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▣ ORIGINAL ARTICLE

Molecular Markers for Sulfadoxine/Pyrimethamine and Chloroquine Resistance in *Plasmodium falciparum* in Thailand

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Abstract: Drug resistance is an important problem hindering malaria elimination in tropical areas. Point mutations in *Plasmodium falciparum* dihydrofolate reductase (*Pfdhfr*) and dihydropteroate synthase (*Pfdhps*) genes confer resistance to antifolate drug, sulfadoxine-pyrimethamine (SP) while *P. falciparum* chloroquine-resistant transporter (*Pfcrt*) genes caused resistance to chloroquine (CQ). Decline in *Pfdhfr*/*Pfdhps* and *Pfcrt* mutations after withdrawal of SP and CQ has been reported. The aim of present study was to investigate the prevalence of *Pfdhfr*, *Pfdhps*, and *Pfcrt* mutation from 2 endemic areas of Thailand. All of 200 blood samples collected from western area (Thai-Myanmar) and southern area (Thai-Malaysian) contained multiple mutations in *Pfdhfr* and *Pfdhps* genes. The most prevalent haplotypes for *Pfdhfr* and *Pfdhps* were quadruple and double mutations, respectively. The quadruple and triple mutations of *Pfdhfr* and *Pfdhps* were common in western samples, whereas low frequency of triple and double mutations was found in southern samples, respectively. The *Pfcrt* 76T mutation was present in all samples examined. Malaria isolated from 2 different endemic regions of Thailand had high mutation rates in the *Pfdhfr*, *Pfdhps*, and *Pfcrt* genes. These findings highlighted the fixation of mutant alleles causing resistance of SP and CQ in this area. It is necessary to monitor the re-emergence of SP and CQ sensitive parasites in this area.

Key words: *Plasmodium falciparum*, dihydropteroate synthase, dihydrofolate reductase, chloroquine-resistant transporter, molecular marker

INTRODUCTION

Although malaria is an ancient disease caused by *Plasmodium* parasite, it remains important to public health to present era. *Plasmodium falciparum* infection causes variable clinical symptoms ranging from asymptomatic to severe manifestations. The emergence of resistance of *P. falciparum* to the available antimalarial drugs is an important factor for malaria control [1]. In Thailand, resistance to many antimalarial drugs, including chloroquine (CQ), sulfadoxine-pyrimethamine (SP), mefloquine, and artemisinin has been reported [2,3]. CQ resistant *P. falciparum* was reported in the early 1960s [4,5]. In 1973, SP replaced CQ as the first-line treatment for uncomplicated falciparum malaria due to widespread resistance [1,6], but after 10 years, SP was ineffective [1,7]. Then, mefloquine was introduced in 1985 and resistance emerged in the same decade [8]. Artemisinin-based combination therapy was introduced as first-line treatment in 1995 [9].

Molecular epidemiological investigation provides information for detecting the emergence and spread of antimalarial drug resistance. Mutations in the *P. falciparum* dihydrofolate reductase (*Pfdhfr*) and *P. falciparum* dihydropteroate synthase (*Pfdhps*) genes (at codons 51, 59, 108, and 164 of *Pfdhfr* and 437, 540, and 581 of *Pfdhps*) are associated with SP treatment failures [10,11]. The mutations in *Pfdhfr* and *Pfdhps* genes were staged, resulting in increased levels of SP drug resistance [12]. The *P. falciparum* CQ resistance transporter gene (*Pfcrt*) K76T mutation has been linked to *P. falciparum* CQ resistance [13].

In some countries, the withdrawal of CQ for *P. falciparum* treatment, *Pfcrt* mutation (K76T) gently decreased and disappeared completely [14-17]. Similar to withdrawal of SP for *P. falciparum* treatment, *Pfdhfr* and *Pfdhps* gene mutations also decreased in some countries [18-21]. Conversely, alleles conferring CQ and SP resistance still occur at high frequency after discontinuation of these drugs [22,23]. However, declining of *Pfdhfr*, *Pfdhps*, and *Pfcrt* mutations might be associated with duration of drug withdrawal and geographical differences.

