Original Article

Genotyping of the *pvmdr1* Gene in *Plasmodium vivax* Isolates from Endemic Areas of Thailand

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Objective: Mutations in the *pvmdr1* gene have been recognized as a possible molecular marker of chloroquine resistance. The aim of this study was to investigate the genotypes of *pvmdr1* in *P. vivax* isolates from Thailand.

Materials and Methods: A total of 32 *P. vivax*-positive blood samples were collected from two high malaria-endemic areas of Thailand, 20 samples from Yala and 12 from Kanchanaburi provinces. The *pvmdr1* polymorphisms were determined using direct sequencing of nested PCR products.

Results: The *pvmdr1* gene mutation was found in 71.9% of 32 isolates. The four *pvmdr1* polymorphic sites composed of Y976F, F1076L and two novel mutations of D932Y and V1008. The double mutation (Y976F-F1076L) was the predominant strain (53.1%). The single mutation (F1076L) represented 12.5%, and the two novel mutants represented 3.1% each. In Yala, 80% of the isolates carried the *pvmdr1* mutation and two genotypes detected were F1076L (15%) and Y976F-F1076L (65%). In Kanchanaburi, 58.3% of isolates were *pvmdr1* mutants and four genotypes including Y976F-F1076L (33.3%), F1076L (8.3%), V1008-F1076L (8.3%) and D932Y-Y976F-F1076L (8.3%) were found.

Conclusion: A high prevalence of *pvmdr1* gene mutation in this study provides valuable information regarding the awareness of *P. vivax* chloroquine resistance spreading in Thailand. The two novel mutations should be noted.

Keywords: *Plasmodium vivax*, *pvmdr1*, Chloroquine resistance, Malaria, Thailand

J Med Assoc Thai 2018; 101 (Suppl. 8): S185-S191
Website: http://www.jmatonline.com

Malaria is an important infectious disease caused by *Plasmodium* parasites. According to the "World Malaria Report 2016", malaria is transmitted in 91 countries, and in 2015, there were an estimated 212 million malaria cases with 429,000 deaths[1]. Most of these deaths were children aged less than 5 years in Africa. Human malaria infection is caused by *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, *and Plasmodium knowlesi*. Most cases of malaria caused by *P. vivax* occur in Southeast Asia (58%)[1]. Although *P. vivax* malaria is considered to be a benign malaria, it has been increasingly reported to cause various manifestations of severe and fatal disease, particularly in pregnant women and young children[2-5]. There were approximately 3,100 deaths related to vivax malaria in 2015 globally[1]. In Thailand, malaria remains a significant public health problem and *P. vivax* was responsible for 74.5% of the malaria cases in 2016[6].

Currently, the spread of *P. vivax* drug-resistant malaria is a challenge facing malaria control. In recent years, there have many reports describing that severe *P. vivax* malaria is often attributed to repeated relapses as a result of chloroquine [CQ] resistance[4,5,7]. CQ-resistant vivax was first reported in Papua New Guinea in 1989[8] and have been increasingly reported in Indonesia, Myanmar, Papua New Guinea, India, and Vietnam[8-15]. In Thailand, a declining trend of *in vitro* sensitivity to CQ has been recognized in some areas especially along the Thai-Myanmar border[16-18]. Aside from the CQ resistance, Chotivanich et al also demonstrated a reduction of *in vitro P. vivax* susceptibility to mefloquine, amodiaquine, and

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How to cite this article: Boonsilp S, Kritsiriwuthinan K. Genotyping of the *pvmdr1* Gene in *Plasmodium vivax* Isolates from Endemic Areas of Thailand. J Med Assoc Thai 2018;101;Suppl.8: S185-S191.

artesunate along the western border of Thailand[19]. Recently, Rungsihirunrat demonstrated that 63.3% of *P. vivax* from Thailand isolates were CQ resistant by the *in vitro* susceptibility test based on the cut-off IC50 (100 nM)[20]. The CQ resistance found in several *in vitro* studies was also demonstrated in a study by Congpuong who tested the 28-day *in vivo* therapeutic efficacy of CQ for *P. vivax* and found 2.5% treatment failure in four bordered provinces of Thailand[17].

