IN VITRO SUSCEPTIBILITY AND GENETIC VARIATIONS FOR CHLOROQUINE AND MEFLOQUINE IN *PLASMODIUM FALCIPARUM* ISOLATES FROM THAI-MYANMAR BORDER

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Abstract. *In vitro* drug susceptibility to chloroquine (CQ) and mefloquine (MF) were assessed in 39 *P. falciparum* isolates from the Thai-Myanmar border area. To further characterize CQ- and MF-resistance profiles in this area, we also analyzed *pfcrt* K76T mutation that is critical for CQ resistance, and *pfmdr1* polymorphism that has an association with MF resistance. Eighteen isolates were successfully examined by *in vitro* tests for CQ, and 17 of them had resistance to the drug. Geometric mean concentration of CQ that inhibited the growth of parasites at 50% (IC_{50}) was 371 ± 227 nM (105-971 nM). Sixteen isolates were successfully examined by *in vitro* tests for MF, and 8 of them were resistant to the drug. Geometric mean of IC_{50} for MF was 41 ± 31 nM (4-125 nM). Genotypes of drug resistance, such as *pfcrt* and *pfmdr1* mutations, were also analyzed. All the 39 isolates had the same haplotype (CVIET) for PfCRT at its 72-76th amino acids. A *pfmdr1* Y86 mutation was found in 95% of isolates. A *pfmdr1* D1042 mutation was also present in 7 isolates, while no *pfmdr1* Y1246 mutation was observed. These results indicated a correlation between CQ resistance and the *pfcrt* T76 and *pfmdr1* Y86 mutations.

INTRODUCTION

The emergence of drug-resistant falciparum malaria is a serious threat to tropical countries. Chloroquine (CQ)-resistant *P. falciparum* was first reported in Southeast Asia in the 1950s and has since become widespread in this region (Looareesuwan and Chongsuphajaisiddlu, 1994; White, 1998; Breman, 2001). Recently, multi-drug resistant falciparum malaria has also become widespread in Southeast Asia, especially in the Thai-Myanmar border areas (Nosten *et al*, 1991; Peters, 1998); clinical efficacy of a number of drugs has been rapidly decreasing. Surveillance for drug-resistant malaria is based on strict *in vivo* criteria for treatment failure and on measurement of the *in vitro* susceptibilities of cultured parasites to antimalarials.

More recently, pathogen genotyping has proven to be useful in assessing resistance to some antimalarial drugs. Molecular methods, such as direct sequencing or restriction fragment-length polymorphism (RFLP) analysis, are currently used (Decuypere *et al*, 2003). Over the past two decades of using the polymerase chain reaction (PCR), numerous molecular markers for drug resistance of falciparum malaria parasites were described (Wongsrichanalai *et al*, 2002). Several genes attracted interest in the quest to elucidate polymorphisms related to antimalarial resistance and that could serve as specific molecular markers. There is consistent evidence that mutations in *pfcrt* (especially at position 76) correlate with *in vitro* and *in vivo* resistance of the parasite to CQ. There is also evidence that mutations in *pfmdr1* are associated with drug resistance (Wongsrichanalai *et al*, 2002). In this study, we investigated the *in vitro* drug susceptibility of CQ and mefloquine (MF), and *pfcrt* and *pfmdr1* mutations of *P. falciparum* isolates from 4 areas on the Thai-Myanmar border. This paper addressed *in vitro* drug resistance and concomitant gene mutations of parasite in the area where drug resistant malaria was reported to be highly endemic.

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MATERIALS AND METHODS

Study samples

The study was conducted at the Hospital for Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok. Isolates of *P. falciparum* were obtained from 39 symptomatic patients admitted to the hospital. These patients had been residing in four different areas of Thailand where malaria was highly endemic: Suan Phueng (western part of central region), Kanchanaburi (western part of central region), Mae Hong Son (northern region), and Tak (northern region) (Fig 1). After confirmation of

the diagnosis, venous blood was collected from each patient in a tube coated with EDTA (Venoject vacuum tube, Terumo, Tokyo, Japan). The patients' guardians gave written consent to this study that was approved by the Ethics Committee of Mahidol University, and we enrolled them. This survey research also followed the ethical guidelines for epidemiological studies set down by the Japanese Ministry of Education, Culture, Sports, Science and Technology; and Ministry of Health, Labor and Welfare.