The objective of the present study was to investigate the

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prevalence of 5 *Pfdhfr* (A16V, N51I, C59R, S108N/T, and I164L), 5 *Pfdhps* (S436A, A437G, K540E, A581G, and A613S/T) and 1 *Pfcrt* (K76T) mutation from 2 different endemic areas of Thailand.

MATERIALS AND METHODS

Ethics approval

The study protocol was reviewed and approved by the Ethics Committee of Thammasat University (COA No. 134/2561). All patients were informed about the study objectives, sampling technique, and the benefits of the study. Informed consents were obtained according to the ethical standards from all patients.

Sample collection

A total of 200 dried blood spot samples were collected during 2007-2017 from patients with *P. falciparum* infection who attended malaria clinics in the western (Tak Province) and southern (Yala Province) regions along the Thai-Myanmar and Thai-Malaysian border, respectively.

Extraction of parasite genomic DNA

Genomic DNA of all blood samples was prepared using a QIAamp DNA extraction mini-kit (QIAGEN, Valencia, California, USA) according to manufacturer's instruction and used as a template for polymerase chain reaction (PCR) amplification.

Amplification and detection of the *Pfdhfr* and *Pfdhps*

Pfdhfr and *Pfdhps* genes were amplified by nested PCR using *Pfdhfr* and *Pfdhps* specific primers (Table 1) according to the previously described methods with some modification [24]. Briefly, the PCR was carried out with the following reaction mixture including 0.25 μM of each primer, 1.5 mM $MgCl_2$ (Thermo scientific, Waltham, Massachusetts, USA), 1×Taq buffered with KCl (Thermo scientific), 200 μM deoxynucleotides (dNTPs) (Bioline, London, UK), 2 μl of genomic DNA in the primary PCR, and 1 μl of primary PCR product in nested PCR and 1 unit of Taq DNA polymerase (Thermo scientific). All of the PCR products were then analyzed on 1% agarose gel and visualized under UV illuminator. PCR products were digested with restriction enzymes (Table 1) [24] then the restriction fragments were analyzed on 1.2% agarose gel and visualized under UV illuminator.

Table 1. The primers and enzymes for genotyping of *Pfdhfr*, *Pfdhps* and *Pfcrt* genes

Gene	PCR	Primer	Primer sequence (5' to 3')	RFLP position	Restriction enzyme	PCR size (bp)	Restriction product size (bp)	
							Wild type	Mutation
Pfdhfr	Primary	M1	TTTATGATGGAACAAGTCTGC					
		M5	AGTATATACATCGCTAACAGA					
	Secondary	M3	TTTATGATGGAACAAGTCTGCGACGTT	A16V	NlaIII	522	376, 93, 53	376, 146
	(16, 51, 108, 164)	F/	AAATTCTTGATAAACAACGGAACCTTTTA	N51I	MluCI		154, 120, 65, 55	218, 120, 65, 55
				S108T	BstNI		522	181, 145
				S108N	BsrI		522	332, 190
				I164L	DraI		245, 171, 107	245, 143, 107, 27
	Secondary	F	GAAATGTAATTCCCTAGATATGGAATATT	C59R	XmnI	326	189, 137	163, 137, 26
	(59)	M4	TTAATTTCCCAAGTAAAACTATTAGAGCTTC					
Pfdhps	Primary	R2	AACCTAAACGTGCTGTTCAA					
		R/	AATTGTGTGATTTGTCCACAA					
	Secondary	K	TGCTAGTGTTATAGATATAGGATGAGCATC	S436A	MnlI	438	317, 121	278, 121, 39
	(436, 437, 540)	K/	CTATAACGAGGTATTGCATTTAATGCAAGAA	A437G	AvaII		438	404, 34
				K540E	FokI		405, 33	320, 85, 33
	Secondary	L	ATAGGATACTATTTGATATTGGACCAGGATTCG	A581G	BslI	161	161	128, 33
	(581, 613)	L/	TATTACAACATTTTGATCATTCGCGCAACCGG	A613S	BsaWI		161	131, 30
				A613T	AgeI		161	128, 33
Pfcrt	Primary	CRTP1	CCGTTAATAATAAATACACGCAG					
		CRTP2	CGGATGTTACAAAACTATAGTTACC					
	Secondary	CRTD1	TGTGCTCATGTGTTTAAACTT	K76T	ApoI	134	100, 34	134
		CRTD2	CAAAACTATAGTTACCAATTTTG					

