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Genetic Structures of Geographically Distinct *Plasmodium vivax* Populations Assessed by PCR/RFLP Analysis of the Merozoite Surface Protein 3 β Gene

Zhaoqing Yang^{1,7}, Jun Miao⁷, Yaming Huang², Xinyi Li⁷, Chaturong Putaporntip³, Somchai Jongwutiwes³, Qi Gao⁴, Rachanee Udomsangpetch⁵, Jetsumon Sattabongkot⁶, and Liwang Cui^{7,*}

¹Parasitology Department, Kunming Medical College, Yunnan, 650031 China ²Malaria Department, Guangxi CDC, Nanning, Guangxi, 530021, China ³Department of Parasitology Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand ⁴Jiangsu Institute of Parasitic Diseases, Wuxi, Jiangsu, China ⁵Department of Pathobiology, Mahidol University, Bangkok 10400, Thailand ⁶Department of Entomology, AFRIMS, Bangkok 10400, Thailand ⁷Department of Entomology, The Pennsylvania State University, University Park, PA 16802, USA

Abstract

The recent resurgence of *Plasmodium vivax* malaria requires close epidemiological surveillance and monitoring of the circulating parasite populations. In this study, we developed a combination of polymerase chain reaction and restriction fragment length polymorphism (PCR/RFLP) method to investigate the genetic diversity of the *P. vivax* merozoite surface protein 3 β (*PvMSP3 β*) gene among four Asian parasite populations representing both tropical and temperate strains with dramatic divergent relapse patterns (N = 143). Using *P. vivax* field isolates from symptomatic patients, we have validated the feasibility of this protocol in distinguishing parasite genotypes. We have shown that PCR alone could detect three major size polymorphisms of the *PvMSP3 β* gene, and restriction analysis detected a total of 12 alleles within these Asian samples. Samples from different geographical areas differed dramatically in their *PvMSP3 β* allele composition and frequency, indicating that complex, yet different parasite genotypes were circulating in different endemic areas. This protocol allowed easy detections of multiple infections, which reached 20.5% in the samples from Thailand. It is interesting to note that samples from one temperate site in China collected during a recent outbreak of the disease also showed a high level of genetic diversity with multiple infections accounting for 5.6% of the samples. When combined with the *PvMSP3 α* locus, this method provides better capability in distinguishing *P. vivax* genotypes and detecting mixed genotype infections.

Keywords

Plasmodium vivax; genotyping; merozoite surface protein 3 β ; mixed strain infection

* Corresponding author: Liwang Cui, Department of Entomology, The Pennsylvania State University, 501 ASI Building, University Park, PA 16802, Phone: 814-863-7663, Fax: 814-865-3048, Email: luc2@psu.edu

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1. Introduction

Of the four human malaria parasite species, *Plasmodium vivax* is the most widespread malaria globally, causing 70–80 million cases annually (Mendis et al., 2001). Outside Africa, it is a major public health problem and an important contributor to the global burden of morbidity. Recently, vivax malaria has re-emerged in many regions such as Korea, certain temperate provinces of China, and some former Soviet republics, where it had been largely eradicated during the global malaria control campaigns (Sleigh et al. 1998; Chai et al., 1999; Sabatinelli et al., 2000; Leclerc et al., 2004). In many regions of *P. vivax* and *P. falciparum* coexistence, *P. vivax* has become more prevalent (Sattabongkot et al., 2004; Zhou et al., 2005). As another serious concern, *P. vivax* has evolved resistance to antimalarial drugs. Although chloroquine (CQ) still constitutes the first-line therapy against vivax malaria, CQ resistance has been reported in both Asia and South America (Rieckman, 1989; Baird et al., 1991; Malar-Than et al., 1995; Phillips et al., 1996). In addition, resistance to the antifolate drug pyrimethamine is also widespread (de Pecoulas et al., 1998; Imwong et al., 2003). Therefore, global resurgence of vivax malaria and escalating drug-resistance demand more extensive research and control efforts.

