CHARACTERIZATION OF MALARIA INFECTION AT TWO BORDER AREAS OF THAILAND ADJOINING WITH MYANMAR AND MALAYSIA

Natthawan Sermwittayawong[1], Mitsuaki Nishibuchi[2], Nongyao Sawangjaroen[1] and Varaporn Vuddhakul[1]

[1]Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand; [2]Center for Southeast Asian Studies, Kyoto, Japan

Abstract. During 2009 to 2010, a total of 408 blood samples collected from malaria patients in Ranong (149) and Yala (259) Provinces, Thailand were investigated for *Plasmodium* spp using microscopic examination. There are no statistical differences in the prevalence of *P. falciparum* and *P. vivax* in samples collected from Ranong and Yala (46% *vs* 52%, and 54% *vs* 45%, respectively). Single nucleotide polymorphism of codon 86 in *pfmdr1* (encoding *P. falciparum* multidrug resistance protein 1) was investigated among 75 samples of *P. falciparum* and 2 samples of *P. knowlesi*. A *pfmdr1* N86Y mutation was detected in 1 out of 29 samples and 45 out of 46 samples obtained from Ranong and Yala Provinces, respectively. It is interesting that *pfmdr1* was detected in two *P. knowlesi* DNA samples obtained previously from Ranong Province which was 99% homologous to *pfmdr1* obtained from falciparum parasites in the same area but the mutation was not observed. The difference in multidrug resistance protein in *Plasmodium* obtained from those two border areas of Thailand will be of use in monitoring drug resistance in these border regions of the country.

Keywords: *Plasmodium falciparum*, *Plasmodium vivax, Plasmodium knowlesi*, *pfmdr1*, *pkmdr*, Thai borders

INTRODUCTION

Malaria is still one of the public health problems in Thailand. The most prevalent species of Plasmodium detected in this country are *Plasmodium falciparum* and *P. vivax* (WHO SEARO, 2011). The main malaria vectors are *Anopheles dirus*, *An. maculatus*, *An. minimus*, *An. aconitus*, and *An. sundaicus* (Ratanatham *et al*, 1988; Rattanarithikul *et al*, 1996). The habitats of these Anopheline mosquitoes are in the area covered with natural forests and hills of the Thai-border regions. In addition, the prevalence of these vectors is influenced by rainfall, which is high in southern Thailand.

Incidence of malaria is high at the border areas of Thailand (WHO SEARO, 2011). In addition, resistance to antimalarial drugs is a major public health problem in the management of malaria (Chareonviriyaphap *et al*, 2000; Mita *et al*, 2009). There are many factors that influence the

Correspondence: Natthawan Sermwittayawong, Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90100, Thailand.
Tel: +66 (0) 74 288339; Fax: +66 (0) 74 446661
E-mail: natthawan.k@psu.ac.th

antimalarial drug resistance, such as polymorphisms and increasing copy number of genes which encode parasite transport proteins. The most recognized genes are *P. falciparum* multidrug resistance protein-1 *(pfmdr1)* encoding a P-glycoprotien homologue 1 (Pgh1) and *P. falciparum* chloroquine resistance transporter gene (*pfcrt*) encoding the transmembrane protein PfCRT involved in transportation or efflux of antimalarial drugs. The lysine to threonine point mutation at codon 76 (K76T) in *pfcrt* have been associated with *in vitro* chloroquine resistance (Babiker *et al*, 2001; Djimde *et al*, 2001; Dorsey *et al*, 2001; Binder *et al*, 2002). However, the association between resistance of *P. falciparum* to antimalarial drugs and *pfmdr1* polymorphisms has been debatable, depending on geographical areas. In addition, the amino acid substitution at codon 86 of *pfmdr1,* from asparagine to tyrosine (N86Y), as well as increase in copy number have been associated with *in vitro* drug resistance to artemisinin, chloroquine, halofantrine and mefloquine (von Seidlein *et al*, 1997; Price *et al*, 1999; Nagesha *et al*, 2001; Price *et al*, 2004; Duraisingh and Cowman, 2005).

