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Genotyping of polymorphic marker (*MSP3α* and *MSP3β*) genes of *Plasmodium vivax* field isolates from malaria endemic of Thailand

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Abstract

Two polymorphic marker genes; merozoite surface protein 3α (*MSP3α*) and merozoite surface protein 3β (*MSP3β*) of *Plasmodium vivax* 100 field isolates have been investigated using PCR-RFLP. Genotyping of *PvMSP3α* and *PvMSP3β* exhibited high polymorphism in length and sequence. Three major types of *PvMSP3α* (Type A, B, and C) and two major types of *PvMSP3β* (Type A, and B) were detected based on length of PCR products. Fourteen alleles of both genes were distinguished by restriction fragment length polymorphism, with difference frequencies. These results strongly support that *P. vivax* isolates are highly diverse. *PvMSP3α* and *PvMSP3β* are reliable polymorphic markers for population genetic analysis of *P. vivax* and PCR-RFLP provide a powerful method for genotyping and identification of mixed parasite infections without the need for sequencing

Keywords*:* polymorphic marker, genotyping, merozoite surface protein, *Plasmodium vivax*

Introduction

The widespread of drug resistant parasites has major consequences for future malaria control. Recently, *Plasmodium vivax* infections have become more prevent than *Plasmodium falciparum* in some regions of Thailand. Increasing trend of *P. vivax* malaria and the emergence of drug resistant parasites are a major concern for future malaria control. Both *P. falciparum* and *P. vivax* infection in malaria endemic region are consisting of multiple genotypes (Bruce *et al.,* 2000). Different genotypes exhibit distinctive biological characteristics. Polymorphic molecular markers such as Circumsporozoite Surface Protein (CSP) (Imwong *et al.,* 2005), Apical Membrane Antigen 1 (*AMA1*), Merozoite Surface Protein (*MSP*), Duffy Binding Protein (*DBP*) and microsatellites have been the powerful and easily deployable tool for assessing parasite genetic variation in *P. falciparum* and also for *P. vivax*. However, some polymorphic markers exhibit limited polymorphism. Molecular epidemiological studies require reliable polymorphic markers to understand the population structure of parasite and disease transmission. Molecular techniques have become more widely apply for field studies with limited resources. Using a combination of molecular markers to investigate the parasite genetic diversity of field populations displayed high level of genetic diversity. Understanding genetic diversity of *P. vivax* field isolate is essential for developing effective antimalarial drugs and vaccine.

The objective of this work was to characterize the polymorphic molecular markers in *P. vivax* isolates collected from malaria endemic areas of Thailand. These selected polymorphic marker genes were extensively investigate for genetic diversity study in *P. vivax* population.

Materials and Methods

1. Sample collection

P. vivax infected blood used for genotyping were collected from patients attending the Malaria Clinics in different geographical malaria endemic areas along the international border of Thailand-Myanmar and Thailand-Cambodia (Tak, Kanchanaburi, Ranong, Ratchaburi, and Chanthaburi provinces). Written inform consent for study participation was

obtained from all patients. This study protocol was reviewed and approved by the Ethics Review Committee for Research Involving Human Research Subjects, Health Science Group, Chulalongkorn University. Following microscopic confirmation of blood films, approximately 200 ul of infected blood sample, irrespective of parasitaemia, were collected from finger-prick and doted onto the filter paper (Whatman 3MM). The dried filter paper samples were stored in small plastic zip lock bags prior to the extraction of parasite DNA and analysis of parasite genotypes by the polymerase chain reaction and Restriction fragment length polymorphism (PCR/RFLP). Extraction of parasite genomic DNA from individual dried blood spots on filter paper was carried out using a QIAamp DNA extraction mini-kit (QIAGEN) and used as template for PCR amplification.

2. Genotyping of *P. vivax MSP3α* and *MSP3β*

MSP3α and *MSP3β* primers and PCR condition were used according to the previous studies (Bruce et al., 1999, Cui et al., 2003, Yang *et al.,* 2006) with some modification. The amplified PCR products were then analyzed on 1.5% agarose gel. Restriction digestion was carried out in 20 ul reaction. The amplified PCR product of *MSP3α* and *MSP3β* were digested with enzymes *Hha I* and *Pst I* (5 units of enzyme/reaction; Promega) respectively, in buffer supplied with the enzymes at 37^{O}C for 4–5 hr. Restriction fragments were analyzed on 2% agarose gel.

Results

Genotyping of *P. vivax MSP-3α* locus.

Polymorphism of the *PvMSP3α* was studied using the PCR-RFLP method The *PvMSP3α* was amplified by a nested PCR and RFLP were analyzed using *Hha* I. The restriction patterns of PCR/RFLP were analyzed by 2% agarose gel electrophoresis. Based on the size of the PCR products, three major types, A (1.9 kb), B (1.5 kb), and C (1.1 kb), were identified with frequency 86%, 12% and 2%, respectively. A total of 100 isolates were classified into 14 distinguishable genotypes by restriction pattern (named subtype A1-A11, B1-B2, and C) (Figure 1, Table 1).

Genotyping of *P. vivax MSP-3β* locus.

PvMSP3 β fragments were amplified from 100 field samples collected from different malaria endemic areas in Thailand. The PCR products showed size polymorphism with two alleles size which were categorized as type A= 1.7-2.2 kb and type B = 1.4-1.5 kb. Type A is a predominant type, accounting for 81% while type B accounting for only 19%. RFLP typing by *PstI* digestion showed the 14 allele subtypes by restriction pattern (named subtype A1-A6, and B1-B8 (Figure 2, Table 1).