In vitro drug susceptibility test

The *in vitro* drug susceptibility test used in this study was a modified semi-micro test described previously (Hatabu *et al*, 2003). Briefly, blood samples were washed three times with RPMI 1640 and re-suspended in RPMI 1640 (GIBCO BRL), pH 7.4, supplemented with 10% human serum (from non-immune Japanese donors without a previous history of malaria), and 25 μg/ml gentamicin (Sigma), 25 mM HEPES, and sodium bicarbonate, at a hematocrit of 5%. Five hundred microliters of the erythrocyte suspension were placed in each well of a tissue culture plate (24-well flat bottom, Corning Costar, New York, NY). Twenty microliters of chloroquine diphosphate or MF was added to each well (for CQ to create a series of 2 × dilutions, from 20 to 10,240 nM; and for MF to create a series of 10 × dilutions, from 0.01 to 1,000 nM). To monitor parasite growth, six wells per plate served as controls without antimalarials. Cultivation of parasites was done using the AnaeroPack® system (Mizuno *et al*, 2000). The AnaeroPack® CO_2 (Mitsubishi Gas, Tokyo, Japan) is a foil-packed paper sachet that, on exposure to air, immediately absorbs atmospheric O_2 and simultaneously generates CO_2 until a condition of 15% O_2 and 5% CO_2 is attained. The microaerophilic atmosphere produced within a sealed jar (AnaeroPack® Kakugata jar, SUGIYAMA-GEN, Tokyo, Japan) can be maintained for at least 24 hours. A portable thermostat incubator (SUGIYAMA-GEN) was carried to the laboratory, and the temperature inside the incubator was adjusted to 37°C. During *P. falciparum* cultivation, the sachet inside the jar was replaced with a new sachet every day when the culture medium was changed. When the schizonts were fully grown in the control wells, the culture plate was removed from the incubator. Thin-smear specimens, stained with Giemsa solution, were made from each well. We first counted the number of erythrocytes microscopically in the control smears until we encountered 50 schizonts. The effect of antimalarials on parasite growth was evaluated by observing the decreased number of schizonts per equal number of erythrocytes counted previously in the control cultures. The percentage of growth inhibition effect was calculated as follows: test well schizont count/control well schizont count (50) × 100.

DNA extraction

Fresh venous blood (0.5 ml) was blotted onto filter paper (Watmann) and dried. The filter paper was kept at room temperature until use. DNA was extracted from blots according to the method of Sakihama *et al*, (2001). Immediately, the dried filter paper was cut into 2 × 2 mm pieces and put into 1.5 ml tubes. Each blotted paper was incubated in 1 ml of Hepes-buffered saline (HBS), containing 0.5% (w/v) saponin (Sigma-Aldrich, St Louis, MO), at room temperature for 90 minutes, and washed twice with 1 ml of HBS. DNA remaining on the filter paper was isolated using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacture's instruction. The eluted DNA was stored at 4°C until use.

Polymerase chain reaction (PCR)

The PCR analysis was performed using published methods for *pfcrt* (Lopes *et al*, 2002) and *pfmdr1* (Contreras *et al*, 2002). DNA fragments were amplified by PCR in 25-μl reaction mixture containing

Fig 1- A map of Thailand showing (black circles) sampling areas.

2.0 μM of each primer, 250 μM dNTPs, 10 × PCR buffer (Invitrogen), 2.5 mM $MgCl_2$, and 1.0 units of Taq polymerase (Invitrogen). All the primers used in sequences and concomitant PCR conditions are shown in Table 1.

Restriction fragment length polymorphism and nucleotide sequencing of *pfcrt*

Restriction enzyme digestions of *pfmdr1* PCR products were carried out as previously described using the restriction enzymes *Afl* III (New England Biolabs, Beverly, MA), *Bgl* II (TAKARA Bio, Shiga, Japan), and *Vsp* I (MBI Fementas, Vilnius, Lithuania). The enzyme *Apo* I (New England Biolabs) was used to digest *pfcrt* PCR products. All fragments were subjected to gel electrophoresis on 2.0% agarose gels containing 0.5 μg/ml ethidium bromide.

Direct sequencing of codons 72 to 76 of *pfcrt* was attempted for all samples. The 206 bp PCR products were purified using QIAquick™ PCR purification system (QIAGEN) and sequenced by a BigDye™ Terminator Cycle Sequencing Ready Reaction kit (PE Biosystems) and the specific primers.