Drug resistant malaria has been studied numerously in Thailand, especially in Thai-Myanmar and Thai-Cambodia border areas. Associations of *pfmdr1* codon 86 mutation and antimalarial drug resistance has been demonstrated (Chaiyaroj *et al*, 1999; Price *et al*, 1999; Wongsrichanalai *et al*, 2001; Lopes *et al*, 2002; Price *et al*, 2004; Vijaykadga *et al*, 2006; Mungthin *et al*, 2010). However, less information regarding drug resistant malaria in southern Thailand has been reported. Therefore, it is of interest to characterize *Plasmodium* spp in the border areas of Thai-Myanmar and Thai-Malaysia. In this study, we compared the incidence of malaria infections between Ranong Province (south-western border between Thai and Myanmar) and Yala Province (southern border between Thai and Malaysia) (Fig 1). In addition, the single nucleotide polymorphism (SNP) of *pfmdr1*was investigated by PCR-restriction fragment polymorphism (RFLP) and DNA sequencing.

MATERIALS AND METHODS

Sample collection

Blood samples (149 and 259) were collected between June 2009 and June 2010 from patients with malaria attending the center of vector-borne diseases control in Ranong and Yala Provinces, respectively. Blood films prepared from finger-prick blood samples were stained with Giemsa and malarial species was identified by microscopic examination. A few drops of blood from each *Plasmodium* infected patient was collected on a filter paper (Whatman 903 Protein Saver Card; GE Healthcare, Franklin Lakes, NJ) and air dried for subsequent DNA extraction. All microscopically confirmed *P. falciparum* and *P. vivax* cases were treated with the first line drugs according to the treatment protocol of the Ministry of Public Health, Thailand. *P. falciparum*-infected patients were treated with 12 mg/kg artesunate, 15 mg/kg mefloquine and 0.5 mg/kg primaquine for three days. *P. vivax*-infected individuals were treated with 25 mg/kg cloroquine and 0.5 mg/kg primaquine for 14 days. This work was approved by the Ethics Committee of the Faculty of Science, Prince of Songkla University (document number 0521.1.09/241).

PCR-RFLP analysis of *pfmdr1*

P. falciparum samples (30 and 50) obtained from Ranong and Yala Provinces, respectively and two *P. knowlesi* DNA obtained from Ranong Province in a

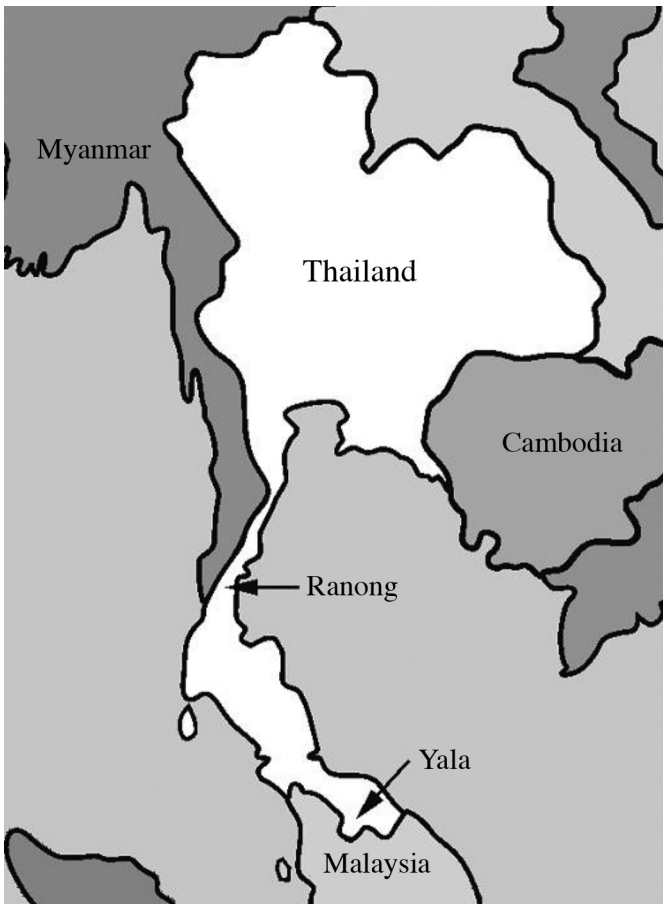

Fig 1–Map of Thailand showing Ranong and Yala Provinces.

