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Genetic diversity of the *msp-1*, *msp-2*, and *glurp* genes of *Plasmodium falciparum* isolates along the Thai-Myanmar borders

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PEER REVIEW

Peer reviewer

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Comments

This is an important paper providing baseline information of the genetic diversity of malaria parasite populations with public health implications on future epidemiological studies on the dynamics of parasite transmission, and its impact on malaria control interventions and the drug resistance situation in the border areas.

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ABSTRACT

Objective: To study the genetic diversity at the *msp-1*, *msp-2*, and *glurp* genes of *Plasmodium falciparum* (*P. falciparum*) isolates from 3 endemic areas in Thailand: Tak, Kanchanaburi and Ranong provinces.

Methods: A total of 144 *P. falciparum* isolates collected prior to treatment during January, 2012 to June, 2013 were genotyped. DNA was extracted; allele frequency and diversity of *msp-1*, *msp-2*, and *glurp* genes were investigated by nested polymerase chain reaction.

Results: *P. falciparum* isolates in this study had high rate of multiple genotypes infection (96.5%) with an overall mean multiplicity of infection of 3.21. The distribution of allelic families of *msp-1* was significantly different among isolates from Tak, Kanchanaburi, and Ranong but not for the *msp-2*. K1 and MAD20 were the predominant allelic families at the *msp-1* gene, whereas alleles belonging to 3D7 were more frequent at the *msp-2* gene. The *glurp* gene had the least diverse alleles. Population structure of *P. falciparum* isolates from Tak and Ranong was quite similar as revealed by the presence of similar proportions of MAD20 and K1 alleles at *msp-1* loci, 3D7 and FC27 alleles at *msp-2* loci as well as comparable mean MOI. Isolates from Kanchanaburi had different structures; the most prevalent alleles were K1 and RO33.

Conclusions: The present study shows that *P. falciparum* isolates from Tak and Ranong provinces had similar allelic pattern of *msp-1* and *msp-2* and diversity but different from Kanchanaburi isolates. These allelic variant profiles are valuable baseline data for future epidemiological study of malaria transmission and for continued monitoring of polymorphisms associated with antimalarial drug resistance in these areas.

KEYWORDS

msp-1 gene, *msp-2* gene, *glurp* gene, *Plasmodium falciparum*, Thai-Myanmar border

1. Introduction

The morbidity and mortality rates due to malaria have been declining gradually in recent years in Thailand, but multidrug resistant *Plasmodium falciparum* (*P. falciparum*) remains one of the major health problems in Thailand. Artesunate-mefloquine combination has been the first line treatment of uncomplicated falciparum malaria in Thailand since 1995. Declining efficacy of this regimen has been observed in provinces along the Thai-Myanmar borders, especially in Kanchanaburi province during 2009-2011. A successful malaria control and prevention require the coordinated use of several strategies, including understanding the nature and extent of genetic diversity within *P. falciparum* which is essential in understanding the mechanism underlying its pathology, the acquisition of immunity, the spread of drug resistance and the infection transmission[1]. The merozoite surface protein 1 (*msp-1*) and *msp-2* are abundant surface protein on the

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blood stage of *P. falciparum*. They are thought to play a role in erythrocyte invasion[2]. Four allelic families had been identified in block 2 of *msp-1* gene: K1, MAD20, RO33, and MR and two allelic families in *msp-2* gene: FC27 and 3D7[3-6]. The *msp-1*, *msp-2* and glutamate-rich protein (*glurp*) have been extensively used as markers to investigate the genetic diversity, multiplicity of infection, the level of malaria transmission, immunity against malaria, as well as a discriminatory tool to distinguish new from recrudescent infections of field parasite population[7-15]. This informative tool has been used for distinguishing individual alleles in the therapeutic drug efficacy monitoring program of the Malaria Control Program of Thailand. It was used to investigate the influence of genetic diversity of *P. falciparum* in a malaria endemic area along the Thai-Cambodia border[16]. However, data were not available from endemic areas along the Thai-Myamar border[17]. The aim of this study was to assess the genetic diversity and allele frequencies of *msp-1*, *msp-2* and *glurp* genes of *P. falciparum* isolates from malaria patients in 3 provinces along the Thai-Myanmar border namely; Tak, Kanchanaburi and Ranong.

