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Prevalence of *Pfcrt* 76 and *Pfmdr1* 86 mutations in *Plasmodium falciparum* isolates from endemic areas of Thailand by multiplex nested PCR-RFLP

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Abstract

Plasmodium falciparum drug resistance is a major factor affecting malaria spread and mortality rates of malaria infections. Several studies have shown that chloroquine and other antimalarial drug resistant *P. falciparum* are associated with a mutation of the *Pfcrt* (K76T) and *Pfmdr 1* (N86Y) genes. The aim of this study is to investigate the prevalence of these two gene mutations in Thailand. A total of 84 *P. falciparum* infected blood samples were collected from eight malaria-endemic provinces. A multiplex-nested PCR-RFLP was used to examine the two gene mutations. The results revealed a very high prevalence (100%) of *Pfcrt* (K76T) gene mutation and a high prevalence (52.4%) of *Pfmdr1* (N86Y) gene mutation. These findings suggest that in Thailand *P. falciparum* is resistant to chloroquine and that there is an increasing trend of other drug-resistant malaria. The highest and lowest prevalence of these double genes mutations were found in Mae Hong Son province (100%) and Trang province (25%). This study is useful for the epidemiology of *P. falciparum* drug resistance, and can support the selection of appropriate antimalarial drug treatment and drug resistance surveillance and control programs.

***Keywords*:** *Plasmodium falciparum*, *Pfmdr1*, *Pfcrt*, *prevalence of gene mutations*, *multiplex-nested PCR-RFLP*

บทคัดย่อ

การดื้อต่อยาต้านมาลาเรียของเชื้อพลาสโมเดียมฟัลซิพารัมเป็นปัจจัยสำคัญของการเพิ่มอัตราการระบาด และการเสียชีวิตของผู้ป่วยโรคมาลาเรีย จากการศึกษาที่ผ่านมาพบว่าการดื้อต่อยาคลอโรควินและยาต้านมาลาเรียชนิดอื่นๆ ของเชื้อพลาสโมเดียมฟัลซิพารัมมีความสัมพันธ์กับการเกิดการกลายพันธุ์ของยีน *Pfcrt* ที่ตำแหน่ง 76 (K76T) และ *Pfmdr1* ที่ตำแหน่ง 86 (N86Y) ผู้วิจัยจึงทำการศึกษาอัตราความชุกของการกลายพันธุ์ของยีนทั้ง 2 ชนิด ด้วยวิธี multiplex-nested PCR-RFLP โดยทำการเก็บตัวอย่างเลือดผู้ติดเชื้อพลาสโมเดียมฟัลซิพารัม จากสถานพยาบาลในเขตชุกชุมโรค 8 จังหวัดของประเทศไทย จำนวนตัวอย่างทั้งหมด 84 ตัวอย่าง พบอัตราความชุกของการกลายพันธุ์ของยีน *Pfcrt* ที่ตำแหน่ง 76 (K76T) สูงมากถึง 100% และ *Pfmdr1* ที่ตำแหน่ง 86 (N86Y) สูงถึง 52.4% แสดงให้เห็นว่าเชื้อพลาสโมเดียมฟัลซิพารัมในประเทศไทย ดื้อต่อยาคลอโรควิน และมีแนวโน้มการดื้อต่อยาต้านมาลาเรียชนิดอื่นๆ เพิ่มมากขึ้น โดยจังหวัดที่พบว่ามีอัตราความชุกของการกลายพันธุ์ของยีนทั้ง 2 ชนิดมากที่สุดคือจังหวัดแม่ฮ่องสอน (100%) และจังหวัดที่พบน้อยที่สุดคือจังหวัดตรัง (25%) จากข้อมูลงานวิจัยนี้สามารถใช้เป็นข้อมูลพื้นฐานทางด้านระบาดวิทยา เพื่อประมาณอัตราความชุกของเชื้อดื้อยา เป็นประโยชน์ในด้านการรักษาโดยเลือกใช้ยาต้านมาลาเรียได้อย่างเหมาะสม และพัฒนาการควบคุม เฝ้าระวังการแพร่ระบาดของเชื้อดื้อต่อยาต้านมาลาเรีย

คำสำคัญ: *พลาสโมเดียมฟัลซิพารัม,ความชุกของยีนดื้อยา, อัตราความชุกของการกลายพันธุ์ของยีน, การเกิดการกลายพันธุ์ของยีน Pfcrt, วิธี multiplex-nested PCR-RFLP*