Understanding population structures of the parasite is important for monitoring the spread of drug resistance and predicting the performance of vaccines under development in a particular parasite population (Cui et al., 2003a). Comparing with *P. falciparum*, the population structures of *P. vivax* are much less understood. With a global distribution ranging from tropical to temperate zones, different *P. vivax* isolates exhibit distinctive biological characteristics (e.g., relapse patterns, transmissibility to mosquitoes), which are often used to distinguish geographical strains and even subspecies (Li et al. 2001). Within a local population, parasite isolates also display extensive genetic diversity as demonstrated by genotyping using various polymorphic molecular markers. These include the circumsporozoite protein (CSP) (Rosenberg et al., 1989), the apical membrane antigen 1 (AMA1) (Figtree et al., 2000), the Duffy binding protein (DBP) (Xainli et al., 2000), the merozoite surface protein 1 (MSP1) (Putapornpip et al., 2002), and the merozoite surface protein 3 α (MSP3 α) genes (Bruce et al., 2000; Mueller et al., 2002; Cui et al., 2003b; Cole-Tobian et al., 2005). However, *PvAMA1* and *DBP* have limited size polymorphism and allele differentiation depends primarily on sequencing (Figtree et al., 2000). *PvCSP* genotyping using polymerase chain reaction (PCR) and molecular hybridization only differentiates the parasites into the VK210, VK247 and the vivax-like types, which possess different repeat sequences in the central domain. In addition, a moderate level of size polymorphism in this region is observed in field isolates due to variation in the number of repeats. Though *PvMSP1* has proven to be more discriminatory, most work relied on sequencing of the amplification products for allele identification (Putapornpip et al., 2002; Zakeri et al., 2006b). Apparently, these methods have limitations for rapid genotyping large field populations. In this regard, a PCR/restriction fragment length polymorphism (RFLP) method for *PvMSP3 α* gene has been validated for genotyping global parasite isolates (Bruce et al., 1999). To date, this marker, allowing detection of as many as 12–14 alleles in a parasite population, has been used widely in genotyping parasite isolates (Bruce et al., 2001; Muller et al., 2002; Cui et al., 2003b). Recently, Imwong et al. (2005) developed a genotyping method to determine size polymorphism in the central domain of *PvCSP* by PCR, and polymorphism in the pre- and post-repeat domains by PCR/RFLP. This method could differentiate the two repeat types and identify 23 allele types from 100 Thai isolates. A similar PCR/RFLP method has also been designed for *PvMSP1* (Imwong et al., 2005). Subsequent identification of polymorphic microsatellite markers has added another set of genotyping markers that are useful for studying *P. vivax* populations (Imwong et al., 2006).

The *P. falciparum* MSP3 and *P. vivax* MSP3 α , β and γ constitute a protein family with limited sequence identity but similar protein structure, with the central Ala-rich region forming an

extensive coiled-coil structure (Galinski et al., 1999;2001). Although *PfMSP3* has limited sequence polymorphism (McColl et al., 1994;McColl and Anders, 1997;Huber et al., 1997), *PvMSP3 α* and β genes are highly polymorphic (Rayner et al., 2002,2004;Mascorro et al., 2005,Ord et al., 2005). Molecular evolution studies suggest that extensive polymorphism of *PvMSP3 α* is probably generated by intragenic recombinations and maintained by balancing selection (Rayner et al., 2002;Mascorro et al., 2005;Ord et al., 2005). Likewise, *PvMSP3 β* sequences from *P. vivax* global isolates are also radically divergent with the majority bearing large insertion/deletion mutations in the central Ala-rich region (Rayner et al., 2004). In this report, we have employed the polymorphic nature of *PvMSP3 β* gene and developed a PCR/RFLP method for genotyping *P. vivax* isolates, which could distinguish 12 alleles within three Asian parasite populations. Combination of this locus with the *PvMSP3 α* gene enables greater capacity in identifying parasite haplotypes and detecting mixed strain infections.