Therefore, there is a critical need to monitor drug resistance of vivax malaria in Thailand closely. Because it is difficult to investigate the drug sensitivity of vivax malaria using *in vitro* culture, several genetic polymorphisms have been tested as genetic markers of *P. vivax* CQ resistance. One polymorphism of interest is the *P. vivax* homologue of the *P. falciparum* multidrug resistance gene 1 (*pvmdr1*). Although there is a controversy regarding the *pvmdr1* polymorphism and the relation of *P. vivax* CQ resistance phenotype[21-23], several studies confirmed the association between *pvmdr1* and CQ resistant vivax malaria. A study in the Brazilian Amazon by Melo et al demonstrated a strong association between the *pvmdr1* expression level with CQ resistance and clinical severity in *P. vivax* infection. The patients with CQ resistance to *P. vivax* presented up to a 2.4-fold increase in the *pvmdr1* expression level compared to the susceptible group[24]. Brega identified the mdr-like gene *pvmdr1* which played a role in drug resistance from different areas of endemicity[25]. In Papua New Guinea[26], Cambodia[27] and Indonesia[10], where CQ resistance is common, they identified a high prevalence of the *pvmdr1* Y976F genotype in *P. vivax* (100%, 89%, 94.6% respectively). Wehereas in areas where *P. vivax* CQ resistance is infrequent, the Y976F and F1076L polymorphisms have a lower prevalence[28].

Many authors reported that *Pvmdr1* Y976F and F1076L mutations were observed in malaria-endemic areas where CQ was used as the first-line treatment[10,20,29], which is likely to be a pressure factor for CQ resistance. A study by Suwanarusk et al reported 96% Y976F mutation in *pvmdr1* in of Papua New Guinea clinical isolates, where there was a high rate treatment failure of CQ for *P. vivax*[29]. This was significantly higher than 25% prevalence found in Thai isolates where CQ treatment is generally effective[29]. The researchers also found that the CQ IC50 in isolates with the Y976F mutation was significantly higher than the wild-type isolates and suggested that the Y976F mutation is a useful genetic marker for CQ resistance monitoring[29]. Another study by Suwanarusk et al confirmed that the *pvmdr1* Y976F mutation was significantly related with an increased IC50 compared to the wild type[10]. A recent report suggested that CQ resistance gene (*pvmdr1*, Y976F and F1076L) mutations are spreading in malaria endemic areas along the Thai-Myanmar border in the Mae Sot district, Tak province of Thailand, where 63.4% (19/30) were classified as CQ-resistant isolates[20].

The aim of this study was to investigate the mutation of CQ resistance markers, *pvmdr1* genotype at expected amino acid codon 976 and 1076, in *P. vivax* isolates collected from Yala and Kanchanburi provinces, Thailand. The single nucleotide polymorphism [SNP] genotypes of the *pvmdr1* gene were examined using nested PCR and nucleotide sequencing.

Materials and Methods

Study area

This study was conducted in Yala and Kanchanaburi provinces which are two of the top ten provinces with the most malaria cases in Thailand. According to the annual malaria reports from 2012 to 2016 by the Bureau of the Vector-borne Diseases, Department of Disease Control, Ministry of Public Health, Thailand[6], nearly half (42.5%) of all malaria cases in 2016 was from Yala and Kanchanaburi provinces. Yala is located in the south of Thailand, near the Thailand-Malaysia border (Figure 1). In 2016, this area had highest prevalence of malaria, with 7,427 cases (39.6% of total cases in Thailand)[6]. Kanchanaburi is a province located in the western part of Thailand, near the Thailand-Myanmar border (Figure 1), and had 545 cases reported in the same year.

Sample collection

A total of 32 *P. vivax*-positive blood samples were collected from subjects living in Yala and Kanchanaburi provinces from 2009 to 2010. EDTA blood samples were performed by thick and thin blood films for Giemsa staining to identify *Plasmodium* spp. under microscopy. Approximately 100 µl was spotted on a piece of filter paper (Whatman™, UK) and air-dried. The dried blood spots were stored in individual plastic bags at room temperature and subsequently transferred to Rangsit University laboratory for DNA extraction and *pvmdr1* genotyping. This study was approved by the ethics committee of Rangsit University, Pathumthani, Thailand (ETHIC RSEC06/52).