1. Introduction

The worldwide spread of drug resistant *Plasmodium falciparum* is a major problem in malaria treatment. Malaria drug resistance has been associated with the spread of malaria to new areas and re-emergence of malaria where elimination was previously confirmed (WHO 2010). Africa and Southeast Asia, including Thailand, were found to be the main drug resistant *P. falciparum* areas (WHO 2010). In Thailand, *P. falciparum* chloroquine (CQ) resistance was first reported in the 1950s, and since then it has spread across the world. Since the 1970s, CQ has no longer been used in Thailand (Parker et al., 2012). Nowadays, artemisinin-based combination therapy (ACT), specifically artesunate-mefloquine combination, is used as the first line drug

for *falciparum* malaria treatment in Thailand, as recommended by the World Health Organization (Na-Bangchang & Karbwang, 2009). However, reports have shown that multidrug resistant *P. falciparum* is a serious problem along the Thai border areas, particularly the Thai-Myanmar and the Thai-Cambodian borders (Parker et al., 2012; Wongsrichanalai, Pickard, Wernsdorfer, & Meshnick, 2002). Several reports have also found that artemisinin resistant *P. falciparum* malaria has appeared in Thailand's other border regions (Carrara et al., 2013; Na-Bangchang & Karbwang, 2013; Noedl et al., 2008). Furthermore, the high number of migrant populations moving across the border results in a high risk of drug resistant malaria transmission among these groups (Khamsiriwatchara et al., 2011; Tipmontree, Fungladda, Kaewkungwal, Tempongko, & Schelp, 2009; Wangroongsarb, Sudathip, & Satimai, 2012; Wiwanitkit, 2002).

A successful control method for this parasite is surveying the spread of drug resistant malaria. One potential surveillance method is detecting drug resistance through genetic markers using molecular methods. Sample collection for such studies is easy as new methods allow us to use dried blood spots for analysis. There are at least two genes that are associated with antimalarial drug resistance: *Pfcrt* (*P. falciparum* chloroquine resistance transporter) and *Pfmdr1* (*P. falciparum* multidrug resistance1). The *Pfcrt* gene is composed of 13 exons exhibiting several mutation points which play a role in CQ resistance (Fidock et al., 2000). Among these, the mutation of an amino acid change from lysine to threonine at codon 76 (*Pfcrt* K76T) is significantly correlated with *P. falciparum* CQ resistant strains from different endemic areas of the world (Babiker et al., 2001; Hatabu et al., 2005; Mungthin et al., 2010b; Severini et al., 2006; Shrivastava, Gupta, Mahanta, & Dubey, 2014; Wongsrichanalai et al., 2002; Zakeri et al., 2008).

Another mutation, observed in the *Pfmdr1* gene, is located on chromosome 5 which encodes a transmembrane glycoprotein (Pgh1, for P-glycoprotein homologue 1). It is a member of the ATP-binding cassette transporter superfamily localized in the parasite vacuole, where it may regulate intracellular drug concentrations (Duraisingh & Cowman, 2005). Five specific point mutations in codons 86, 184, 1034, 1042 and 1246 of *Pfmdr1* have been reported. Several field studies have shown that the mutation of asparagine to tyrosine at codon 86 of the *Pfmdr1* (N86Y) were found to be associated to CQ resistance, as well as related to other antimalarial drugs responses such as quinine (QN), amodiaquine (AQ), lumefantrine (AL), mefoquine (MQ) and artemisinin in various regions (Duraisingh, Roper, Walliker, & Warhurst, 2000; Duraisingh & Cowman, 2005; Mungthin et al., 2010a; Phompradit, Wisedpanichkij, Muhamad, Chaijaroenkul, & Na-Bangchang, 2011; Poyomtip et al., 2012).

2. Objective

The purpose of this study is to explore the prevalence of drug resistant *P. falciparum* from three different regions in Thailand at the same period in 2009. We used a multiplex nested PCR and restriction fragment length polymorphism (RFLP) to detect two molecular markers, *Pfcrt* 76 and *Pfmdr1* 86 mutations.

