endemic areas are much higher than those in Korean isolates [17-19,24,26,30,31]. Considering the rapid genetic dissemination observed in Korean *P. vivax* population, we also could not exclude possibility of the appearance of new genotype of *P. vivax* probably due to genetic recombination and/or introduction of new genotypes from other endemic countries, which may affect population structure of the Korean *P. vivax*. Therefore, continuous molecular epidemiological surveillance to supervise the genetic variation of the parasite in Korea is warranted. The PCR-RFLP methods developed in this study can be applied as simple and powerful tools for this purpose.

ACKNOWLEDGMENT

This research was supported by the grant of Institute of Health Sciences, Gyeongsang National University, Jinju, Korea (no. IHS GNU-2014-1).

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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