Original Article

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Molecular Analysis of Chloroquine and Sulfadoxine/Pyrimethamine Resistant Markers in Plasmodium Falciparum Isolated from Three Provinces in Southern Thailand

Autchariya Thodsapol, B.Sc.[1], Nongyao Sawangjaroen, Ph.D.[1], Waenurama Chemoh, Ph.D.[2], Mathirut Mungthin, M.D., Ph.D.[3], Hansuk Buncherd, Ph.D.[4], Supinya Thanapongpichat, Ph.D.[4]

[1]Division of Biological Science, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand.

[2]Department of Microbiology, Faculty of Medicine, Princess of Naradhiwas University, Narathiwat 96000, Thailand.

[3]Department of Parasitology, Phramongkutklao College of Medicine, Bangkok 10400, Thailand.

[4]Faculty of Medical Technology, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand.

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Abstract:

Objective: This study investigated mutations in various genes associated with chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) resistance in *P. falciparum*; including the *P. falciparum* chloroquine resistance transporter *(pfcrt)*, *P. falciparum* multidrug resistance 1 *(pfmdr1)*, *P. falciparum* dihydrofolate reductase *(pfdhfr)*, and *P. falciparum* dihydropteroate synthase *(pfdhps)*.

Material and Methods: A total of 104 *P. falciparum* samples were obtained from patients across three (Ranong, Surat Thani and Yala) provinces of southern Thailand; between 2012 and 2019. To assess the genetic polymorphisms, *pfcrt* K76T and *pfmdr1* N86Y were identified using PCR-RFLP assay, and *pfdhfr* C59R and *pfdhps* K540E was identified using Semi-nested PCR and nucleotide sequencing.

Results: Genetic analysis revealed that 61 (58.65%) isolates were positive for *pfcrt* and 55 (52.88%) for *pfmdr1*. Notably, the Ranong province isolates showed high prevalence of *pfdhfr* 51I, 59R, 108N, and 164L mutations (IRN-L) along with *pfdhps* 540E mutation. The Surat Thani province isolates exhibited the highest frequency of quadruple mutations in both *pfdhfr* and *pfdhps* genes.

Conclusion: The surveillance guidelines and policy formulation of appropriate Malaria treatment strategies must be implemented in these locations.

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Contact: Supinya Thanapongpichat, Ph.D.
Faculty of Medical Technology, Prince of Songkla University,
Hat Yai, Songkhla 90110, Thailand.
E-mail: supinya.th@psu.ac.th

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Introduction

Malaria remains a health issue that requires constant monitoring since it is life-threatening if not cured rapidly. The overall incidence of Malaria has diminished globally; however, Malaria cases in Thailand have increased from 3,266 in 2021 to 10,156 in 2022—an increase of 211%[1]. *Artemisinin-based combination therapies* are currently used for the treatment of uncomplicated falciparum Malaria in most endemic countries as front-line drugs of choice, such as artemether-lumefantrine, artesunate-amodiaquine, artesunate-mefloquine, artesunate-sulfadoxine-pyrimethamine and dihydroartemisinin-piperaquine[2]. Antimalarial drugs entering *P. falciparum* food vacuole are thought to be modulated by *P. falciparum* chloroquine (CQ) resistance transporter *(pfcrt)* and *P. falciparum* multidrug resistance 1 *(pfmdr1)* genes, which code for the transmembrane protein *pfcrt* and a P-glycoprotein homolog 1 respectively[3].