3. Materials and methods

3.1. Study areas and blood sample collection

A total of 95 *P. falciparum* positive blood samples were collected from nine hospitals (located in eight provinces) in Thailand in 2009. Both genes (*Pfcrt* 76, *Pfmdr1* 86) were successfully collected in only 84 samples. The 84 samples were obtained from three highly malaria-endemic regions of Thailand (Figure 1). From northern Thailand (N=13), samples were taken from Lampang, Mae Hong Son and Tak provinces. Mae Hong Son and Tak, provinces are located on the Thai-Myanmar border. From western Thailand, (N=48) samples were collected from Kanchanaburi province (Thai-Myanmar border). From southern Thailand (N=23), samples were collected from Surat Thani, Chumphon, Songkhla, and Trang provinces. Blood samples were performed by thick and thin blood film for Giemsa staining and about 100 µl was spotted on a piece of Whatman 3M filter paper and air-dried. The dried filters were stored in individual plastic bags at room temperature until DNA extraction and genotyping.

The study was reviewed and approved by the Ethics Committee of Rangsit University.

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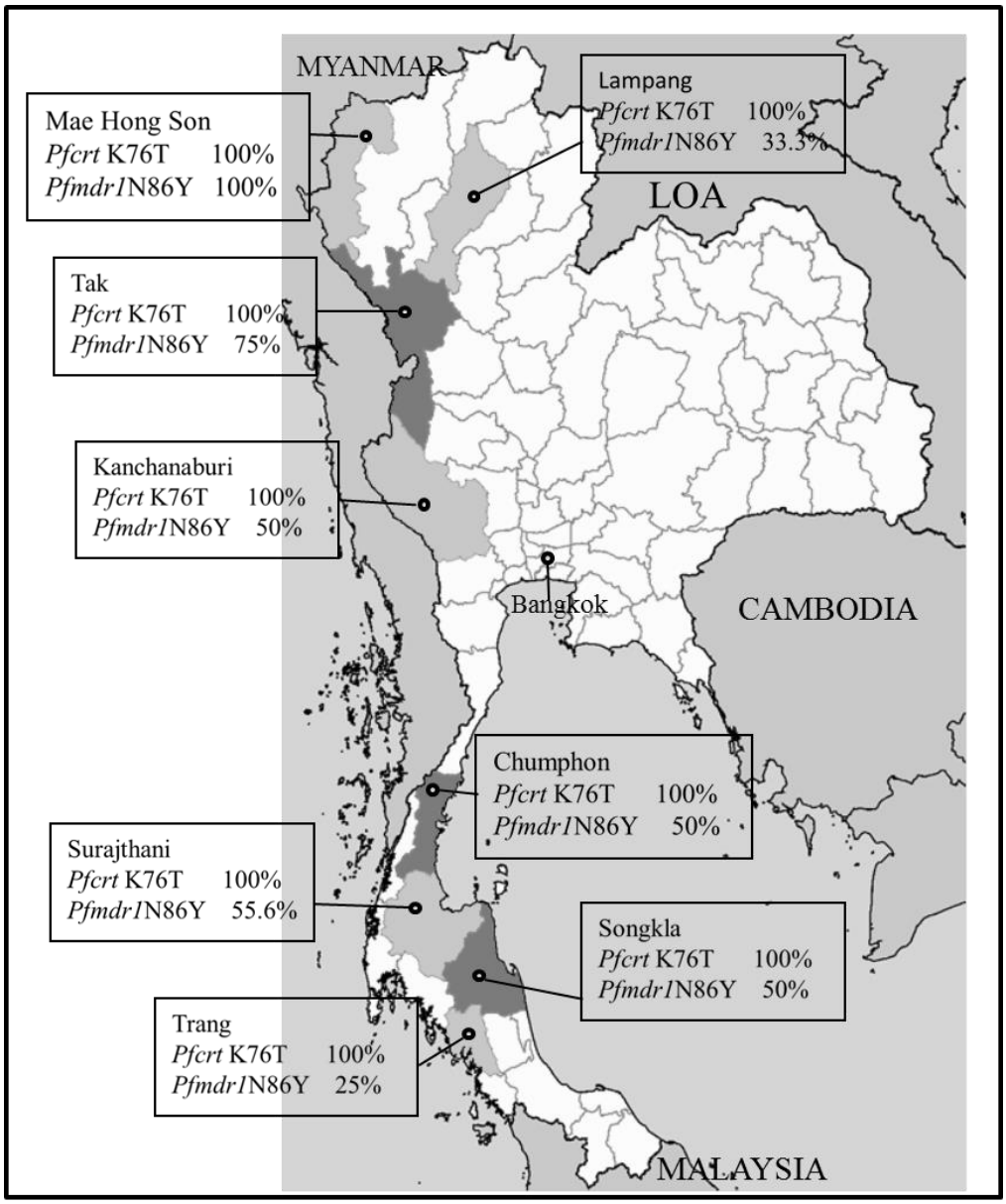