Polymerase chain reaction [PCR] amplification for pvmdr1

P. vivax-positive dried blood spots were investigated for *pvmdr1* SNPs at the 976 and 1076 amino

acid codons. Genomic DNA from the dried blood spots was extracted using the Genomic DNA Mini Kit (Fermentas Life Science, St. Leon-Rot, Germany) according to the manufacturer's instructions and stored at -20°C for further use as a DNA template for *pvmdr1* genotyping by nested PCR. The nested PCR protocol was described by Mint Lekweiry et al[30] and used with slight modifications. The primer sequences and thermocycles are presented in Table 1. The first round PCR was conducted using the reaction mixture in a total volume of 25 µl, containing 3 µl of DNA template with 0.02 U of Taq DNA polymerase (Q5 High-Fidelity DNA Polymerase, New England Biolabs, UK). The second round mixture of PCR reaction was performed in a final volume of 50 µl containing 0.5 µl of the first round PCR product. The PCR amplicons were visualized by 1.2% agarose gel electrophoresis followed by staining with ethidium bromide. A positive PCR *pvmdr1* amplicon was defined as the visualization of an expected band of size ~547 bp.

Figure 1. Map of Kanchanaburi and Yala provinces in Thailand where *P. vivax* infected blood samples were collected.

Nucleotide sequencing and sequence analysis

The PCR products were purified and sequenced by Ward Medic Ltd., Part Thailand using the F2pvmdr1 and R2pvmdr1 primers. Then, the sequences were analysed and edited using SeqManII software (DNASTAR Inc., Wisconsin, and USA). The nucleotide and amino acid sequences of *pvmdr1* were aligned and compared with the published wild-type sequence (accession no. AY618622) and Y976F, F1076L mutant (accession no. AY643799) genotypes acquired from the GenBank database using the software Mega 6.0.

Results

From 53 *P. vivax* infected blood samples, 32 samples were successfully amplified and sequenced for analysis of *pvmdr1* genotype. There were 20 and 12 isolates from Yala and Kanchanaburi provinces, respectively. Table 2 presents the four *pvmdr1* mutation genotypes found in this study. These genotypes were the common mutation reported codons of Y976F (56.3%), F1076L (71.9%) and two novel mutation genotypes of nonsynonymous D932Y (3.1%) and synonymous V1008 (3.1%). In Table 3, we revealed that the double mutation (Y976F-F1076L) was the predominant strain (53.1%). The single mutation of F1076L represented 12.5%, and the two novel mutants represented 3.1% each. The wild type was observed in 28.1% of isolates. In Yala, 80% of isolates carried the *pvmdr1* mutation. Two *pvmdr1* mutation patterns were

Table 1. Primer sequences and PCR Thermocycle

PCR	Primer sets	Primer sequence (5′ to 3′)	PCR Thermocycle
First round	F1pvmdr1 R1pvmdr1	GCGAACTCGAATAAGTACTCCCTCTA GGCGTAGCTTCCCGTAAATAAA	pre-denature 98°C, 30s followed by 30 cycles of 98°C, 30s, 68°C, 30s, 72°C, 45s, final extension at 72°C,10 min
Second round	F2pvmdr1 R2pvmdr1	GGATTGCTGTCAGCACATATTAACA AGAGGGATTTCATAAAGTCATC CAGT	pre-denature 98°C, 3 min followed by 35 cycles of 98°C, 45s, 64°C, 30s, 72°C, 45s, final extension at 72°C, 10 min

Table 2. Point mutations present in the *pvmdr1* gene amplified from 32 *P. vivax*-positive samples