Data analysis

The drug concentration inhibiting parasite growth by 50% (IC_{50}) was calculated by the probit method (Inaba *et al*, 2001). Isolates with IC_{50} values for CQ more than 114 nM were regarded as resistant (Inaba *et al*, 2001). The threshold of the IC_{50} value for MF resistance was considered to be 40 nM (Price *et al*, 1999). Non-normally distributed data were described by median, range, and interquartile range (IQR); comparisons were made by Mann-Whitney *U* test. IC_{50} comparisons were made using Student's *t*-test or Welch's *t*-test. Statistical significance was assumed if the p-value was < 0.05.

RESULTS

***In vitro* drug susceptibility**

In total, 39 samples were analyzed for their *in vitro* drug susceptibility to CQ and MF. Eighteen of 39 samples were successfully examined for CQ. The geometric mean concentration of CQ that inhibited the growth of parasites at 50% (IC_{50}) was 371 ± 227 nM (105-971 nM). One of the 18 isolates was susceptible to CQ, and the IC_{50} value of this isolate was 105 nM. The highest IC_{50} to CQ (971 nM) was recorded for an isolate from Kanchanaburi. Isolates from the western part of the central region (Kanchanaburi and Suan Phueng) showed various IC_{50} values in the range between 105-971 nM, with a geometric mean (±SD) of 375 (±220) nM. Isolates from the northern region (Tak and Mae Hong Song) had IC_{50} values of 149-706 nM, with a geometric mean (±SD) of 352 (±236) nM. The differences between IC_{50} values of these areas were not significant.

Sixteen of 39 samples were also successfully examined for MF. Eight of 16 isolates (50%) were

Table 1
Polymerase chain reaction for amplification of fragments containing *pfcrt* and *pfmdr1* gene polymorphisms.

Primer	Sequence (5´ to 3´)	PCR program
pfcrt 76		
TCR-PA	ATGGCTCACGTTTAGGTGGAG	92°C, 30 sec; 45°C, 30 sec; 65°C, 45 sec;
TCR-P2	CGGATGTTACAAAACTATAGT	45 cycles
pfmdr1 86		
MDR-A	TTGAACAAAAAAGAGTACCGCTG	92°C, 30 sec; 45°C, 30 sec; 65°C, 45 sec;
MDR-B	TCGTACCAATTCCTGAACTCAC	45 cycles
pfmdr1 1042		
1042F	TATGTCAAGCGGAGTTTTTGC	94°C, 30 sec; 50°C, 30 sec; 68°C, 60 sec;
1042R	TCTGAATCTCCTTTTAAGGAC	45 cycles
pfmdr1 1246		
1246A	ATGACAAATTTTCAAGATTA	92°C, 30 sec; 45°C, 30 sec; 65°C, 45 sec;
1246B	ACTAACACGTTTAACATCTT	45 cycles

susceptible to MF. The geometric mean of the IC_{50} values was 41 ± 31 nM (4-125 nM). Six of 10 isolates (60%) from Kanchanaburi were MF-resistant. These isolates had IC_{50} values 10-125 nM, with a geometric mean (±SD) of 47 (±36) nM. Isolates from western and northwestern areas had IC_{50} values of 4-125 and 14-67 nM, with a geometric mean (±SD) of 41 (±32) and 41 (±27) nM, respectively. The statistical difference among IC_{50} values of these areas was not significant, either.

Of 16 CQ-resistant isolates, 8 (50%) were also resistant to MF. There was a significant positive correlation between the IC_{50} values of CQ and those of MF (r^2 = 0.848, $p < 0.0001$) (Fig 2).

pfcrt and *pfmdr1* polymorphisms in the isolates

pfcrt and *pfmdr1* polymorphisms were examined in 39 *P. falciparum*-positive samples (Fig 3). Mutations in both *pfcrt* and *pfmdr1* were quite common. RFLP analysis detected the presence of the mutant K76T allele in all samples. The CVIET sequence of codons 72 to 76 was found in all the 39 samples. N86Y and N1042D were present in 72% and 18% of the samples, respectively, while no polymorphism was found at position 1246.

Correlation between *pfcrt* and *pfmdr1* polymorphisms and *in vitro* antimalarial susceptibility

The prevalence of *pfmdr1* and *pfcrt* mutations in isolates that were successfully tested for drug susceptibility is presented in Table 2. The *pfcrt* K76T mutation was detected in all isolates. The N86Y mutation and mixed polymorphism (N86 and Y86) in the *pfmdr1* gene were also detected in 78% (14 of 18) and 22% (4 of 18) of isolates, respectively. The N1042D mutation was detected 28% (5 of 18) of isolates. However, a correlation between drug resistance and these mutations in *pfmdr1* was not found (Fig 4).