2. Materials and methods

2.1. Sample collection

Parasites were obtained from symptomatic patients attending hospitals or malaria clinics in four geographical regions of Asia. Forty-four finger-prick blood samples were collected from Mae Sot, Thailand in 2000–2001 during an early study (Cui et al., 2003b). These samples were used to assess the genetic diversity of the *MSP3 α* gene. Three groups of samples were collected in China, including 54 samples from Bengbu city, Anhui Province in 2004; 31 samples from Hongshuihe Town, Luodian County, Guizhou Province, in 2005; and 14 samples from residents of Guangxi Province in 2004. Among the 14 Guangxi samples, six were local infections, and the rest were from Guangxi residents, who had contracted malaria while traveling in other malarious regions in China. Ethical clearance was obtained from the Ethical Review Committee of the Armed Forces Institute of Medical Sciences, Thailand, and provincial ethical review committees in China.

2.2. DNA purification

Parasite DNA was extracted from dried blood filters (~100 μ l of blood) using a QIAamp DNA Mini kit (Qiagen, Germany) following the manufacturer's instruction and DNA was eluted in 100 μ l of water.

2.3. PCR of *PvMSP3 β* genes

Based on published *PvMSP3 β* sequences (Galinski et al., 2001;Rayner et al., 2004) and those obtained from 58 Thai isolates (Cui, unpublished), we designed primers from the conserved sequences to amplify the 5' region of the gene containing most of the insertions/deletions. Primary PCR of *PvMSP3 β* was performed in 20 μ l using 3 μ l of DNA using primers F1 (5' GGTATTCTTCGCAACACTC 3') and R1 (5' GCTTCTGATGTTATTTCAG 3'). Nested reactions were done in 20 μ l with primers F2 (5' CGAGGGGCGAAATTGTAAACC 3') and R2 (5' GCTGCTTCTTTGCAAAGG 3') using 1 μ l of the primary PCR product as the template. PCR was performed with advantage *Taq* (Clontech, CA) using the following cycling conditions: 94°C for 20 sec, 54°C for 30 sec, 68°C for 2.5 min, 35 cycles. PCR products (2–3 μ l) were separated on 0.7% agarose gel, stained with ethidium bromide, visualized under UV illumination, and documented using a Kodak digital system.

2.4. RFLP analysis of *PvMSP3 β* PCR products

Based on *in silico* analysis of aligned *PvMSP3 β* sequences, we selected *Pst* I enzyme, which could differentiate the parasites into at least 5 different types. Typically, 3–5 μ l of the PCR products were digested with 5 units of *Pst* I in 10–15 μ l for >3 hr. The DNA fragments were resolved on 1.5% agarose gels and the restriction patterns were recorded.

2.5. Statistical analysis

Chi square test was used to compare the frequencies of individual alleles among the sample sets using Minitab Software 13.0 (MINITAB Inc., PA). The test was done for all alleles at the three sites both simultaneously and in pair. Due to their diverse origins, the Guangxi samples were excluded from statistical analysis.

3. Results

3.1. Size polymorphism of PvMSP3 β and RFLP analysis

Comparing the 16 published *PvMSP3 β* sequences (Galinski et al., 2001; Rayner et al., 2004) and those obtained from 58 Thai *P. vivax* isolates, we designed conserved primers for nested PCR amplifications of the 5' region of this gene, which corresponds to nucleotide positions 81-1515 of the Belem strain (Fig. 1). With these primers, we were able to amplify the *PvMSP3 β* fragments from all 44 Thai and 98/99 Chinese *P. vivax* samples, suggesting that the protocol is suitable for amplifying *PvMSP3 β* gene at these study sites. The only failed sample may be due to low parasite density, since we did not obtain a PCR product using nested primers for the *PvMSP3 α* gene (Cui et al., 2003b). The *PvMSP3 β* PCR products clearly showed size polymorphism with three allele sizes of 1.7–2.2, 1.4–1.5, and ~0.65 kb, which were categorized here as type A, B, and C, respectively (Fig. 2). Type B is in agreement with the reference Belem strain, while type A corresponds to the sequences with different insertions in the central Ala-rich domain of the gene (Rayner et al., 2004). These are the predominant types, accounting for >98% of Asian *P. vivax* isolates in this study. The minor C-type has a large deletion (~780 bp) in the central Ala-rich region, which was not found in the earlier sequencing survey of global *P. vivax* isolates (Rayner et al., 2004). Among the samples analyzed, only two type-C isolates were found in the Thai samples.