In Thailand, molecular markers of Malaria drug resistance have been reported, including CQ, sulfadoxine-pyrimethamine (SP), mefloquine and artemisinin. A mutation in the *pfcrt* gene that results in an amino acid substitution from lysine (K) to threonine (T) at position 76 has been identified as a critical determinant of CQ resistance among parasite isolates from many countries[4-8]. Also, a point mutation in the *pfmdr1* gene, resulting in the substitution of asparagine (N) to tyrosine (Y) at codon 86 (N86Y), has been implicated in conferring a high level of CQ resistance[9]. SP is an antimalarial drug that targets the folate metabolism of the Malaria parasite. However, documented failures of Malaria treatment had been reported[10-11]. Mutations in the target enzymes (DHFR and DHPS), which are vital for folate biosynthesis by the parasite are encoded by the *P. falciparum* dihydrofolate reductase *(pfdhfr)* and *P. falciparum* dihydropteroate synthase *(pfdhps)* genes, respectively. These mutations reduce the ability of DHFR and DHPS to bind with SP, leading to further occurrences of resistant *P. falciparum* isolates[12-13]. The amino acid change from serine (S) to N for codon *pfdhfr* 108 (S108N) was identified as a principal marker conferring pyrimethamine resistance *in vitro,* along with the conversion of isoleucine (I) to leucine (L) at position 164 (I164L) (Wernsdorfer and Noedl, 2003). The degree of pyrimethamine resistance often increases when other mutations are present at codons 51 (N51I) and/or 59 (C59R). Additionally, point mutations on the *pfdhps* gene at codons 437 (A437G) and 581 (A581G) confer resistance to sulphadoxine, while mutations S436A, K540E, and A613S decreased the sensitivity of the parasite to pyrimethamine[14-15].

The characterization of SNPs in various antimalarial drug-resistant genes could provide genetic epidemiological data on the drug resistance of *P. falciparum* that are important for tracking and preventing the spread of drug-resistant parasites. The polymorphisms in *pfcrt*, *pfmdr1*, *pfdhfr* and *pfdhps* have been identified in many studies in Thailand. The mutations in *pfdhfr* and *pfdhps* have been reported from Ubonratchathani province close the Thailand–Cambodia borders with high levels of *pfdhfr* and *pfdhp* (≥ 70% of samples)[16]. Genotyping of *pfcrt* gene from falciparum parasites circulating around northeastern Thailand close to the Thai-Cambodia border showed all mutant haplotypes at positions 72–76 (CVIET), which is associated with CQ resistance[17]. The *pfcrt* 76 T allele is ubiquitous across all regions of Thailand, whereas *pfmdr1* 86Y is more prevalent in the lower southern areas. Mutations

in the *PfKelch13* propeller region are usually associated with artemisinin resistance, particularly the C580Y and P574L mutations, which have been identified in many provinces including Chumphon, Ranong and Phang Nga in southern Thailand and Ubon Ratchathani in northeastern Thailand[16,18].

Therefore, we examined point mutations in the *pfcrt, pfmdr1, pfdhfr,* and *pfdhps* genes associated with CQ and SP resistance in *P. falciparum* isolates collected from patients in three Malaria-endemic regions in southern Thailand, which have not been previously described. These results would provide insights into the different predominance of four antimalarial drug resistance markers in *P. falciparum* field isolates. The present findings may also offer baseline data to establish an antimalarial drug policy and update guidelines for appropriate Malaria control in these regions.

Material and Methods

Blood collection and DNA extraction

Between 2012 and 2019, a total of 104 blood samples were collected from Malaria clinics at Ranong (n=74), Surat Thani (n=14) and Yala (n=16) provinces in southern Thailand. These samples were identified under the microscope as being infected by *P. falciparum*. The blood was spotted onto a Whatman No.3 filter paper. The genomic DNA of Malaria parasites was extracted from individual dried blood spots using the QIAamp DNA Extraction Mini Kit (QIAGEN, Hidden, Germany), following the manufacturer's guidelines. Malaria species in all samples were confirmed using a nested-PCR assay targeting on the *18sRNA* gene, following a previously reported method[19]. All participants gave written informed consent before the study, which was approved by the Human Ethics Review Committee for Research in Human Subjects, Research and Development Office, Prince of Songkla University (Hsc-HREC-61-002-02-1).

The PCR amplification of drug resistance markers in *P. falciparum* isolates

The amplification of the *pfcrt* and *pfmdr1* genes followed protocols described in previous studies[20-22] with minor adjustments. Briefly, the amplification was performed in a total volume of 20 µl using a T100 thermal cycler (Bio-Rad, California, USA). PCR reactions were carried out with 5X MyTaq reaction buffer, 0.25 µM of each forward and reverse primer, 1 unit of MyTaq DNA polymerase (Bioline, United Kingdom), 3 µl of extracted DNA and nuclease-free sterile water. For the amplification of the *pfcrt* gene, a nested-PCR technique was employed. The expected sizes of the PCR amplicons were 145 bp for *pfcrt* and 504 bp for *pfmdr1*, respectively.