	Point mutation in *pvmdr1* gene			
Codon position	932	976	1008*	1076
Wild type/mutant	GAT/TAT	TAC/TTC	GTC/GTG	TTT/CTT
Amino acid changes	Asp (D)/Tyr (Y)	Tyr (Y)/Phe (F)	Val (V)/Val (V)	Phe (F)/Leu (L)
Number (%)	1 (3.1)	18 (56.3)	1 (3.1)	23 (71.9)

* Indicates synonymous mutation

Table 3. Distribution of *pvmdr1* point mutations in *P. vivax* clinical isolates in the Yala and Kanchanaburi provinces, Thailand

Genotype	*pvmdr1* Amino acid codon				The number of isolates (%)		
	D932Y	Y976F	V1008*	F1076L	Yala (n = 20)	Kanchanaburi (n = 12)	Total (n = 32)
Wild type (D932,Y976, V1008*, F1076)	D	Y	V	F	4 (20.0)	5 (41.7)	9 (28.1)
Single mutant(Y976F)	D	F	V	F	0 (0.0)	0 (0.0)	0 (0)
Single mutant(F1076L)	D	Y	V	L	3 (15.0)	1 (8.3)	4 (12.5)
Double mutant (Y976F, F1076L)	D	F	V	L	13 (65.0)	4 (33.3)	17 (53.1)
Double mutant(V1008*, F1076L)	D	Y	V	L	0	1 (8.3)	1 (3.1)
Triple mutant(D932Y, Y976F, F1076L)	Y	F	V	L	0	1 (8.3)	1 (3.1)

* Indicates synonymous mutation

detected, a single mutation (F1076L, 15%) and a double mutation (Y976F-F1076L, 65%). In Kanchanaburi, 58.3% were *pvmdr1* mutants, and four mutation patterns were detected. These patterns were 33.3% double mutation (Y976F-F1076L), 8.3% single mutation (F1076L), 8.3% double mutation (V1008-F1076L) and 8.3% triple mutation (D932Y-Y976F-F1076L). The single mutation (Y976F) was not observed in this study.

Discussion

The high prevalence of the *pvmdr1* double mutant genotypes (Y976F-F1076L) in this study might indicate the ongoing spread of CQ resistant vivax malaria in these areas. This statement was supported by the evidence from previous reports demonstrating that Y976F and F1076L mutant strains were possible genetic markers associated with CQ resistance in vivax malaria. Moreover, treatment failure found in Thai *P. vivax* in Kanchanaburi province along the Thai-Myanmar border was also reported[17]. In addition, recent *in vitro* data showed that Thai *P. vivax* isolates along the Thai-Myanmar border (Tak province) presented a high prevalence of the isolates with an IC50 for CQ resistance of 63.4%[20].

The *pvmdr1* F1076L mutant is the primary change followed by an acquirement of Y976F; these changes were responsible for the decrease in CQ susceptibility[25]. Therefore, the detection of F1076L single mutants might provide an early checkpoint for the CQ resistance before the drug-resistant phenotype occurs. The overall F1076L mutant in this study was 71.9%, of which Y976F was carried in 53.1% and only 12.5% carried the single F1076L mutation. This finding might imply the period of changing to the CQ resistance phenotype. An awareness for the spreading of CQ resistance phenotype vivax should be considered in these regions. However, *in vivo* and *in vitro* CQ susceptibility studies are required to confirm our findings.

In the present study, we observed a rather

high prevalence of the *pvmdr1* double mutant phenotype (Y976F-F1076L) in Yala (65%). These results might indicate the trend of CQ resistance spreading in Yala. Unfortunately, data of CQ response or resistant *P. vivax* reports at this site are limited. A study by Congpuong found the prevalence of CQ treatment failure based on a 28-day *in vivo* therapeutic efficacy of 2.5% for *P. vivax* in four border provinces (Mae Hong Son, Kanchanaburi, Yala, Chanthaburi) of Thailand, but did not demonstrate CQ treatment failure in Yala (0%)[17]. However, further study should explore the CQ susceptibility of *P. vivax* and the association of *pvmdr1* polymorphism in this predominantly *pvmdr1* CQ resistance marker site.