Figure 1 Distribution of *P. falciparum Pfcrt 76* and *Pfmdr1 86* genotype isolated from various endemic areas in Thailand

3.2. Microscopy

The *P. falciparum* diagnosis and parasite density investigation were performed by microscopic examination of Geimsa-stained blood film. Thick and thin blood films were stained with 3% Giemsa solution (Merck, Gemany) for 30 min and microscopically examined at ×1,000 for species identification to confirm *P. falciparum*, and parasitemia density estimation by two well-trained microscopists. The parasitemia density (parasites/µl) was done by counting 10,000 red blood cells from each positive thin blood film. The number of red blood cells (RBCs/µl) was estimated as 5.5×10^6.

$$\textbf{\textit{Calculation of parasitemia/}}\textbf{µl} = \frac{\text{The number of parasites seen} \times 5.5\times10^6}{\text{The number of RBC seen (10,000)}}$$

3.3. DNA extraction

DNA extraction was carried out by the Chelex method with minor modification (Polski, Kimzey, Percival, & Grosso, 1998). Each filter paper punch of a dried blood spot was placed in a micro centrifuge tube, soaked in 300 µl of distilled water, and incubated at room temperature for 30 min. The punch was then pressed gently at the bottom of the tube several times, then the supernatant was taken and added with 1 ml of distilled water. After centrifugation for 3 min at 12,000 rpm, most of the supernatant was removed and 250 µl of 5% Chelex-100 Resin (Bio-Rad Laboratories, Hercules, CA) was added. The tubes were then incubated for 30 min at 56°C, vortexed for 10 sec, boiled for 8 min, vortexed again for 10 sec, and centrifuged for 2 min at 12,000 rpm. Then the supernatant was collected and stored at -20°C until used as the PCR DNA template.

3.4. Determination of *Pfcrt* K76T and *Pfmdr1* N86Y genes mutation by multiplex nested PCR-RFLP

The target regions of *Pfcrt* K76T and *Pfmdr1* N86Y were amplified by multiplex nested PCR and the restriction fragment length polymorphism (RFLP) screening was performed as previously described with some modifications (Maria, Pedro, Anders, & Jose, 2006). Two sets of outer and inner primer sequences, expected product sizes of amplicons, and the PCR conditions are illustrated in Table 1. The multiplex nested PCR products were analyzed using 1.5% agarose gel electrophoresis and visualized with 1 µg/ml ethidium bromide under UV transilluminator. The expected product sizes were two bands of 481 and 145 bp for *Pfmdr1* 86 and *Pfcrt* 76 target genes respectively. Then RFLP analysis of *Pfcrt* codon 76 and *Pfmdr1* codon 86 was conducted. The *Pfmdr1* 86 and *Pfcrt* 76 PCR products were digested with *Apo*I restriction enzyme (Fast Digest Xapl Fermentas/Canada), according to the manufacturer's recommendations. All digested products were electrophoretic with 2.2 % agarose gels, paralleled with untreated samples and visualized with 1 µg/ml ethidium bromide under UV transilluminator. After *Apo*I digestion, the amplicons of the expected product sizes were visualized of 145 bp for *Pfcrt* 76T mutant and 481 bp for *Pfmdr1* 86Y mutant alleles (undigested product for mutant). The amplicons of the expected two sizes of 239 and 135 bp bands indicated the presence of the *Pfmdr1* N86 wild type codon, and two sizes of 98 bp and 47 bp bands presenting *Pfcrt* Y76 wild type codon (digested product for wild type).

3.5. Data analysis

Data were analyzed using SPSS software package (SPSS 11.5 for Windows). The MannWhitney U-test was used to compare the parasitemia density between the different groups. Comparisons of prevalence of *Pfcrt* K76T and *Pfmdr1* N86Y mutations among regions were analyzed using Chi-square test. A p-value ≤ 0.05 was considered statistically significant.

4. Results

4.1. Sample data

All *P. falciaparum* isolates from each location in Thailand is shown in Table 2. A majority of the samples came from the western (57.1%, 48/84) part of Thailand (Thai-Myanmar border), followed by the southern (23/84, 27.4%) and northern (13/84, 15.5%) parts. The overall range of parasitemia was 12.6-1,037.8 $x10^4$/µl. The geometric mean (GM) of parasitemia in all isolates was 45.8 $x10^4$/µl. The GM was highest in northern (170.5 $x10^4$/µl) followed by western (2.4 $x10^4$/µl) and southern (2.3 $x10^4$/µl) regions.