The fragments of *pfdhfr* (499 bp) and *pfdhps* gene (682 bp) were amplified by nested PCR and semi-nested assay following a previously established protocol[23], with minor modifications. In brief, the primary PCR reactions for each gene were performed in 20 µl reaction volume with 0.2 µM primers, 0.75 unit (Bioline, United Kingdom) MyTaq DNA polymerase, and 2 µl genomic DNA. A one-microliter aliquot of the initial PCR product was transferred into the nested PCR reaction, which comprised the same reagents as the primary PCR reaction. *P. falciparum* laboratory parasite isolate 3D7, Dd2, K1 clone were used as a positive control in the PCR amplifications. All amplified PCR products were visualized by electrophoresis on 1.5–2% agarose gel stained with ethidium bromide.

Genotyping of *pfcrt* and *pfmdr1* genes by PCR-RFLP

Genotyping was conducted using PCR-RFLP method[21-22], with the *P. falciparum* 3D7 laboratory strain serving as the wild type of control. For the analysis of SNPs in the *pfcrt* gene and *pfmdr1* gene, Dd2 and K1 strains were used as mutant controls. Under a digestion

reaction with *XapI*-restriction endonucleases (Thermo Scientific FastDigest, MA), the wild-type allele of the *pfcrt* gene exhibited cleavage into two fragments, measuring 111 bp and 34 bp; unlike the cleavage pattern observed in the *pfmdr1* gene, where it produced fragments of 249 bp and 255 bp, indicative of the wild-type allele. Another independent amplicon was selectively sequenced to detect mutations in the targeted genes if RFLP results contained ambiguous patterns.

Mutation analysis of pfdhfr and pfdhps genes by DNA sequencing

The mutations of the *pfdhfr* (C59R) and *pfdhps* (K540E) genes were analyzed through DNA sequencing conducted by Macrogen Inc. (Seoul, South Korea). The sequences were assembled and aligned against the 3D7 *pfdhfr* and *pfdhps* reference sequences (GeneDB PF3D7_0417200, GeneDB PF3D7_0810800), converted to amino acid sequences and compared using BioEdit software.

Statistical analysis

Data were analyzed using IBM statistics package for social science version 26.0. The prevalence of genes associated with antimalarial resistance and polymorphisms was computed as frequencies and proportions. Differences in prevalence between sites in each drug-resistant gene of *P. falciparum* isolates were assessed using the Chi-square or Fisher's exact test when appropriate. A p-value of less than 0.05 was considered statistically significant.

Results

Polymorphisms in *pfcrt* and *pfmdr1* genes

All samples in this study were diagnosed as mono-*P. falciparum* infections by nested PCR. Among the 104 samples, 61 (58.6%) were positive and successfully analyzed for *pfcrt* polymorphisms at codon 76 (K or T allele) using PCR-RFLP assays. All alleles were mutant genotypes. Out of 55 samples positive for *pfmdr1* at codon 86 (N or Y alleles) from PCR-RFLP or DNA sequencing, 47 were successfully genotyped. From Ranong 6/29 samples (20.6%) harbored a *pfmdr1* 86Y mutation; from Surat Thani 2/7 (28.5%), and from Yala, 11/11 (100.0%). The prevalence of the *pfmdr1* 86Y mutation was, therefore, significantly higher in samples from Yala than in samples from Ranong and Surat Thani (p-value<0.001) (Table 1).

Analysis of *pfdhfr* and *pfdhps* mutations

A total of 32 samples out of 104 (30.7%) were positive for the *pfdhfr* gene and successfully genotyped. The mutation frequency of the four codons containing N51I, C59R, S108N and I164L in samples from each province is indicated in Table 2. From Ranong, all samples (26/26, 100.0%) harbored mutations at the N51I, C59R, and S108N codons and 20/26 (76.9%) harbored additional mutations at the I164L codon. From Surat Thani, 3/5 samples (60.0%) harbored mutations at codon N51I, 5/5 (100.0%) at C59R, 5/5 (100.0%) at S108N, and 2/5 (40.0%) at I164L. The mutant *pfdhfr* 51I was significantly more prevalent in the samples from Ranong and Yala than in Surat Thani (p-value=0.030). Although, the I164L mutant allele was not observed in samples from Yala.