DISCUSSION

In Thailand, malaria continues to be a major public health problem due to the emergence of multidrug-resistant parasites. An increasing prevalence of *P. falciparum* resistance to CQ and MF have been reported. Our *in vitro* observations found CQ- and MF-resistant isolates to have been 94% and 50%, respectively, which indicated an alarmingly high prevalence of multidrug-resistant falciparum malaria on the Thai-Myanmar border.

Several studies have reported that the susceptibility of *P. falciparum* to CQ increased after the use of antimalarial was stopped for several years (Mita *et al*, 2003). Although mono-therapy with either CQ or MF for uncomplicated falciparum malaria has ceased for the past decades in Thailand, our results indicated that highly CQ-resistant falciparum malaria parasites were still prevalent in the present study areas. On Thai-Myanmar border, *P. vivax* is also highly prevalent, and CQ or CQ + primaquine is commonly used for the treatment of vivax malaria (Pukrittayakamee *et al*,

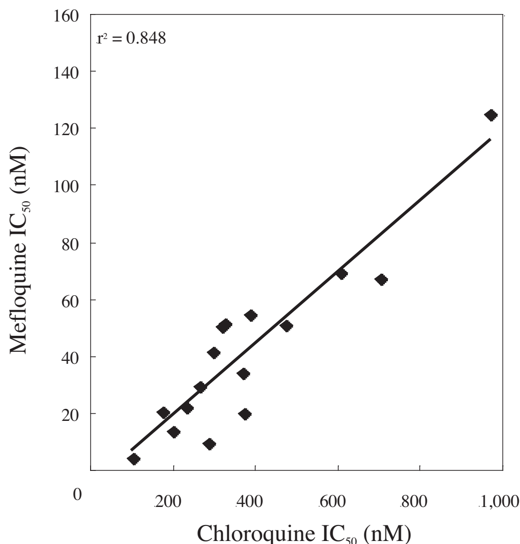

Fig 2- Linear regression analysis of the relationship between the IC_{50} values of chloroquine and mefloquine.

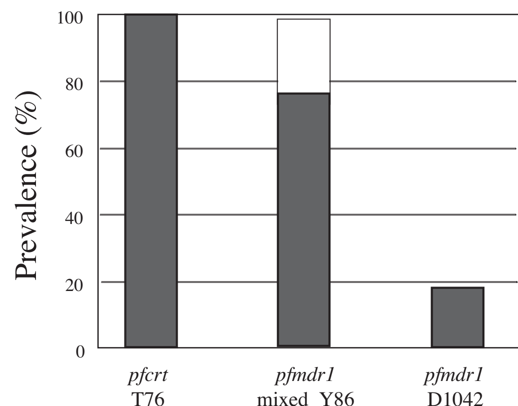

Fig 3- Prevalence of specific point mutations in *pfcrt* and *pfmdr1*. No mutations were observed at *pfmdr1* position 1246 (data not shown). Closed column indicated mutation in *pfcrt* and *pfmdr1*; open column indicated mixed polymorphism.

Table 2
pfcrt and *pfmdr1* mutations associated with chloroquine or mefloquine response *in vitro*.

Allele		Chloroquine *in vitro* (n=18)			Mefloquine *in vitro* (n=16)		
		Susceptible	Resistant	Total	Susceptible	Resistant	Total
pfcrt 76	Mutant (T)	1	17	18	8	8	16
	Wild (K)	-	-	-	-	-	-
pfmdr1 86	Mutant (Y)	1	13	14	6	6	12
	Wild (N)	-	-	-	-	-	-
	Mixed (N/Y)	-	4	4	2	2	4
pfmdr1 1042	Mutant (D)	-	5	5	4	1	5
	Wild (N)	1	11	12	4	7	11
	Mixed (N/D)	-	-	-	-	-	-
pfmdr1 1246	Mutant (Y)	-	-	-	-	-	-
	Wild (D)	1	17	18	8	8	16
	Mixed (D/Y)	-	-	-	-	-	-