Amplification and detection of the *Pfcrt*

Amplification of K76T was performed by nested PCR using *Pfcrt* specific primers (Table 1) according to the previously described methods with some modification [25-27]. Briefly, the PCR was carried out with the following reaction mixture including 0.1 μM of each primer, 2.5 mM $MgCl_2$ (Thermo scientific), 1×Taq buffered with KCl (Thermo scientific), 100 μM deoxynucleotides (dNTPs) (Bioline), 0.5 μl of genomic DNA in the primary PCR, and 0.5 μl of primary PCR product in nested PCR and 0.5 unit of Taq DNA polymerase (Thermo scientific). All of the PCR products were then analyzed on 1.5% agarose gel and visualized under UV illuminator. PCR products were digested with restriction enzymes ApoI (New England Biolabs Inc., Hertfordshire, UK) (Table 1), as described by the manufacturer. Then the restriction fragments were analyzed on 2.0% agarose gel and visualized under UV illuminator.

Statistical Analysis

Data analysis was performed by SPSS software version 21.0

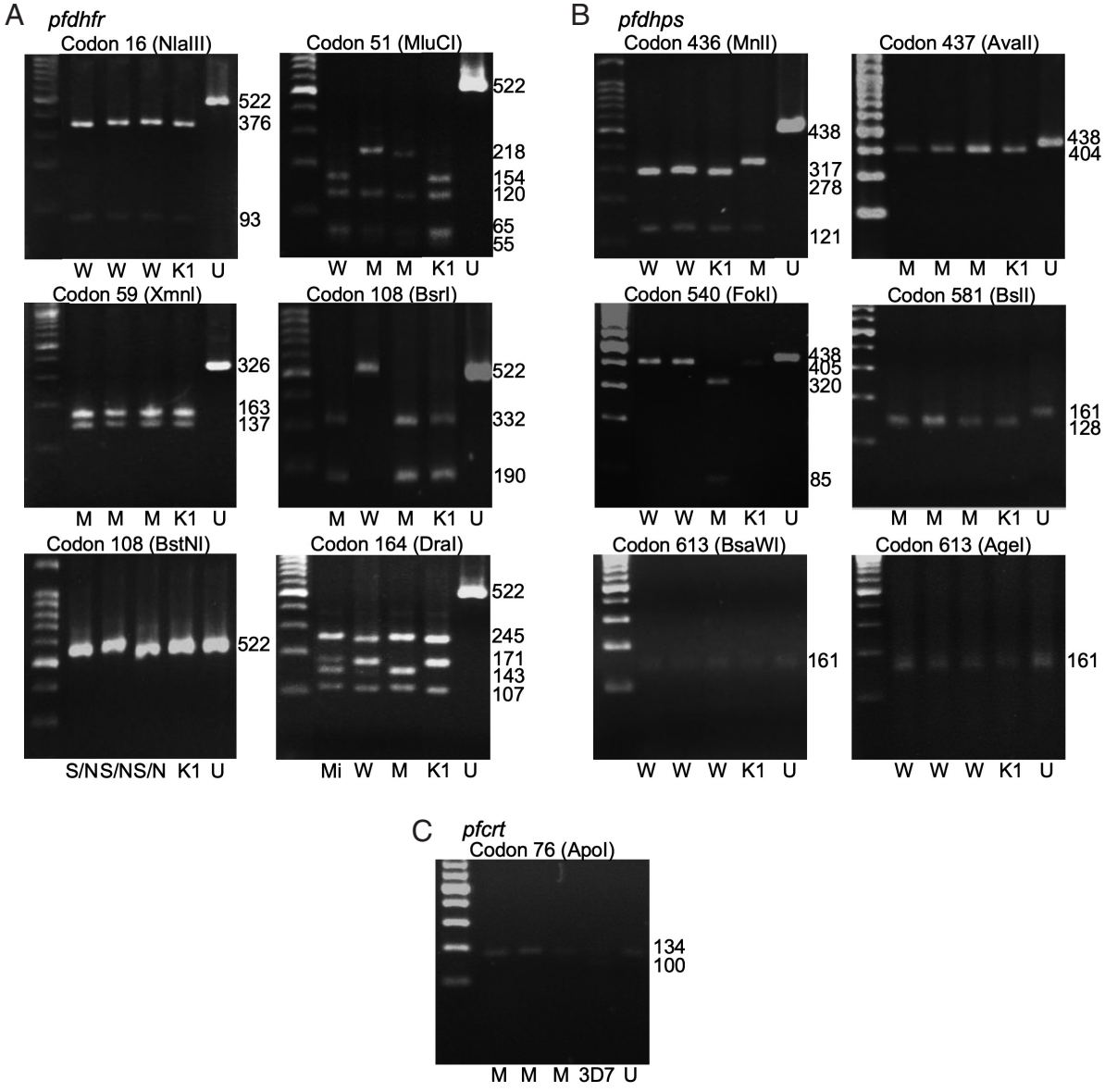

Fig. 1. The polymorphism of *pfdhfr* (A), *pfdhps* (B), and *pfcrt* (C) gene by gel electrophoresis. W-wildtype, M-mutant, S/N-serine/threonine, Mi-mixed, K1-*P. falciparum* K1 strain, 3D7- *P. falciparum* 3D7 strain, U-undigested fragment. Fragment sizes in base pair (bp) are shown.