Restriction analysis of the PCR products by *Pst* I showed that the A and B types could be divided into five A alleles and six B alleles, whereas the minor C-type lacked a *Pst* I site (Fig. 3A). These alleles probably represented the majority of *P. vivax* genotypes existing in the vivax-endemic areas of China, since RFLP typing 14 additional samples from five Chinese provinces did not identify novel alleles. Altogether, a combination of PCR with RFLP distinguished 12 alleles from the Asian field isolates, indicating that this protocol can be used for differentiating *P. vivax* isolates in epidemiological studies.

3.2. Geographical variations in allele distribution

Analysis of *P. vivax* isolates from symptomatic patients from three geographical regions in Asia revealed that the population structure of *P. vivax* varied greatly. In western Thailand, B type is more abundant (60.4%) than other types, whereas in the Chinese Bengbu and Guangxi samples, both A and B types were similarly prevalent (Table 1). In Hongshuihe town of Guizhou province of China, only the A type was detected in *P. vivax* parasites isolated in 2005. In addition, RFLP analysis also revealed different allele compositions at each site. Ten (four A, five B, and 1 C), eight (five A and three B), and three (A) alleles could be distinguished from the Thai, Chinese Bengbu, and Hongshuihe *P. vivax* isolates, respectively. Among the 14 Guangxi samples of various origins, three alleles were detected: 2, 5, and 7 isolates harbored allele A2, A3, and B1, respectively. Two alleles were present in the Chinese samples but absent in the Thai samples, whereas the Thai samples had four unique alleles. Besides variation in allele type composition, the frequencies of individual alleles also differed significantly among the three parasite populations ($\chi^2=116$, $df=22$, $P\leq 0.001$). In the Thai samples, A5, B1, B2 and B4 were the most abundant alleles, which accounted for 66% of all isolates. In the Chinese Bengbu *P. vivax* samples, the frequency of four alleles (A2, A5, B1 and B2) reached 80.8%. In contrast, the Hongshuihe *P. vivax* population had a predominating A1 allele with a frequency of 66.7%. Interestingly, despite this difference of individual allele types, the Thai and Chinese

Bengbu *P. vivax* populations shared three major alleles (A5, B1 and B2) (Table 1). The frequencies of these three alleles were not significantly different between the Thai and Chinese Bengbu samples ($\chi^2=5.142$, $df=2$, $P=0.076$). These results indicated that the PCR/RFLP method for *PvMSP3β* could be used to differentiate spatially-separated parasite populations.

3.3. Mixed strain infections

Size polymorphism associated with the *PvMSP3β* PCR products allowed easy detection of mixed genotype infections. Mixed infections were assigned when a single sample resulted in more than one PCR product of different sizes (Fig. 2) or when the summed size of the restriction fragments of a single PCR band exceeded the size of the uncut PCR band (Fig. 3B). PCR alone detected mixed infections of A and B types in 15.9% of the Thai samples, and 5.6% of the Chinese Bengbu samples. RFLP analysis detected two additional mixed type B infections (e.g., sample 465 in Fig. 3B) in the Thai population, giving a final mixed infection of 20.5%.

4. Discussion

Polymorphic molecular markers that differ in size and restriction patterns have been employed to evaluate genetic diversity and distinguish parasite isolates in epidemiological studies of malaria parasites. In addition, drug efficacy studies for *P. vivax* often rely on *in vivo* trials, which require markers for differentiating recrudescences (treatment failures) from re-infections (successful treatments). To date, PCR/RFLP methods to distinguish *P. vivax* isolates are available for genotyping the highly polymorphic genes such as *PvMSP3α*, *PvCSP* and *PvMSP1* genes (Bruce et al., 1999; Imwong et al., 2005). In this report, we designed a similar PCR/RFLP method for genotyping *PvMSP3β* gene. The *PvMSP3β* gene was shown to have three major size types in tropical and temperate parasite strains from Asia, comparing with six size polymorphisms found in global samples (Rayner et al., 2004). This protocol, enabling us to distinguish 12 *PvMSP3β* alleles in 142 Asian samples, has a similar resolving power to that of the *PvMSP3α* gene (Cui et al., 2003b). For example, PCR/RFLP method could differentiate the Thai samples into 13 and 10 alleles at the *PvMSP3α* and *3β* loci, respectively (Cui et al., 2003b). This study revealed that the three geographically distinct *P. vivax* sample sets differed in *PvMSP3β* allele composition and frequency, suggesting that this method is suitable for genotyping *P. vivax* parasites in these areas.