Regarding the *pfdhps* gene, a total of 39 *P. falciparum* samples were successfully sequenced. The K540E mutation was found in samples from Ranong only (20/30, 66.6%). The A581G mutant allele was found in 8/30 samples (26.6%) from Ranong, 4/6 samples (66.6%) from Surat Thani and 3/3 samples (100.0%) from Yala. Quintuple mutations of 4 *pfdhfr* mutant alleles (N51I, C59R, S108N, and I164L) with 1 *pfdhps* genotype (K540E) were also found in 8 out of the 17 samples from Ranong (47.0%).

Table 1 Prevalence of *pfcrt* and *pfmdr1* mutations in 104 *P. falciparum* isolates from three provinces in southern Thailand, identified using PCR-RFLP or DNA sequencing

Gene	No. of PCR positive (%)	No. successfully genotyped	Haplotypes	No. of wild-type or mutant isolates/ total no. of isolates in each area positive with genotyping (%)				p-value
				Ranong	Surat Thani	Yala	Total	
pfcrt	61/104 (58.6)	61	K76	0/46 (0)	0/6 (0)	0/9 (0)	0/61 (0)	
			76T	46/46 (100.0)	6/6 (100)	9/9 (100.0)	61/61 (100)	
pfmdr1	55/104 (52.8)	47	N86	23/29 (79.3)	5/7 (71.43)	0/11 (0)	28/47 (59.5)	<0.001*
			86Y	6/29 (20.6)	2/7 (28.57)	11/11 (100.0)	19/47 (40.4)	

pfcrt=P. falciparum chloroquine resistance transporter, *pfmdr1=P. falciparum* multidrug resistance transporter I
Amino acid: K=lysine, T=threonine, N=asparagine, Y=tyrosine.
*Statistically significant differences between the three regions were evaluated by Fisher's exact test (p-value<0.05),

Table 2 The prevalence of pfdhps genes mutations and Combined mutations between *pfdhfr* and *pfdhps* genes in plasmodium falciparum found each year from 3 provinces in southern Thailand

Gene	No. of PCR positive (%)	No. successfully genotyped	Haplotypes	No. of wild-type or mutant isolates/ total no. of isolates in each area positive with genotyping (%)			
				Ranong	Surat Thani	Yala	Total
pfdhfr	32/104 (30.7)	32	N51	0/26 (0)	2/5 (40.0)	0/1 (0)	2/32 (6.3)
			51I	26/26 (100.0)	3/5 (60.0)	1/1 (100.0)	30/32 (93.8)
			59R	0/26 (0)	0/5 (0)	0/1 (0)	0/32 (0)
			S108	26/26 (100.0)	5/5 (100.0)	1/1 (100.0)	32/32 (100.0)
			108N	0/26 (0)	0/5 (0)	0/1 (0)	0/32 (0)
			I164	26/26 (100.0)	5/5 (100.0)	1/1 (100.0)	32/32 (100.0)
			164L	6/26 (23.1)	3/5 (60.0)	1/1 (100.0)	10/32 (31.3)
pfdhps	39/104 (37.5)	39	K540	20/26 (76.9)	2/5 (40.0)	0/1 (0)	22/32 (68.8)
			540E	10/30 (33.3)	0/6 (0)	0/3 (0)	10/39 (25.6)
			A581	20/30 (66.7)	6/6 (100.0)	3/3 (100.0)	29/39 (74.4)
			581G	22/30 (73.3)	2/6 (33.3)	0/3 (0)	24/39 (61.5)
pfdhfr/pfdhps	8/18 (44.4)	18	IRN-LE	8/30 (26.7)	4/6 (66.7)	3/3 (100.0)	15/39 (38.5)

pfdhfr=P. falciparum dihydrofolate reductase, pfdhps=P. falciparum dihydropteroate synthase
Amino acid: A=alanine, C=cysteine, E=glutamic acid, G=glycine, I=isoleucine, K=lysine, L=leucine, N=asparagine, R=arginine, S=serine