4.2. Prevalence of wild type and mutant alleles of *Pfcrt* 76 and *Pfmdr1* 86 in *P. falciparum* isolates from endemic area of Thailand

Out of the 95 samples examined, we were unable to amplify one or both genes of 11 (11.6%)

samples. The remaining 84 (78.5%) samples gave amplification products of both genes and were further analyzed. The 84 samples from different endemic areas of Thailand were analyzed by multiplex nested PCR-RFLP. The prevalence of both alleles in field isolates is shown in Table 3. Forty four (44) isolates carried *Pfmdr1* 86Y mutant alleles (52.4%), while all of isolates were *Pfcrt* 76T mutants (100%). The prevalence of parasites that carried both mutant genes; *Pfcrt* 76 and *Pfmdr1* 86, was 52.4%. The highest prevalence of mutant *Pfmdr1* 86Y isolates was found in northern Thailand, 69.2% (9/13). Of these, 100% prevalence was found in Mae Hong Son (2/2) and 75% (6/8) in Tak provinces. The lowest *Pfmdr1* 86Y prevalence was found in southern Thailand, 47.83% (11/23). Fifty (50%) of isolates from Kanchanaburi province, western Thailand (Thai-Myanmar border) were *Pfmdr1* 86Y mutant.

4.3. Severity and mutation

The severity of *P. falciparum* was analyzed using the parasitemia of each location and statistical results from the t-test (Table 1). The data showed that higher parasitemia was found in the northern than in the western and southern regions. There were statistically significant differences of parasitemia between the areas of north and west (P=0.004) and between north and south (P=0.004), but no significant difference between west and south (P=0.655). The prevalence of wild type and mutant alleles of *Pfmdr1* 86 in *P. falciparum* isolates from Thailand were classified into three levels of parasite densities as seen in Table 4. The results showed that in high and medium parasite density levels, the frequencies of mutant was found to be higher than wild type isolates (62.5%/37.5%). In low parasite density levels, we found higher frequencies of wild type than mutant parasites (55%/45%). However, we found no significant differences using Chi-square (P>0.05). The mean parasite density was also analyzed by grouping mutant and wild type *Pfmdr1* 86 alleles in all *P. falciparum* isolates (Table 5). A higher GM was found in mutant than wild type, but it was not tested for statistical difference by Mann-Whitney U-test (P=0.334).

Table 1 Sequences of the primers and PCR conditions used for the detection of the *Pfcrt* 76 and *Pfmdr1* 86 by multiplex PCR

Primer	–3' Sequence 5'-	Size (bp)	PCR condition
First round *Pfcrt* 76	C1Fw ATTTCGTACCAATTCCTGAACT C1Rev CGGATGTTACAAAACTATAGTTACC	538	94ºC, 3 min followed by 45 cycles (94ºC, 30s; 56ºC, 30s; 60ºC, 30s); 60ºC,3 min
***Pfmdr1* 86**	M1Fw AAGAGGTTGAAAAAGAGTTGAAC M1Rev CCGTTAATAATAAATACACGCAG	447	
Second round *Pfcrt* 76	C2Fw TGTGCTCATGTCTTTAAACTT C2Rev CAAAACTATAGTTACCAATTTTG	145	94ºC, 3 min followed by 40 cycles (94ºC, 30 s; 47ºC, 30s; 64ºC, 30s); 64ºC, 3 min
***Pfmdr1* 86**	M2Fw AGAGTACCGCTGAATTATTTAG M2Rev CCTGAACTCACTTGTTCTAAAT	418	

Table 2 *Plasmodium falciparum* isolated from each location in Thailand

Data	Location			Total
	North	West	South	
Sample No. (%)	13(15.5)	48 (57.1)	23 (27.4)	84 (100.0)
Parasite/µl ($\times 10^4$) Max.	1,037.8	52.4	63.8	1,037.8
Parasite/µl ($\times 10^4$) Min.	12.6	0.4	0.3	0.3
Geometric Mean Parasite/µl ($\times 10^4$)	170.5	2.4	2.3	4.6
SD of mean ($\times 10^4$)	295.6	10.3	14.2	155.1

The mean were significant compared between north and west (p=0.004), north and south (p=0.004)
Not significant compared between west and south (p=0.655)

Table 3 Distribution of wild type and mutant alleles of *Pfcrt* 76 and *Pfmdr1* 86 in *P. falciparum* isolates from three regions of Thailand