Compared to other studies evaluating the prevalence of *pvmdr1* polymorphism CQ-resistant markers in Thailand, our results demonstrated the highest frequency of Y976F mutant (53.1%). Previous studies in Thailand in different geographic areas reported Y976F mutation varied from 18% to 25%[20,26,29,31]. A recent report found that the *pvmdr1* double mutant genotype (Y976F-F1076L) represented 23.3% in the Thai-Myanmar border[20], a similar location to Kanchanaburi this study, though there was a higher double mutant genotype (Y976F-F1076L) frequency of 33.3%.

Compared to nearby countries, where CQ resistance was also reported but with higher prevalence of the Y976F mutation in *pvmdr1* than that found in Thailand. These countries include Indonesia, Papua New Guinea and Cambodia, with Y976F prevalences of 96%[10], 100%[26] and 89%[27], respectively. However, studies in Myanmar which is close to the Thai border (western region), the prevalences of CQ resistant vivax malaria of 1.7 to 26.7% were lower than that found in this study[12,26].

In addition to the Y976F and F1076L mutations, two novel *pvmdr1* mutations were detected from the Kanchanburi isolates in our study. One mutation was a non-synonymous G2794T in the nucleotide sequence, corresponding to D932Y in the amino acid sequence, which corresponded to the triple mutant genotype (D932Y-Y976F-F1076L). Another mutation was a synonymous mutation C3024G in the nucleotide sequence, corresponding to valine at the V1008 amino acid codon. The synonymous V1008 mutation coexists with an F1076L mutation. These mutations resulted in 4 different patterns of *pvmdr1* mutant observed in the *P. vivax* isolates in Kanchanaburi. The *P. vivax* population in Yala province revealed only 2 genotype patterns. The different *pvmdr1* mutation patterns in each endemic area might depend on the local epidemiological and demographic situation, such as the incidence of infected humans, the mosquito transmission intensity, and migration of infected inhabitants.

Conclusion

The two novel *pvmdr1* gene mutations described in this study may indicate the spreading of *P. vivax* CQ resistance in Thailand, and should be further observed. A high prevalence of *pvmdr1* gene mutations might serve as a CQ resistance marker alert. The precise mutations in *P. vivax* CQ susceptibility warrant further investigation, particularly in a combination of *in vitro* and clinical response.

What is already known on this topic?

Plasmodium vivax is a widely distributed malaria parasite present in Thailand. CQ is the first-line therapy used for malaria treatment; however, CQ-resistant *P. vivax* has been reported in Thailand. Little is known regarding the spread of CQ resistant *P. vivax* because the culture method used to determine drug susceptibility is difficult. Mutations in *pvmdr1* gene have been recognized as a possible molecular marker of *P. vivax* CQ resistance. The aim of this study was to investigate the *pvmdr1* mutation in *P. vivax* isolates from endemic areas of Thailand.

What this study adds?

This study demonstrated a high prevalence of a possible CQ resistant resistance genetic marker (*pvmdr1* gene mutation) in the southern and western part of Thailand and found two novel mutations sites in *pvmdr1*. This finding provides valuable information regarding the awareness of CQ resistance in *P. vivax*. CQ susceptibility with molecular marker association requires further research in Thailand to determine the effectiveness of malaria therapy and control programmes.

Acknowledgements

The authors would like to thank Ms. Aneesa Etae and Ms. Anna Jehmah for their kind assistance of blood sample collection. We also wish to thank Mr. Gorchat Sabangban, Ms. Piyachat Thengchittrakool, Ms. Sudarat Nartnamphong, Mr. Api Kullasettawoot, and Mr. Apiwat Pornsiriwatanakul for the laboratory assistance. We thank Mr. Jason Douglas Cullen, language advises at Academic Unit, Faculty of Medicine Vajira hospital, Navamindradhiraj University

for critical reading of the manuscript. This study, partially supported by Rangsit University and Navamindradhiraj University, Thailand.

Authors contributions

KK designed the study, performed laboratory experiments and edited final manuscript. SB performed the sequence analysis, analyzed the data, wrote draft manuscript, and edited final manuscript. Both authors read and approved the final manuscript.

Potential conflicts of interest

The authors declare no conflict of interest.

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