2. Materials and methods

2.1. Study sites and samples

A total of 144 *P. falciparum* infected blood were collected from uncomplicated falciparum malaria adult patients enrolled into the therapeutic efficacy monitoring of artemisinin derivatives antimalarial drugs during January, 2012 to June, 2013 in malaria endemic areas along the western border of Thailand with Myanmar. Ethical approval was obtained from the Research Ethics Committee of the Department of Disease Control, Ministry of Public Health, Thailand. Pre-treatment blood samples collected on Whatmann 3MM filter paper for DNA extraction was obtained from patients in 3 areas, 1) Maesot district of Tak province (57 cases), 2) Saiyok district of Kanchanaburi (55 cases), and 3) Kraburi district of Ranong (32 cases) (Figure 1).

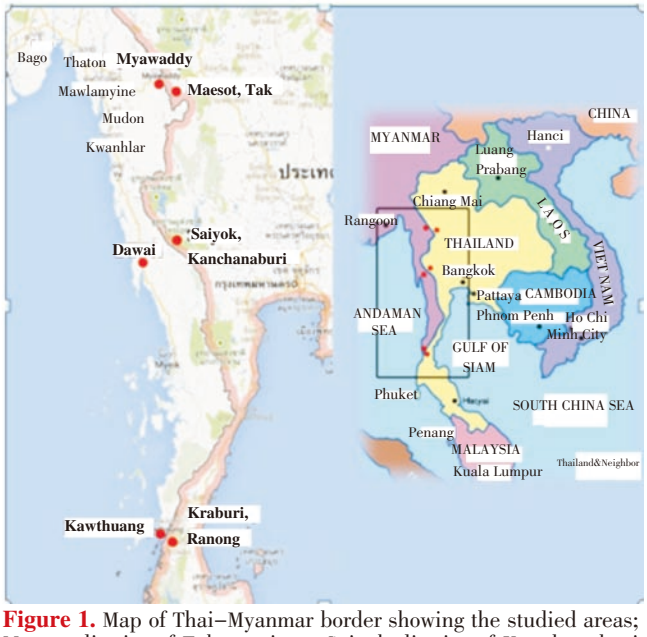

Figure 1. Map of Thai-Myanmar border showing the studied areas; Maesot district of Tak province, Saiyok district of Kanchanaburi province, and Kraburi district of Ranong province.

2.2. Genotyping of *P. falciparum msp-1, msp-2 and glurp genes*

Parasite DNA for PCR was extracted from dried blood spot using the QiaAmp DNA mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Polymorphic regions from *P. falciparum msp-1*, *msp-2*, and *glurp* genes were used as genetic markers for the genotyping of parasite populations. For *msp-1* and *msp-2*, the presence of unique sequences was used to divide the variants into distinct allelic families. The polymorphic repetitive regions Block 2 of *msp-1*; Block 3 of *msp-2* and RII repeat region of *glurp* were amplified by nested PCR using the primers and methods as recommended genotyping protocol[18]. In brief, the primary PCR, primer pairs corresponding to conserved sequences spanning the polymorphic regions of each gene were included in separate reactions. The product generated in the primary PCR was used as a template in six separate nested PCR, using in each case a specific primer pair in order to determine the presence of allelic variants of the K1, MAD20 and RO33 families of *msp-1* block2; the 3D7 and FC27 families of *msp-2* repeats, and the RII blocks of *glurp*. Each polymorphic domain was amplified from 5 μL of DNA solution in a 20 μL reaction mixture containing 0.12 μmol/L of each primer, 2 mmol/L $MgCl_2$, 125 μmol/L of each deoxyribonucleoside triphosphate, 0.4 U Amplitaq Gold® 360 Master Mix (Invitrogen) and PCR buffer.

The thermocycling conditions in the thermocycler (Thermo Scientific Hybaid Px2 Thermal Cycler, Fisher Scientific) for *msp-1*, *msp-2* and *glurp* primary PCR and *glurp* nested PCR were as follows: 5 min at 95 °C, followed by 30 cycles for 1 min at 94 °C, 2 min at 58 °C and 2 min at 72 °C and final extension of 10 min at 72 °C. For the *msp-1* and *msp-2* nested PCR, conditions were as followed: 5 min at 95 °C, followed by 30 cycles for 1 min at 94 °C, 2 min at 61 °C and 2 min at 72 °C and final extension of 5 min at 72 °C. The amplified products were either stored at 4 °C or analyzed immediately by electrophoresis on a 2% molecular grade agarose gel and visualized by UV transilluminator after gel SYBR® safe staining. The sizes of the PCR products were estimated using Imagelab software version 3.0 (Biorad) with the size computed automatically by the software based on the 100 base pairs DNA ladder calibrator (Real Biotech Corporation). Standard 3D7, Dd2 and RO33 clones were used as positive controls for 3D7 and K1; FC27 and MAD20; and RO33 alleles, respectively.