Location/ province	Number of Sample	Genotyping of *Pfmdr1* 86 and *Pfcrt* 76 N (%)				
		***Pfmdr1* 86 (mutant)**	***Pfmdr1* 86 (wild type)**	***Pfcrt* 76 (mutant)**	***Pfcrt* 76 (wild type)**	***Pfmdr1*86/ *Pfcrt* 76 (mutant/mutant)**
North	13(15.5)	9 (69.2)	4 (30.8)	13 (100)	0	9 (69.2)
Mae Hong Son	2	2(100)	0	2 (100)	0	2 (100)
Lampang	3	1(33.3)	2(66.7)	3(100)	0	1(33.3)
Tak	8	6(75.0)	2(25.0)	8(100)	0	6 (75.0)
West	48(57.1)	24(50.0)	24(50.0)	48(100)	0	24 (50.0)
Kanchanaburi	48	24(50.0)	24(50.0)	48(100)	0	24 (50.0)
South	23(27.4)	11(47.8)	12(52.2)	23(100)	0	11 (47.8)
Chumphon	6	3(50.0)	3(50.0)	6(100)	0	3(50.0)
Surajthani	9	5(55.6)	4(44.4)	9(100)	0	5(55.6)
Trang	4	1(25.0)	3(75.0)	4(100)	0	1(25.0)
Songkla	4	2(50.0)	2(50.0)	4(100)	0	2(50.0)
Total	**84(100)**	**44 (52.4)**	**40 (47.6)**	**84 (100)**	**0**	**44 (52.4)**

Table 4 Prevalence of wild type and mutant alleles of *Pfmdr1* 86 in *P. falciparum* isolates from Thailand classified by 3 level parasite densities

Parasite Density (µl)	*Pfmdr1* genotype		Total
	Mutant (N/%)	Wild (N/%)	
High (>100,000)	15 (62.5)	9 (37.5)	24 (28.6)
Medium (10,000-100,000)	20 (50.0)	20 (50.0)	40 (47.6)
Low (≤10,000)	9 (45.0)	11 (55.0)	20 (23.8)
Total	44 (100.0)	40 (100.0)	84 (100.0)

Table 5 Parasite densities; geometric mean and prevalence of wild type and mutant alleles of *Pfmdr1* 86 in *P. falciparum* isolates from Thailand

	Pfmdr1 genotype		P-value
	Mutant (N=44)	Wild type (N=40)	
Parasite Density ($\times 10^4$/µl) GM (SD 0f mean)	5.8 (20.2)	3.5 (11.2)	0.334

5. Discussion

P. falciparum resistant malaria is a major problem in the North, West and South of Thailand. The spread of drug resistance to CQ, MQ, SP and ATS have been widely reported, especially along the Thai-Myanmar, Thai-Cambodian and Thai-Malaysia borders (Carrara et al., 2013; WHO 2010; Wiwanitkit, 2002; Wongsrichanalai et al., 2002; Wongsrichanalai & Meshnick., 2008). Drug resistance in Thailand has been found to fluctuate, and surveillance and monitoring of drug resistant *Plasmodium* is needed in order to design effective policies and treatment plans.

Pfcrt K76T and *Pfmdr1* N86Y polymorphisms have been found to be potential molecular markers for CQ and other drug resistant *P. falciparum*. Many studies have confirmed that *Pfcrt* K76T mutation is strongly associated with CQ resistant *P. falciparum.* Previous reports have detected a correlation of CQ resistance with the *Pfcrt* K76T mutation in several areas. A study of *P. falciparum* isolates from the Thai-Myanmar border area indicated an association between CQ resistance and the *Pfcrt* 76T polymorphism (Hatabu et al., 2005). Results in Southeastern Iran projected a strong association between *Pfcrt* 76T mutation and in vivo CQ resistance from malaria infected patients (Zakeri et al., 2008). All CQ resistant isolates in malaria endemic states of Assam and Arunachal Pradesh, Northeast India also showed the presence of

the *Pfcrt* 76T mutation (Shrivastava et al., 2014). Moreover, *Pfcrt* 76T mutation has also been found to be related with AQ resistance (Folarin et al., 2011).

We found that *P. falciparum* carried *Pfcrt* K76T allele in all isolates (100%) from the three (north, west, and south) studied areas. These imply that *P. falciparum* in Thailand is still resistant to CQ, though CQ has not been used for treating falciparum malaria in the past decades. Our findings contradict some reports that found that the frequency of *Pfcrt* K76T mutation declined after CQ was no longer used as a therapeutic drug (Kublin et al., 2003; Mita et al., 2003). However, our results correspond with a report on field *P. falciparum* isolates in the Yunnan province of China, which found that 90.3% of the parasites still carried the *Pfcrt* K76T mutation, despite the termination of CQ from falciparum malaria therapy (Yang et al., 2007).