Genetic diversity of the malaria parasites is associated with the levels of endemicity and transmission intensity (Babiker et al., 1997; Anderson et al., 2000). In hyperendemic areas such as Papua New Guinea, *P. vivax* is highly diverse and multiplicity of infections (MOI, >1.4) is high (Kolakovich et al., 1996; Bruce et al., 2000). However, in hypoendemic areas such as Thailand, *P. vivax* also appear to have complex genetic structures (Cui et al., 2003b; Zakeri et al., 2003; Leclerc et al., 2004; Imwong et al., 2005). We have shown high levels of genetic diversity and mixed genotype infections (MOI, 1.2–1.3) of the *P. vivax* population in western Thailand by PCR/RFLP analysis of *PvMSP3α* and *β* loci (Cui et al., 2003b), and by direct sequencing of *PvMSP3α* from 17 isolates (Mascorro et al., 2005). In the present study, the Chinese *P. vivax* samples representing strains from a temperate zone, where malaria is seasonal and experiences a long break during the winter, also displayed considerable genetic diversity at the *PvMSP3β* locus. Although it is not clear how genetic diversity of *P. vivax* in these temperate sites is maintained, it may be associated with increased transmission intensities during the recent upsurge of vivax malaria in a large geographical area of China that covers five northern provinces (Sheng et al., 2003). In other hypoendemic regions of the world where temperate strains of *P. vivax* parasite persist, the relatively high levels of diversity were speculated to be partially due to increased travel, resulting in the introductions of diverse parasite strains from neighboring endemic countries (Zakeri et al., 2003; Leclerc et al., 2004).

We have noticed a dramatic difference in *PvMSP3β* allele composition and frequency between the Bengbu samples (eight alleles) and the Hongshuihe samples (three alleles). Although sample sizes may be responsible for this difference, dissimilar epidemiological settings at these two sites may be a more plausible explanation for the divergent parasite population structures. The Bengbu samples were collected from a much larger area in eastern China, where *P. vivax* had largely been eradicated, but reappeared and became increasingly prevalent for the past ten years (Sleigh et al., 1998; Xu et al., 2003, 2006). In contrast, Hongshuihe town is located in a remote area of Guizhou province, where malaria has persisted at low endemicity. The mountainous environments might have helped isolating this area from other large malarious regions in China. Our analysis indicated that parasite population in this area was reminiscent of clonal infections with lower genetic diversity, which resembles that of recently resurgent or introduced *P. vivax* cases in Korea and Northern Iran (Lim et al., 2000; Han et al., 2004; Zakeri et al., 2006b). Therefore, more detailed studies are needed to understand the population structures of *P. vivax* in large temperate zones.

Other than the influence of transmission intensity on allele diversity, the different genetic structures of three *P. vivax* populations observed in this study may also be the result of additional factors. With large geographical separation, the Thai and Chinese samples represent tropical and temperate *P. vivax* strains, which differ radically in terms of relapse patterns. While the tropical strains of the parasite relapse in about a month after the first episode, the temperate strains in China have a long incubation period that often exceeds eight months (Garnham, 1988). Moreover, these endemic areas have different mosquito vector species. In western Thailand, *Anopheles dirus*, *An. minimus*, and *An. maculatus* are the major malaria vectors. In comparison, in Bengbu area of Anhui province, China, *An. sinensis* and *An. anthropophagus* are the main vectors and the latter has become more abundant in many districts (Kan et al., 1999). It is noteworthy that *An. anthropophagus* is highly anthropophilic with a vectorial capacity ~20 times higher than that of *An. sinensis* (Xu et al., 2006). In Hongshuihe town of Guizhou, *An. minimus* is the predominant vector species (Xu et al., 2000). Whether these mosquito species vary in transmission efficiency for certain *P. vivax* genotypes remains to be determined (Rodriguez et al., 2000).