The detection of a single PCR fragment for each locus was classified as an infection with one parasite genotype (monoclonal infection) for that locus. Isolates with more than one genotype were considered as polyclonal infection[10]. Alleles in each family were considered the same if fragment size were within 20 bp interval for *msp-1* and *msp-2* genes[11], and 50 bp interval for *glurp* gene. In each isolate, number of genotypes and allelic type (or family) of each gene were described.

2.3. Statistical analysis

The *msp-1* and *msp-2* allele frequencies were calculated as the proportion of alleles found for the allelic family out of the alleles detected in isolates. The proportions of alleles observed at each genetic locus within each group were compared using the *Chi*-square test statistics. Multiplicity of infection (MOI) was defined as the number of parasite genotypes per infection.

MOI was calculated for each gene (*msp-1*, *msp-2*, and *glurp*) independently. Estimation of the overall MOI of the isolates was also calculated by combining the three markers, namely by using the highest number of bands detected in one marker. The maximum number of bands detected whatever the locus was considered as the MOI of that infection. Mean MOI was calculated by dividing the total number of fragments detected in *msp-1*, *msp-2* or *glurp* by the number of samples positive for the same marker. The median MOI was compared among isolates from Tak, Kanchanaburi and Ranong using the non-parametric Kruskal Wallis H test. Statistical significance was defined as a P-value$\leq$0.05. All statistical analyses were performed using SPSS statistical software, version 17.0.

3. Results

The study population comprised *P. falciparum* isolated collected from 144 uncomplicated falciparum malaria patients before treatment; 57, 55, and 32 samples were from Tak, Kanchanaburi and Ranong provinces, respectively.

3.1. Distribution of block 2 of msp-1 gene

Allelic families of *msp-1* were not evenly distributed among the isolates from Tak, Kanchanaburi, and Ranong provinces (P<0.000 1). Forty-one (74.5%) *P. falciparum* isolates from Kanchanburi province were positive for the K1 allele, in contrast to 23 (40.4%) of Tak and 14 (43.8%) of Ranong isolates (Table 1). Isolates from Kanchanaburi also carried the highest proportion of RO33 allele (45.5%), followed by isolates from Tak (12.3%) and Ranong (9.4%) but carried significantly the lowest proportion (21.8%) of MAD20 allele (P=0.000 1).

Table 1

Distribution of merozoite surface protein-1 (*msp-1*) allelic family of *Plasmodium falciparum*.

Allelic family of *msp-1* gene	Number (%) of *P. falciparum* isolates				P-value
	Tak	Kanchanaburi	Ranong	Total	
K1	23 (40.4)	41 (74.5)	14 (43.8)	78 (54.2)	0.001
MAD20	34 (59.6)	12 (21.8)	17 (53.1)	63 (43.8)	0.000 1
RO33	7 (12.3)	25 (45.5)	3 (9.4)	35 (24.3)	0.000 1

3.2. Distribution of block 3 of msp-2 gene

FC27 and 3D7 had similar distribution pattern in the samples from Tak, Kanchanaburi, and Ranong provinces (Table 2). Forty-two (73.7%) *P. falciparum* isolates from Tak province were positive for the 3D7 allele and 34 (59.6%) for FC27. Kanchanaburi isolates had slightly lower proportion of FC27 allele (45.5%) but higher 3D7 allelle (80.0%). Ranong isolates had quite similar proportion of FC27 (59.4%) to Tak isolates but had higher proportion of 3D7 (93.8%). However, there was no significant difference (P>0.05).

3.3. Genetic diversity of infection

Of the 144 *P. falciparum* isolates genotyped, 139 (96.5%) comprised multiple genotypes (Table 3). A high degree of genetic diversity was detected at the *msp-1* and *msp-2* genes whereas less diversity was observed at the *glurp* gene. MOI calculated for each gene showed that the mean MOI for *msp-2* was the highest (2.93), followed by *msp-1* (2.15), and *glurp* (1.26). The difference in MOI of *msp-1* among the isolates from Tak, Kanchanaburi and Ranong was statistically significant (P=0.015). The mean MOI of *msp-1* was the highest in Kanchanaburi isolates (2.47), followed by Ranong (2.19) and Tak (1.89). Proportion of isolates having MOI$\geq$3 was the highest in Ranong isolates (25.0%), followed by Kanchanaburi (23.7%) and Tak (15.8%).