The high prevalence of *Pfcrt* 76T mutant in our study might be because the majority of parasite isolates were derived from the area near the Thai-Myanmar border. A high level of CQ resistance and multidrug resistant malaria is found in this region (WHO, 2010). In addition, asymptomatic malaria infections among migrant workers in Thailand have been reported (Kritsiriwuthinan & Ngrenngarmlert). Large people movements along the Thai-Myanmar border and the Thai-Cambodian border may enhance transmission of drug resistant *Plasmodium* in Thailand (Khamsiriwatchara et al., 2011; Tipmontree et al., 2009; Wangroongsarb et al., 2012; Wiwanitkit, 2002). Furthermore, a high prevalence of *P. vivax* and mixed infections have also been reported on the Thai-Myanmar border (Konchom et al, 2003; Zhou et al, 2005), and the use of CQ for *P. vivax* infected patients that may have mixed infection with *P. falciparum*, could exert selective pressure for a CQ *P. falciparum* resistant strain.

Our high prevalence of *Pfcrt* 76T *P. falciparum* mutant supports previous studies in Thailand. Congpuong et al. (2005) found that 100% of *P. falciparum* isolates from malaria endemic areas carried the mutant allele 76T of the *Pfcrt* gene. Rungsihirunrat et al. (2009) found that 99.1% of *P. falciparum* isolates from various endemic areas of Thailand throughout 2002-2004 carried the *Pfcrt* 76T allele. Muhamad et al. (2013) reported a 100% frequency of *Pfcrt* 76T *P. falciparum* isolates along the Thai-Myanmar border. Moreover, our survey shows concordance with a recent study that demonstrated increased CQ resistant *P. falciparum* in Thai-Myanmar border provinces, Tak and Kanchanaburi, where also included in this study (Parker et al., 2012).

With regards to *Pfmdr1* mutation, out of the several codons in *Pfmdr1* gene mutations described, the mutation in codon 86 (*Pfmdr1* N86Y) appears to be the most important position that is correlated to *P. falciparum* response to antimalarial drugs. Some reports indicate that *Pfmdr1* N86Y mutant is associated with CQ resistance. Some groups suggest that the mutation is related with multi-drug-resistant *P. falciparum*. Several studies have shown that the *Pfmdr1* gene copy number could be used as a predictor of some drug resistant *P. falciparum.* However, other studies have shown conflicting results on the association between polymorphisms in the *falciparum* drug susceptibility.

Previous studies have suggested an association between N86Y mutation and resistance to CQ (Andriantsoanirina et al., 2010). A project was recently carried out to examine *P. falciparum* isolates in northeast India in line of the relation of N86Y mutations with CQ resistance (in vitro). They found all CQ resistant isolates displayed the incidence of *Pfmdr1* N86Y mutations (Shrivastava et al., 2014).

In addition to the relation of *Pfmdr1* N86Y mutation to CQ resistance, there are reports that have described the association of *Pfmdr1* N86Y mutation with AQ resistant parasites. Research conducted by Holmgren et al. (2006) indicated that in vivo AQ resistant *P. falciparum* malaria was associated with selection of *Pfmdr1* 86Y. Similarly, an observation from Nigeria exhibited decreasing susceptibility of *P. falciparum* parasites to AQ in *Pfmdr1* N86Y polymorphism (Folarin et al., 2011).

An association between *Pfmdr1* and QN resistance has also been reported. A report done by Poyomtip et al. (2012) found significant modulate associations between the *Pfmdr1* mutations at codons N86Y and N1042D and in vitro QN susceptibility in Thai isolates of from the Thai-Myanmar and Thai-Cambodia borders. Similar to Cheruiyot et al. (2014) report, they demonstrated significant correlations between polymorphism in *Pfmdr1* 86Y, *Pfmdr1*-184F, or *Pfcrt*-76T and reduced in vitro QN activities of *P. falciparum* isolates from western Kenya.