Table 2. Prevalence of *Plasmodium falciparum* dihydrofolate reductase (*Pfdhfr*) and dihydropteroate synthase (*Pfdhps*) single nucleotide polymorphisms (SNPs) in 200 *P. falciparum* isolates from 2 endemic areas of Thailand

Gene	Amino acid position	SNPs	Prevalence (%)			*P*-value
			Total *n*=200	Tak Province *n*=100	Yala Province *n*=100	
Pfdhfr	16	A (wild-type)	200 (100.0)	100 (100.0)	100 (100.0)	-
		V (mutant)	0 (0.0)	0 (0.0)	0 (0.0)	
	51	N (wild-type)	3 (1.5)	3 (3.0)	0 (0.0)	0.001[a]
		I (mutant)	187 (93.5)	87 (87.0)	100 (100.0)	
		M (mix)	10 (5.0)	10 (10.0)	0 (0.0)	
	59	C (wild-type)	0 (0.0)	0 (0.0)	0 (0.0)	-
		R (mutant)	200 (100.0)	100 (100.0)	100 (100.0)	
	108	S (wild-type)	0 (0.0)	0 (0.0)	0 (0.0)	-
		T (mutant)	0 (0.0)	0 (0.0)	0 (0.0)	
		N (mutant)	200 (100.0)	100 (100.0)	100 (100.0)	
	164	I (wild-type)	84 (42.0)	6 (6.0)	78 (78.0)	<0.001[a]
		L (mutant)	103 (51.5)	86 (86.0)	17 (17.0)	
		M (mix)	13 (6.5)	8 (8.0)	5 (5.0)	
Pfdhps	436	S (wild-type)	158 (79.0)	79 (79.0)	79 (79.0)	0.946
		A (mutant)	29 (14.5)	14 (14.0)	15 (15.0)	
		M (mix)	13 (6.5)	7 (7.0)	6 (6.0)	
	437	A (wild-type)	0 (0.0)	0 (0.0)	0 (0.0)	-
		G (mutant)	200 (100.0)	100 (100.0)	100 (100.0)	
	540	K (wild-type)	116 (58.0)	16 (16.0)	100 (100.0)	<0.001[a]
		E (mutant)	83 (41.5)	83 (83.0)	0 (0.0)	
		M (mix)	1 (0.5)	1 (1.0)	0 (0.0)	
	581	A (wild-type)	7 (3.5)	7 (7.0)	0 (0.0)	0.007[a]
		G (mutant)	193 (96.5)	93 (93.0)	100 (100.0)	
	613	A (wild-type)	100 (100.0)	100 (100.0)	100 (100.0)	-
		S/T (mutant)	0 (0.0)	0 (0.0)	0 (0.0)	