previous study (Sermwittayawong *et al*, 2012) were investigated for PCR-RFLP analysis of *pfmdr1* as described previously (Duraisingh *et al*, 2000). Briefly, total DNA was extracted using a DNA extraction kit (QIAGEN, Hilden, Germany) and used as DNA template for nested PCR. First-round PCR was performed using primers, Pfmdr1-n1F (5′-TGTTGAAA-GATGGGTAAAGAGCAGAAAGAG-3′) and Pfmdr-n1R (5′-TACTTTCTTATTA-CATATGACACCACAAACA-3′). PCR was conducted in a 20-μl reaction mixture containing 1x *Pfu* buffer, 2 mM $MgSO_4$, 0.2 mM dNTPs, 0.25 μM each primer, 0.5 U *Pfu* DNA polymerase (Promega, Madison, WI) and 2 μl of DNA template. PCR was performed in a thermal cycler (ASTEC PC-818; Fukuka, Tokyo, Japan) with conditions as follows: 94°C for 4 minutes, followed by 40 cycles at 94°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute, with a final step at 72°C for 4 minutes. The first-round PCR amplification product was 10-fold diluted and used as DNA template for the second-round PCR employing primers Pfmdr-n2F (5′-GTCAAAC-GTGCATTTTTTATTGAC-CATTTA-3′) and Pfmdr-n2R (5′-AAAGATGGTAACCT-CAGTATCAAAGAAGAG-3′). The PCR conditions were as described above except the annealing temperature was 55°C. An amplicon (560 bp) was analyzed on 1% agarose gel-electrophoresis and ethidium bromide staining.

Nested PCR amplicon was subjected to *Apo*I digestion. The reaction was conducted in a 10-μl mixture containing 1x NEB3 buffer, 100 μg/ml bovine serum albumin (New England Biolabs, Beverly, MA), 2 U *Apo*I (New England Biolabs), and 5 μl of nested PCR product. The reaction mixture was incubated at 50°C for 5 hours and analyzed on 2% agarose gel-electrophoresis as described above. DNA fragments of 79 and 481 bp indicated a SNP in codon 86 of *pfmdr1*.

DNA sequencing

SNP of *pfmdr1* was confirmed by DNA sequencing. In brief, nested PCR amplicon was extracted using phenol-chloroform-isoamyl alcohol (Sambrook and Russell,

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PF M8/70   Yala     FFISVFGVILKNMYLGDDINPIILSLVSIGLVQFILS
PF M8/79   Yala     FFISVFGVILKNMYLGDDINPIILSLVSIGLVQFILS
PF M10/11  Ranong   FFISVFGVILKNMNLGDDINPIILSLVSIGLVQFILS
PF M10/21  Ranong   FFISVFGVILKNMNLGDDINPIILSLVSIGLVQFILS
PF M10/50  Ranong   FFISVFGVILKNMNLGDDINPIILSLVSIGLVQFILS
PK M2/20   Ranong   FFISVFGVILKNMNLGDDINPIILSLVSIGLVQFILS
PK M2/51   Ranong   FFISVFGVILKNMNLGDDINPIILSLVSIGLVQFILS
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Fig 2–Deduced amino acid sequences of *P. falciparum* MDR1 and *P. knowlesi* MDR. Box indicates amino acid residue 86 of MDR. PF, *P. falciparum*; PK, *P. knowlesi*.

2001) and subjected to direct nucleotide sequencing using an automated ABI PRISM 3730XL DNA Sequencing System (Applied BioSystems, Foster City, CA). Homology search was conducted using BlastN program (National Center for Biotechnology Information, Washington, D.C.). The nucleotide and deduced amino acid sequences were aligned using ClustalW program (www.ch.embnet.org/software/ClustalW.htm). The deduced amino acid sequences of *pkmdr* were compared to those in PlasmoDB, a genome database for the genus *Plasmodium* using BlastP 2.2.28+ program (Schaffer *et al*, 2001).

RESULTS

Identification of *Plasmodium* spp

By microscopic examination, 69 (46.3%), 77 (51.7%) and 3 (2%) blood samples collected from Ranong Province were identified as *P. falciparum, P. vivax* and mixed infection (*P. falciparum* and *P. vivax*), respectively, and 140 (54%), 118 (45.6%) and 1 (0.4%) blood samples collected from Yala Province were *P. falciparum, P. vivax* and mixed infection (*P. falciparum* and *P. vivax*), respectively.

SNP analysis of *pfmdr1*

The 560 bp amplicon of *pfmdr1* was obtained from 29/30 and 46/50 DNA samples from Ranong and Yala Provinces, respectively. In addition, this 560 bp amplicon was also generated from the two *P. knowlesi* DNA samples (M2/20 and M2/51) previously obtained from Ranong Province (Sermwittayawong *et al*, 2012) (Table 1).