Moreover, data supports the idea that the *Pfmdr1* gene is involved in the response to QN, LF and artemisinin derivatives (Duraisingh et al., 2000;

Mungthin et al., 2010a; Poyomtip et al., 2012). The associations of *Pfmdr1* N86 wild allele with decreased sensitivity of *P. falciparum* to MQ, AQ, QN and artemisinin have been reported (Duraisingh et al., 2000; Sidhu, Valderramos, & Fidock, 2005). Additionally, reduced susceptibilities to MQ, QN and AS in isolates with increased *Pfmdr1* copy numbers have been described (Duraisingh, & Cowman, 2005; Phompradit et al., 2011; Price et al., 2004).

In this study, the overall frequency of *Pfmdr1* 86Y mutant genotype was 52.4% among field isolates from the three regions. The highest prevalence was found in the north area (69.2%), and particularly high frequencies of *Pfmdr1* mutant was found in Mae Hong Son (100%) and Tak (75%) provinces. Fifty per cent (50%) *Pfmdr1* 86Y was found in Kanchanaburi; west isolates. The lowest *Pfmdr1* 86Y mutation frequency (47.8%) was found in the southern part of Thailand. The distribution of mutant is comparable with the GM of the parasite densities in each region. The GM of parasitemia found in the Northern part (170.5×10^4) was significantly higher than that in west (2.4×10^4), and south (2.3×10^4) ($p=0.004$). The results may imply more severe falciparum malaria exists in the north than other areas. In addition, the results demonstrated that *Pfmdr1* 86Y mutant isolates had higher parasitemia than wild type isolates. This implies that the severity of the parasite might be associated with the *Pfmdr1* 86Y mutation. However, there was no significant difference between parasitemia of mutant and wild type isolates tested by t-test ($p>0.05$).

The prevalence of *Pfmdr1* 86Y mutant in this study (52.4%) appears to be higher than previous studies in Thailand. Mungthin et al. (2010b) found *Pfmdr1* 86Y prevalence of 9% from *P. falciparum* isolates collected between the years 1988 to 2003. A report done by Rungsihirunrat et al. (2009) indicated that *Pfmdr1* 86Y mutant alleles were found in 20% of field isolates from various endemic areas of Thailand between the years 2002-2004. This is similar to a report by Chaijaroenkul, Wisedpanichkij, and Na-Bangchang (2010), which found low *Pfmdr1* 86Y mutant prevalence of only 7.7% *P. falciparum* isolates from Thai-Myanmar border. However, a high prevalence of *Pfmdr1* 86Y allele has been reported. Hatabu et al. (2005) reported that there were presented a high prevalence of *Pfmdr1* 86Y (95%) in parasites isolated from Thai-Myanmar border. This is similar to a report done by Mungthin et al. (2014) which presented a high *Pfmdr1* 86Y allele (96.3%) of *P. falciparum* isolates collected from Yala, Narathiwas and Songkhla, three provinces along the Thai-Malaysia border. The different frequencies of *Pfmdr1* 86Y genotypes might be due to the differences in study locations, drug pressures, and study duration. These variations encourage further studies to clarify the prevalence of *Pfmdr1* N86Y and its association to drug resistant malaria. This data would be a very useful molecular marker during surveillance.

The full picture of resistant *P. falciparum* in this study could not be concluded using *Pfmdr1* 86Y, due to the dissimilarity of the relation of *Pfmdr1* 86Y mutation to drug resistant *falciparum* malaria. However, based on evidence found in this study, the high frequency of *Pfmdr1* 86Y (52.4%) isolates from all sites may indicate that *P. falciparum* isolates in the studied areas would be resistant to CQ and/or other antimalarial drugs. Moreover, the 52.4% of *P. falciparum* observed in our study carrying both *Pfcrt* 76T and *Pfmdr*1 86Y mutation genes, implies more CQ resistance, and have been shown to increase the risk of *in vivo* resistance to chloroquine as previously described (Babiker et al., 2001; Picot et al., 2009).

6. Conclusion

The findings of a very high prevalence of *Pfcrt* K76T mutation (100%) and a high *Pfmdr1* 86Y mutation (52.4%) found in *P. falciparum* isolated from three regions of Thailand are alarming. These imply that there is still a high prevalence of CQ resistant parasites in these areas. Approximately half, or 52.4% of isolates were found to carry both *Pfmdr1* 86Y+*Pfcrt* 76T mutant, which may suggest a high level CQ resistance of the parasites. In addition, a high prevalence of *Pfmdr1* 86Y mutant and its different distribution suggests increasing CQ and/or other drugs resistance that should be studied further. Our data will be useful in the continued mapping of the epidemiology of *P. falciparum* drug resistance, and can potentially be used for selecting appropriate antimalarial drug treatments and aiding drug resistance surveillance and control programs.

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