Large epidemiological and *in vivo* drug efficacy studies require highly polymorphic markers for distinguishing parasite genotypes. These markers are often combined to provide higher resolving power. For instance, *MSP1*, *MSP2* and *GLURP* are often used in combination to genotype *P. falciparum* isolates (Meyer et al., 2002). Multiple microsatellites are also used for such purposes (Anderson et al., 2000; Imwong et al., 2006). Our analysis of parasite diversity at the *PvMSP3β* locus detected multiple genotypes and mixed-genotype infections in several *P. vivax* endemic regions. The levels of mixed-genotype infections were correlated with the levels of endemicity. Genotyping the Thai samples at *PvMSP3β* detected 20.5% mixed infections, whereas combination with genotyping *PvMSP3α* increased the level to 29.5% (Cui et al., 2003b). Similarly, the combination of the *PvCSP* and *PvMSP1* markers allowed the detection of 26% of Thai samples with mixed-genotype infections (Imwong et al., 2005). While this is common for tropical malarious regions, our finding of 5.6% mixed-strain infections in the Bengbu sample set is the first record for a temperate zone, which may serve as an indicator of increased prevalence of the disease in this area. Mixed-strain infections favor genetic recombination in vectors, which in turn contributes to higher genetic diversity of the parasite population. Relapses of *P. vivax* malaria undoubtedly increase mixed-genotype infections, recombination, and genetic diversity of the parasite in hypoendemic areas. The lack of mixed-genotype infections in the Hongshuihe samples could be due to low endemicity in this area and/or the inability of the genotyping method to differentiate closely related clones. This resembles the scenarios of the resurgent or introduced *P. vivax* malaria in Korea and Iran, where mixed infections were very low (Lim et al., 2000; Han et al., 2004; Zakeri et al., 2006b). In conclusion, we have shown that the PCR/RFLP protocol for *PvMSP3β* gene could be used as

a convenient way to differentiate *P. vivax* isolates in different geographical regions. The polymorphic nature of *PvMSPβ* should also make it a useful marker for distinguishing relapses (and recrudescences) from new infections.

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Fig 1.

Schematic drawing of the *P. vivax* *MSP3β* gene of the Belem strain. The outer (F1 and R1) and inner (F2 and R2) primers are indicated with the numbers referring to their positions (in base pairs) within the Belem sequence. Filled, open, and hatched boxes indicate conserved, less conserved and highly variable Ala-rich central domain of the gene, respectively.

Fig 2. PCR amplifications of *PvMSP3β* gene showing three major size polymorphisms. Numbers indicate the malaria cases. Samples containing mixed-genotype infections are marked with asterisks.

Fig 3.

A. RFLP patterns of the *PvMSP3β* PCR products digested with *Pst* I showing the 12 alleles detected in the Thai and Chinese *P. vivax* samples studied. M – DNA markers in bp. **B.** An example of PCR/RFLP detection of mixed-genotype infection. Digestion of PCR product from sample 465 revealed the presence of two allele types B1 and B2.

Table 1
PvMSP3β RFLP allele types and their distribution frequencies in three Asian *P. vivax* populations

Alleles	Allele frequencies (%) in populations*		
	Mae Sod, Thailand (N=44)	Bengbu, Anhui (N=54)	Hongshuihe, Guizhou (N=31)
A1	7.5	2.1	66.7
A2	0	14.9	26.7
A3	9.4	4.3	6.7
A4	1.9	6.4	0
A5	17.0	23.4	0
B1	13.2	31.9	0
B2	20.8	10.6	0
B3	0	6.4	0
B4	15.1	0	0
B5	5.7	0	0
B6	5.7	0	0
C	3.8	0	0

* χ^2 analysis detected significant differences among the three samples at individual alleles ($\chi^2=116$, df=22, $P\leq 0.001$).