RFLP using *Apo*I to identify SNP at *pfmdr1* codon 86 (N86Y) revealed 1 and 45 samples from Ranong and Yala Provinces, respectively were positive (Table 1). DNA sequencing of *pfmdr1* 560 bp amplicons confirmed the nucleotide change from AAT to TAT (Fig 2). *Apo*I digestion of the two *P. knowlesi* samples generated fragments of approximately 79, 231 and 250 bp indicating the wild type sequence (86N) (Table 1). Confirmation by DNA sequencing indicated that the two putative *pkmdr* amplicons were 99% homologous to *pfmdr1* deposited in GenBank as well as the three DNA sequences of the wild type *pfmdr1* obtained from Ranong Province (Fig 2). Nucleotide sequences of five *pfmdr1* amplicons (two from Yala and three from Ranong) and two *P. knowlesi* samples were deposited in GenBank (Accession nos. JN819292-JN819296 and JF923563-JF923564, respectively).

DISCUSSION

Malaria is typically diagnosed by the microscopic examination of blood films, in which *P. falciparum* and *P. vivax* are clearly differentiated. In this study, there is no

Table 1
Single nucleotide polymorphism analysis of *pfmdr1* and *pkmdr1* by PCR-RFLP.

Province	Species (No. of samples)	PCR positive for *mdr1*	PCR-RFLP result	
			Wild type (86N)*	Mutant (N86Y)**
Ranong	*P. falciparum* (30)	29 (97%)	28 (97%)	1 (3%)
	P. knowlesi (2)	2 (100%)	2 (100%)	0 (0%)
Yala	*P. falciparum* (50)	46 (92%)	1 (2%)	45 (98%)

*PCR-RFLP fragment sizes of 79, 231 and 250 bp generated by *Apo*I digestion. **PCR-RFLP fragment sizes of 79 and 481 bps generated by *Apo*I digestion.

significant difference in the prevalence of *P. falciparum* and *P. vivax* between the Thai-Myanmar and Thai-Malaysian border areas. Investigation of the presence of *pfmdr1* in 80 *P. falciparum* DNA samples obtained from both areas revealed 5 samples were negative for PCR amplification. This may be due to loss of target sequence in these samples as determination of *pfmdr1* in *P. falciparum* isolates from the western border of Thailand demonstrated that their copy numbers ranging from 0.4 to 4.1 (Price *et al*, 1999).

In this work, two DNA samples of *P. knowlesi* were included because they were obtained from Ranong Province (Sermwittayawong *et al*, 2012). It has been demonstrated that early trophozoites of *P. knowlesi* are indistinguishable from *P. falciparum* (Singh *et al*, 2004). Thus, primers specific to *pfmdr1* was able to amplify *pkmdr* from those two *P. knowlesi* DNA samples. The 560-bp amplicons obtained were 99% identical to wild type *pfmdr1* from *P. falciparum* samples from the same province. In addition, the deduced amino acid sequences of *pkmdr,* when compared to *P. knowlesi* and *P. falciparum* sequences deposited in the PlasmoDB, showed that the pkMDR sequences obtained from this study were 71% and 94% identical to *P. knowlesi* (strain H) multidrug resistance protein (PKH_100920) (accession number XM_002259545.1) and *P. falciparum* (3D7) multidrug resistance protein (PfMDR1) (accession number XM_001351751.1), respectively. Thus, this indicates close relationship between both *Plasmodium* species and possible horizontal drug-resistant gene transfer among *P. faciparum* and *P. knowlesi,* which poses a public health concern.

Point mutation of asparagine to tyrosine at codon 86 of *pfmdr1* has been reported to modulate the sensitivity to chloroquine, mefloquine and quinine (Reed *et al*, 2000), and is associated with antimalarial resistance in many countries of Africa, South America and Asia, including Thailand (Price *et al*, 1999; Babiker *et al*, 2001; Djimde *et al*, 2001), although contradictory findings have been reported (Povoa *et al*, 1998; Chaiyaroj *et al*, 1999; Dorsey *et al*, 2001; Pillai *et al*, 2001). In Thailand, 86N and increase in copy number of *pfmdr1* have been reported as associated with multidrug-resistant phenotype (Chaiyaroj *et al*, 1999; Price *et al*, 1999; Mungthin *et al*, 2010). In this study, N86Y in PFMDR1 was detected in 98% of samples collected from Yala Province but only 3% of samples were observed from

Ranong Province, suggesting a higher, prevalence of multidrug resistant malaria in the Thai-Myanmar border than in the Thai-Malaysian border area. However, the same protocol of antimalarial treatment for falciparum malaria is used in these two regions. Further studies will be needed to clarify this observation. In addition the close relationship between *pfmdr1* and *pkmdr* demonstrated in this study will provide useful information for treatment guidance and assessment of antimalarial drug efficacy in both Thai border areas.

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