Similar difference among the locations was also observed in median MOI for *msp-2* gene (P=0.013). Isolates from Ranong had the highest mean MOI of 3.34, followed by Tak (2.95) and Kanchanaburi (2.62). The highest proportion of isolates having MOI$\geq$3 was also found in Ranong isolates (81.2%), followed by Tak (61.4%) and Kanchanaburi (49.2%). *P. falciparum* isolates in this study had less complexity if using only *glurp* as a marker gene. Most isolates (84.7%) had only one allele. Isolates from Ranong had no diversity at the *glurp* locus; all isolates carried only one genotype. Estimation of the overall MOI of the isolates showed that the mean MOI was the highest in Ranong isolates (3.47), followed by Kanchanaburi isolates (3.15) and Tak isolates (3.14) but the difference was not statistically significant (P=0.164).

Table 2

Distribution of merozoite surface protein-2 (*msp-2*) allelic family of *Plasmodium falciparum*.

Allelic families of *msp-2* gene	Number (%) of P. falciparum isolates				P-value
	Tak	Kanchanaburi	Ranong	Total	
FC27	34 (59.6)	25 (45.5)	19 (59.4)	78 (54.2)	0.257
3D7	42 (73.7)	44 (80.0)	30 (93.8)	116 (80.6)	0.071

Table 3

Multiplicity of infection (MOI) of *msp-1*, *msp-2* and *glurp* genes.

Markers/Provinces		MOI=1	MOI=2	MOI$\geq$3	P-value
msp-1	Tak	19 (33.3)	29 (50.9)	9 (15.8)	0.015
	Kanchanaburi	12 (21.8)	30 (54.5)	13 (23.7)	
	Ranong	4 (12.5)	20 (62.5)	8 (25.0)	
	Total	35 (24.3)	79 (54.9)	30 (20.8)	
msp-2	Tak	8 (14.0)	14 (24.6)	35 (61.4)	0.013
	Kanchanaburi	8 (14.5)	20 (36.3)	27 (49.2)	
	Ranong	1 (3.2)	5 (15.6)	26 (81.2)	
	Total	17 (11.7)	39 (27.1)	88 (61.2)	
glurp	Tak	47 (82.5)	6 (10.5)	4 (7.0)	0.022
	Kanchanaburi	43 (78.2)	7 (12.7)	5 (9.1)	
	Ranong	32 (100.0)	0	0	
	Total	122 (84.7)	13 (9.0)	9 (6.3)	
All 3 markers	Tak	3 (5.3)	16 (28.1)	38 (66.6)	0.164
	Kanchanaburi	1 (1.8)	16 (29.1)	38 (69.1)	
	Ranong	1 (3.1)	2 (6.3)	29 (90.6)	
	Total	5 (3.5)	34 (23.6)	105 (72.9)	

4. Discussion

In this study we have examined the allelic diversity of *P. falciparum* and attempted to understand whether the polymorphism and frequency of alleles of *msp-1*, *msp-2* and *glurp* varies with locations of the parasite isolates. *P.*

falciparum isolates from 3 locations along the western Thailand bordered to Myanmar, *i.e.* Tak, Kanchanaburi, and Ranong provinces were studied. *P. falciparum* isolates in these areas had very high complexity of infection; 95% of the isolates had multiple genotype infection (75.7%, 88.3%, and 15.3% for *msp-1*, *msp-2*, and *glurp*, respectively). The highest number of alleles per isolate was 3, 5, and 5 for the *msp-1*, *msp-2*, and *glurp*, respectively. Overall mean MOI was 3.21. K1 and MAD20 were the predominant allelic families at the *msp-1* gene, whereas alleles belonging to 3D7 were more frequent at the *msp-2* gene. Lower polyclonal infected *P. falciparum* isolates was found in the western Cambodia[16]; 31% of the isolates had polyclonal infection (17%, 26%, and 8% for *msp-1*, *msp-2*, and *glurp*, respectively). Genotypic similarity of *P. falciparum* isolates in the eastern provinces of Thailand bordered to Cambodia such as Chanthaburi and Trat provinces is interesting to further study and compare with the isolates from those obtained from the western border of Thailand demonstrated in this study.

In the Thai-Mynmar border, population structure of *P. falciparum* isolates from Tak and Ranong provinces were similar as revealed by the presence of similar proportions of MAD20 and K1 alleles at *msp-1* loci, 3D7 and FC27 alleles at *msp-2* loci as well as comparable mean multiplicity of infections. In contrast, isolates from Kanchanaburi had different structure; the most prevalent alleles were K1 and RO33. The difference is probably due to the local factors as previously described[19], such as vector population, human host immunity, local transmission, as well as drug susceptibility pattern of the parasites in the areas. A recent efficacy study of artesunate-mefloquine combination in these 3 provinces showed that the cure rate declined rapidly in Kanchanaburi province from 92.7% in 2009 to 80% in 2011. High proportion of RO33 allele found in isolates from Kanchanaburi (45.5%) may relate with the reduction in treatment outcome. It is interesting to further monitoring the association of RO33 allelic family with the treatment outcome and drug resistant malaria.