[a]*P*-value were statistically significant between 2 areas.

Table 3. *Plasmodium falciparum* dihydrofolate reductase (*Pfdhfr*) and dihydropteroate synthase (*Pfdhps*) alleles in 170 *P. falciparum* isolates from 2 endemic areas of Thailand

Pfdhfr haplotypes[a]	Amino acid position					Prevalence (%)		
	16	51	59	108	164	Total *n*=170	Tak Province *n*=81	Yala Province *n*=89
Triple mutation	A	I	R	N	I	78 (45.9)	6 (7.4)	72 (80.9)
Triple mutation	A	N	R	N	L	3 (1.8)	3 (3.7)	0 (0.0)
Quadruple mutation	A	I	R	N	L	89 (52.4)	72 (88.9)	17 (19.1)
Pfdhps haplotypes[a]	Amino acid position					Prevalence (%)		
	436	437	540	581	613	Total *n*=170	Tak Province *n*=81	Yala Province *n*=89
Double mutation	S	G	E	A	A	2 (1.2)	2 (2.5)	0 (0.0)
Double mutation	S	G	K	G	A	85 (50.0)	11 (13.6)	74 (83.1)
Triple mutation	A	G	E	A	A	2 (1.2)	2 (2.5)	0 (0.0)
Triple mutation	A	G	K	G	A	16 (9.4)	1 (1.2)	15 (16.9)
Triple mutation	S	G	E	G	A	57 (33.5)	57 (70.4)	0 (0.0)
Quadruple mutation	A	G	E	G	A	8 (4.7)	8 (9.9)	0 (0.0)

[a]*P*-value were statistically significant between 2 areas.

(IBM Corporation, Armonk, New York, USA). The chi-square test was used to compare the frequencies and correlations of all data. The level of significance was set at $P<0.05$.