Discussion

Mutations in the *pfcrt, pfmdr1, pfdhfr,* and *pfdhps* genes associated with antimalarial drug resistance were determined in 104 blood samples from patients infected with *P. falciparum*. The patients were from the provinces of Ranong, Surat Thani and Yala in southern Thailand. Ranong Province borders Myanmar, Yala Province borders Malaysia and Surat Thani Province is located on the western coast

of the Gulf of Thailand. The K76T mutation in *pfcrt* and the *N86Y* mutation in *pfmdr1* gene are strong indicators of CQ resistance in *P. falciparum*[24–26]. These findings were consistent with earlier observations conducted between 2009 and 2016 from southern Thailand and other areas of the country[7,27–29]. The *pfcrt* 76T allele has occasionally been identified in neighboring countries, where 6.9% of isolates collected from Malaysia and 100% of isolates from Myanmar were positive[30–31]. The 100% prevalence of the mutation in the *pfmdr1* gene observed in samples from Yala is also like results from previous studies[7–8,18]. On the other hand, the lower prevalence of *pfmdr1* gene mutation in samples from Ranong and Surat Thani, 20.6% and 28.5% respectively, indicated the transmission of *P. falciparum* isolates with the *pfmdr1* 86Y allele by perennial human migration from endemic areas[7].

The *pfdhfr* and *pfdhps* genes are widely used as molecular markers of SP resistance. The high prevalence in blood samples from Ranong of four-point mutations (N51I, C59R, S108N, I164L) of *pfdhfr* and two codon mutations (K540E, A581G) of *pfdhps* (76.9–100% and 26.6–66.6% respectively) implied the spread and circulation of *P. falciparum* isolate from areas where the parasite is SP-resistant. This predominance was slightly different from the results of previous monitoring in Myanmar, showing that rates of those mutation points ranged from 69.5–98.9% in *pfdhfr* and 38.7–90.3% in *pfdhps* genes[31]. Mutations at *pfdhfr* S108N combined with N51I, C59R and I164L have been strongly correlated with increased resistance to pyrimethamine[32–34].

Our findings reveal a high level of pyrimethamine resistance in Ranong Province, with point mutations like N51I, C59R, S108N, and I164L[35], which reported the prevalence of the SP resistance marker *pfdhfr* across various regions, including Thailand-Myanmar, Thailand-Cambodia and the Thailand-Malaysia border, from 2008 to 2016. The result shows that *P. falciparum* isolates from Surat Thani exhibited a 100% prevalence of *pfdhfr* C59R and S108N, a 66.67% prevalence of *pfdhps* A581G mutations, along with 40 and 60% prevalences, respectively, of *pfdhfr* triple IRN and RNL mutations indicated a high frequency of SP-resistant alleles.

As the number of Malaria cases reported from Surat Thani was very low for many years[36], the high prevalence of *pfdhfr* and *pfdhps* mutations in this area may be due to the movement of infected people from Malaria-endemic regions such as Ranong, which is only 238 km from Surat Thani. In this study, the highest frequency of single mutations in three codons of *pfdhfr* (N51I, C59R, S108N) and *pfdhps* A581G was found in samples from Yala, suggesting that the SP mutant alleles have remained in the province. The observed results were similar to those of previous reports[35,37].

Conclusion

The prevalence of mutations in *pfcrt* haplotypes at codon 76 was 100%, whereas the prevalence of *pfmdr1* mutant-type alleles was 40.43%. Among *pfdhfr* and *pfdhps* mutations, the *pfdhfr* quadruple mutations (IRN-L) showed the highest prevalence in Ranong Province. *P. falciparum* isolates from Ranong not only contained mixed mutant alleles of *pfdhps* at codons K540E and A581G (EG) but also high rates of quadruple and quintuple mutations in *pfdhfr/pfdhps* genes. This study has demonstrated the persistent circulation and spread of CQ- and SP-resistant *P. falciparum* throughout the studied regions. Consequently, continuous investigation of antimalarial drug resistance genes remains necessary to identify parasites with diminished drug sensitivity to know in advance where increasing trends of drug resistance are likely to arise.

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Conflict of interest

There are no potential conflicts of interest to declare.

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