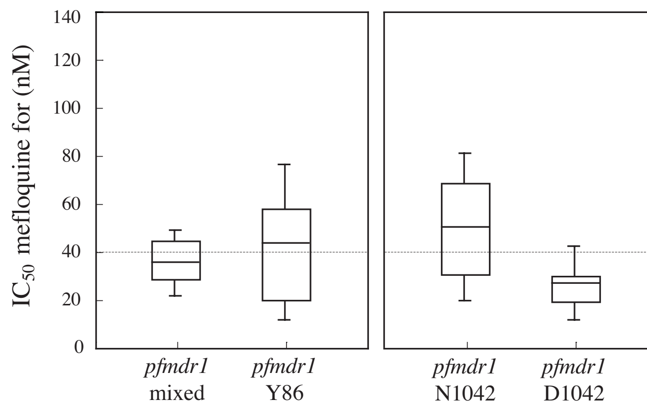

Fig 4- Box plots of median, range, and IQR according to *pfmdr1* mixed, Y86, *pfmdr1* N1042 and D1042 alleles.

2004; Konchom *et al*, 2003). Because a mixed infection of *P. falciparum/P. vivax* is commonly observed in the present study area, CQ used for *P. vivax* might be keeping pressure on the coexistent *P. falciparum*; thus affecting the recovery of CQ susceptibility.

The significant positive correlation between the IC_{50} values of CQ and MF was also observed in this study area, although several reports suggested an inverse relationship between CQ and MF resistance (Cowman *et al*, 1994; Ringwald *et al*, 1999). This may be attributed to dual drug pressures of CQ and MF in the highly endemic areas.

Although clinical study with adequate periods of patient follow-up is important for the assessment of optimal treatment of the drug resistant falciparum malaria, *in vivo* drug susceptibility testing is frequently difficult to implement. It is also difficult to perform parasite cultivation for *in vitro* drug susceptibility testing at hospitals or clinics in malaria endemic areas. In this situation, molecular genotyping of the isolates for an assessment of drug resistance can be an alternative means to indicate a need for a shift in antimalarial treatment regimens.

Recently, a strong association between mutant alleles of two genes (*pfcrt* and *pfmdr1*) and a high-level *in vitro* resistance to CQ in *P. falciparum* has been reported. Especially, a mutation in the *pfcrt* gene at

codon 76, with a change from lysine to threonine, has been invariably found in CQ-resistant laboratory strains and also in CQ-resistant field isolates from Southeast Asia, including Lao PDR, Thailand; and South America (Fidock *et al*, 2000; Wongsrichanalai *et al*, 2002). As several investigators have reported that the haplotype of *pfcrt* position 72-76 was CVIET in Thailand (Chen *et al*, 2001; Labbe *et al*, 2001), our results also indicated that all isolates tested in the present study area had a CVIET haplotype. On the other hand, point mutations in *pfmdr1*, especially at codon 86, have been known to associate with decreased CQ susceptibility (Duraisingh *et al*, 1997). In this study, 14 of 18 (78%) isolates that were successfully examined for CQ resistance, had a Y86 mutation (4 of these 18 isolates were mixed haplotype). Thirty-seven of 39 (95%) isolates that analyzed PCR-RFLP had Y86 mutation. These results suggested that there is a correlation between CQ resistance, and *pfcrt* T76 and *pfmdr1* Y86 mutations.

Mefloquine-resistant falciparum malaria has increased and presents a real threat to the control of malaria on the Thai-Myanmar border (Boudreau *et al*, 1982; Harinasuta *et al*, 1983). In this study, 8 of 16 (50%) isolates had MF-resistance and they also had the *pfmdr1* Y86 mutation. However, PCR-RFLP demonstrated that MF-susceptible isolates also had Y86 mutation; thus, the correlation between MF-resistance and *pfmdr1* mutations was not seen. Recently, several studies have reported that increased copy numbers of *pfmdr1* correlated with MF resistance (Pickard *et al*, 2003; Price *et al*, 1999, 2004). To understand more of the relationship between MF-resistance and *pfmdr1*, it may be necessary to assess the *pfmdr1* copy numbers.

In conclusion, highly CQ-resistant falciparum malaria parasites that have *pfcrt* CVIET haplotype were prevalent in Thai-Myanmar border areas. In addition, correlations between CQ resistance and mutations of *pfcrt* (T76) and *pfmdr1* (Y86) were observed. It is necessary to assess the new molecular techniques in the surveillance of antimalarial drug resistance in various epidemiological settings because the associations among *pfcrt* haplotype, *pfmdr1* copy numbers, and the levels of drug-resistance are still unclear. Further studies are also needed to clarify whether the drug susceptibility of *P. falciparum* might be influenced by the treatment measures against other human malaria parasites that are not falciparum malaria.

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