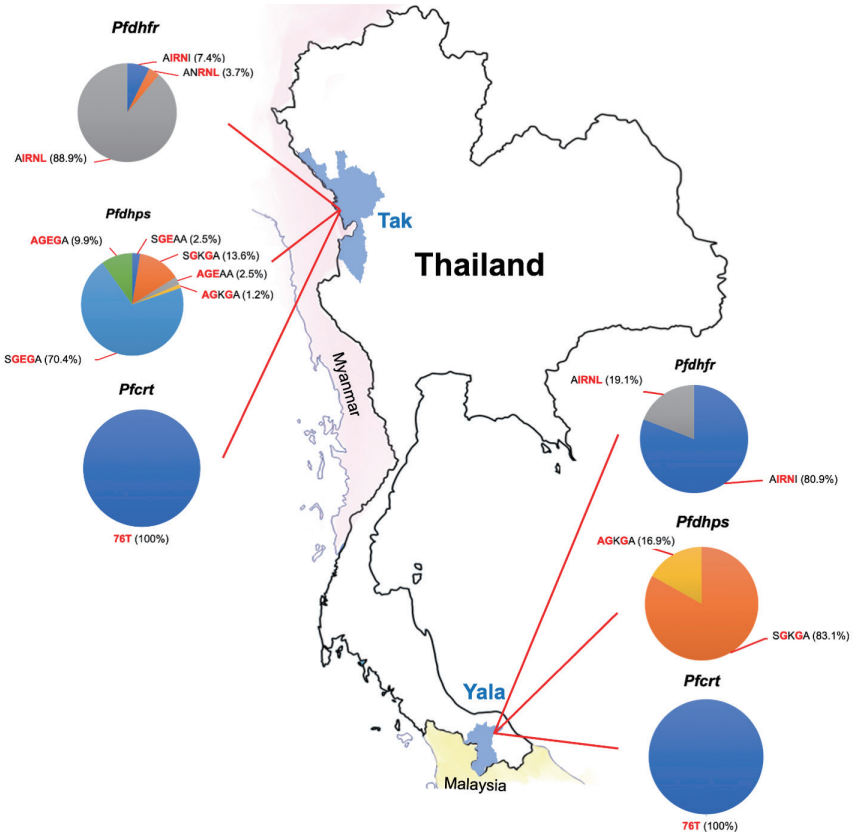

Fig. 2. The proportions of mutations in 3 resistance genes (*pfdhfr*, *pfdhps*, and *pfcrt*) observed in *P. falciparum* isolates in this study.

RESULTS

Analysis of *Pfdhfr* and *Pfdhps* mutations

A total of 200 *P. falciparum* samples were successfully amplified and analyzed for both the *Pfdhfr* and *Pfdhps* genes. The polymorphisms at each codon were demonstrated by restriction fragments (Fig. 1A, B). The frequencies of *Pfdhfr* and *Pfdhps* mutations was summarised in Table 2. All samples had at least 1 codon mutation in the *Pfdhfr* (A16V, N51I, C59R, S108N/T, and I164L) and *Pfdhps* (S436A, A437G, K540E, A581G, and A613S/T). Four codon mutations were detected in *Pfdhfr* (51I, 59R, 108N, and 164L) in samples from 2 areas. All isolates carried mutations at codon 59 and 108 in *Pfdhfr*. Four codon mutations were detected in *Pfdhps* (436A, 437G, 540E, and 581G) in samples from Tak Province, whereas 3 codon mutations were detected from Yala Province. All isolates carried wild-type alleles at codon 613 in *Pfdhps*. Mixed genotypes were detected in thirty isolates by codon 51 and 164 of *Pfdhfr*, and 436 and 540 of *Pfdhps* that were no processed further. There was no wildtype allele ANCSI, but 3 alleles (AIRNI, AIRNL, and ANRNL) of *Pfdhfr* were identified in this study (Table 3). AIRNL was the most prevalent allele (52.4%) in all *P. falciparum* isolates collected from Tak Province (88.9%) (Fig. 2). AIRNI was the most prevalent allele in *P. falciparum* isolates

Table 4. Allele combinations of *Plasmodium falciparum* dihydrofolate reductase (*Pfdhfr*) and dihydropteroate synthase (*Pfdhps*) in 170 *P. falciparum* isolates from 2 endemic areas of Thailand

Pfdhfr-Pfdhps allele combinations	Prevalence (%)		
	Total *n*=170	Tak Province *n*=81	Yala Province *n*=89
AIRNI-AGEGA	1 (0.6)	1 (1.2)	0 (0.0)
AIRNI-AGKGA	13 (7.6)	0 (0.0)	13 (14.6)
AIRNI-SGEGA	4 (2.4)	4 (4.9)	0 (0.0)
AIRNI-SGKGA	60 (35.3)	1 (1.2)	59 (66.3)
AIRNL-AGEGA	7 (4.1)	7 (8.6)	0 (0.0)
AIRNL-AGKGA	3 (1.8)	1 (1.2)	2 (2.2)
AIRNL-SGEAA	2 (1.2)	2 (2.5)	0 (0.0)
AIRNL-SGEGA	52 (30.6)	52 (64.2)	0 (0.0)
AIRNL-SGKGA	25 (14.7)	10 (12.3)	15 (16.9)
ANRNL-AGEAA	2 (1.2)	2 (2.5)	0 (0.0)
ANRNL-SGEGA	1 (0.6)	1 (1.2)	0 (0.0)