Table 1. *PvMSP3α,* and *PvMSP3 β* RFLP alleles types and their frequency

PvMSP3α	A1=19	A2=3	***PvMSP3 β***	A1=18	A2=9
	A3=4	A4=3		A3=14	A4=11
	A5=5	A6=3		A5=21	A6=8
	A7=26	A8=2		B1=2	B2=1
	A9=14	A10=1		B3= 1	B4=3
	A11=6	B1=10		B5=4	B6=3
	B2=2	C=2		B7= 3	B8=2

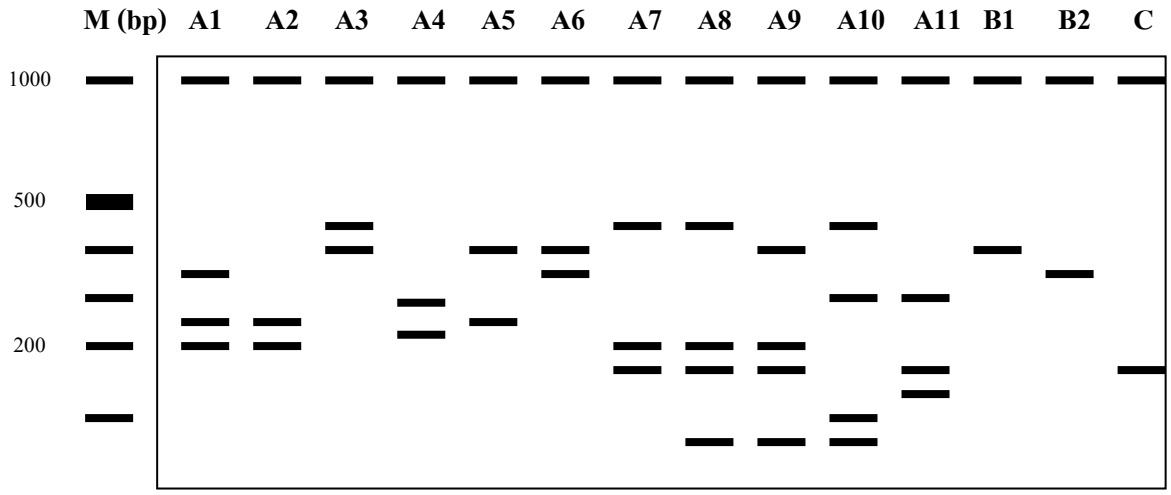

Figure 1. Schematic representation of *PvMSP3α* restriction patterns digested with *HhaI*

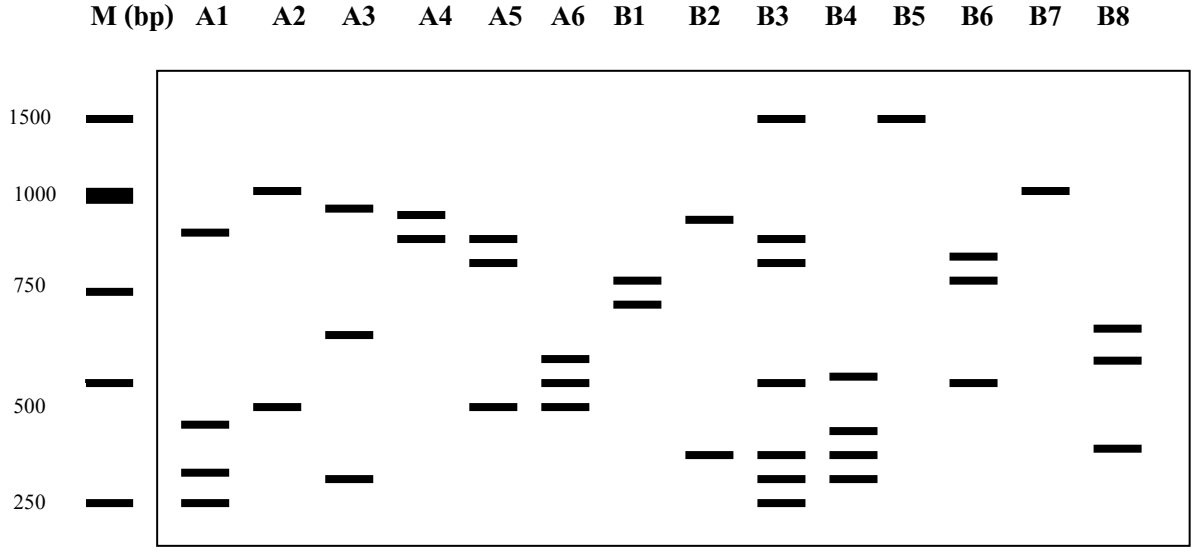

Figure 2. Schematic representation of *PvMSP3β* restriction patterns digested with *PstI*

Discussion

Polymorphic molecular markers have been described in malaria parasite, particularly polymorphic markers encoding parasite surface antigens that play an importance role in vaccine development. In addition, polymorphic molecular markers have been employed to evaluate genetic directly and distinguish parasite isolates for differentiating relapse and re-infections. PCR-RFLP methods are simple and available for parasite genotyping such as *PvMSP1, PvMSP3α, PvMSP3β*. and *PvCSP* genes.

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