As shown in the map, Kanchanaburi situates between Tak and Ranong. It was surprised to see the similar allelic patterns of *msp-1* and *msp-2* genes of *P. falciparum* isolates from Tak and Ranong, instead of Tak and Kanchanaburi or Kanchanburi and Ranong where there are shared border. In order to explain the 2 different allelic patterns, Tak and Ranong in one group and Kanchanaburi in another group, infection history of the patients were reviewed from the malaria case investigation forms recorded by the malaria clinics in the areas. It was found that most Kanchanaburi isolates were obtained from indigenous malaria cases contracted the disease in the villages or nearby forest in Bongti Subdistrict of Saiyok District. The parasite genotypes found in Kanchanaburi isolates were thus the genotypes circulated in the area. In contrast, most patients in Tak and Ranong were non-permanent Myanmese immigrants crossing border to seek employment or health facilities in Thai side.

Although Tak and Ranong had similar allelic patterns of *msp-1* and *msp-2* genes, Ranong had higher diversity as measured by MOI. This may be resulted from higher malaria transmission in the area. High diversity of parasite isolates may also affect the treatment outcome. Data from artesunate-mefloquine combination efficacy monitoring in 2009 of these 3 provinces showed that the cure rate in Ranong (87%) was lower than that in Tak (90.4%) and Kanchanaburi (92.7%). In addition, efficacy study of artemether-lumefantrine in 2012 in Tak and Ranong also showed lower cure rate in Ranong (88.4%) compared to 93.8% in Tak.

There was a limitation in the analysis of PCR products by gel electrophoresis. Varies in measuring of PCR product by gel electrophoresis was common. There was no standardized criterion to define distinct genotype. In the present study, lower number of distinct alleles was demonstrated in isolates from Thai-Myanmar border compared to the isolates from the western Cambodia near the eastern border of Thailand[16]. However, it was not able to conclude that the parasite from the Thai-Myanmar border had lower diversity because different setting criterion was used to define different alleles. A minimum of ten base pairs size difference was required to define an additional genotype in the study in western Cambodia[16], while 20 base pairs size difference was used in this study.

The present study shows that *P. falciparum* isolates from Tak and Ranong provinces had similar allelic pattern of *msp-1* and *msp-2* and diversity but different from Kanchanaburi isolates. These allelic variant profiles are valuable baseline data for future epidemiological study of malaria transmission and for continued monitoring of polymorphisms associated with antimalarial drug resistance in these areas.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

This study aims to assess the genetic diversity and allele frequencies of *msp-1*, *msp-2* and *glurp* genes of *P. falciparum* isolates from malaria patients in 3 provinces along the Thai-Myanmar border. These markers are used to investigate the genetic diversity, multiplicity of infection, and to distinguish new from recrudescent infections in the therapeutic efficacy monitoring of the Malaria Control Program of Thailand.

Research frontiers

Such genetic diversity of *P. falciparum* has been observed in a malaria endemic area along the Thai-Cambodia border,

but this is the first study along the Thai-Myanmar border. It would shed light on how divergent parasite populations affect the transmission dynamics in this part of the country.

Related reports

The genotyping of *P. falciparum msp-1*, *msp-2* and *glurp* genes follows the standard protocols for DNA extraction, PCR and sequencing for allelic variants. The multiplicity of infections and frequency of alleles can vary with different locations of the parasite isolates.

Innovations and breakthroughs

This is the first study on the genetic diversity of *P. falciparum*, comparing families of allelic variants from 3 different malaria endemic provinces along the western border of Thailand. It showed a high multiple genotype of infection and a significantly different distribution of allelic families of *msp-1* from the 3 locations.

Applications

The allelic variant profiles are valuable baseline information for future epidemiological studies of malaria parasite populations and for continued monitoring of polymorphisms associated with antimalarial drug resistance in these border areas with a worsening drug resistance situation.

Peer review

This is an important paper providing baseline information of the genetic diversity of malaria parasite populations with public health implications on future epidemiological studies on the dynamics of parasite transmission, and its impact on malaria control interventions and the drug resistance situation in the border areas. It also depicts a healthy collaborative partnership between the academe and the Ministry of Public Health of Thailand, with the application of basic science research in public health issues.

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