from Yala Province (80.9%). Statistical significance was found between 2 study areas ($P<0.001$). For *Pfdhps*, 6 alleles were identified (Table 3). SGKGA was the most prevalent allele (50.0%) found in *P. falciparum* isolates from Yala Province (83.1%). SGEGA was the most prevalent allele in *P. falciparum* isolates from Tak Province (70.4%). Statistical significance was found between 2 study areas ($P<0.001$). Eleven allele combination of *Pfdhfr-Pfdhps* were found in this study (Table 4). The quintuple mutation (AIRNI-SGKGA), which comprise triple mutations in *Pfdhfr* and 2 mutations in *Pfdhps* were found to be most prevalent in study population (35.3%) and in isolates from Yala Province (66.3%). The septuple mutation (AIRNL-SGEGA), which comprised quadruple mutations in *Pfdhfr* and 3 mutations in *Pfdhps* were most frequent in isolates from Tak Province (64.2%).

Analysis of *Pfcrt* mutations

A total of 187 samples (93.5%) were analyzed by nested PCR for the *Pfcrt* K76T gene. The *Pfcrt* mutation resulting in substitution of threonine (T) for lysine (K) at position 76 was present in all studied samples from 2 endemic areas (Figs. 1C, 2).

DISCUSSION

Mutations on *Pfdhfr* and *Pfdhps* genes associated with SP resistance have been reported in several malaria endemic areas such as Guinea [28], Indonesia [29], Malaysia [30], Myanmar [31], and Thailand [32,33]. In Thailand, a previous study revealed that the change of *Pfdhfr* point mutations from double mutations to triple and quadruple mutations in some areas [34,35] which the number of mutations is correlated with increased level of SP resistance [12]. In the present study, all *P. falciparum* isolates had at least three mutation point in *Pfdhfr* genes, indicated persistence of highly mutations on SP resistant markers. The *Pfdhps* mutation studies conducted between 2001 and 2007 in Thailand indicated that there has been fluctuation of the *Pfdhps* mutations between triple and quadruple mutations. In this study, predominant frequency of double mutation was found especially in isolates from southern endemic area. The predominance of triple mutations was also found in isolates from western area. This result indicated that high mutation in *Pfdhfr* and *Pfdhps* genes with different frequency existed in these 2 different localities. In Thailand, SP was withdrawn from *P. falciparum* treatment for many years. Although decreased of *Pfdhfr* and *Pfdhps* mutations were reported from some countries after the withdrawal of these drugs, high frequency of *Pfdhfr* and *Pfdhps* mutations still present in Thailand. This existence of mutations on *Pfdhfr* and *Pfdhps* genes may be associated with using of other antifolate drugs that can also induce pressure on *Pfdhfr* and *Pfdhps*.

A previous study has demonstrated that high prevalence of *Pfcrt* K76T mutation in study isolates might contribute to CQ resistance to *P. falciparum* [36]. A high prevalence rate of *Pfcrt* K76T mutation was previously observed in several countries such as Mali [25], Kenya [37], Indonesia [26], Philippines [38] and Thailand [39-41]. Declining of *Pfcrt* mutations after withdrawal of CQ has been reported in Malawi [14], Tanzania [15], Kenya [16], and China [17]. However, even CQ was withdrawn from Thailand for long period, the CQ resistance allele still remains with high frequency. This complete fixation of CQ resistance in *P. falciparum* is might due to the co-existence of *P. falciparum* and *P. vivax* infections in this country while CQ is a standard regimen for *P. vivax* malaria treatment, leading to the phenomenon of continuous exposure to drug pressure in *P. falciparum*.

Our study demonstrates a high prevalence of *Pfdhfr*, *Pfdhps*, and *Pfcrt* mutations of *P. falciparum* isolates from 2 endemic areas in Thailand, emphasizing the fixation of mutant *Pfdhfr*, *Pfdhps* and *Pfcrt* alleles that confer consistent resistance of SP and CQ. SP and CQ drugs are still not appropriate for *P. falciparum* treatment in Thailand and other antimalarial groups should be considered.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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