

## RESEARCH ARTICLE

***Pvmdr1* Polymorphisms of *Plasmodium vivax* Isolates from Malaria Endemic Areas in Southern Provinces of Thailand****Kanchana Rungsihirunrat<sup>1</sup>, Jiraporn Kuesap<sup>2</sup>, Wanna Chaijaroenkul<sup>3</sup>, Mathirut Mungthin<sup>4</sup>, Kesara Na-Bangchang<sup>3</sup>**<sup>1</sup> College of Public Health Sciences, Chulalongkorn University, Bangkok, Thailand<sup>2</sup> Faculty of Allied Health Sciences, Thammasat University, Pathumthani, Thailand<sup>3</sup> Chulabhorn International College of Medicine, Thammasat University, Pathumthani, Thailand<sup>4</sup> Department of Parasitology, Phramongkutklao College of Medicine, Bangkok, Thailand**Abstract**

Since chloroquine is the mainstay for treatment of *Plasmodium vivax* infection, monitoring of chloroquine drug resistance marker provides useful information for effective malaria control program. The aim of the study was to investigate the genetic polymorphisms of *pvm-dr1* in *Plasmodium vivax* isolates collected from Southern Thailand. A total of 70 *P. vivax* isolates were collected by finger-prick from patients. DNA from dried blood spot samples were extracted and analyzed for *pvm-dr1* polymorphisms. Seven non-synonymous and one synonymous mutations were identified in *pvm-dr1* gene. Five haplotypes of *pvm-dr1* were observed with different frequencies. However, 21 isolates (30%) carried 4 mutations of 515R, 698S, 908L, and 958M. Amino acid Y976F and F1076L mutations, key point mutations associated with *P. vivax* chloroquine resistance, were detected in 17 (24.3%) and 38 (54.3%) isolates, respectively. The prevalence and pattern of mutations of *pvm-dr1* obtained from this study suggest the spreading of chloroquine resistance alleles in *P. vivax* isolates from Southern part of Thailand. The monitoring of chloroquine drug resistance marker in *P. vivax* can provide useful information for early warning system and for developing the appropriate drug policy.

**Keywords:** *Plasmodium vivax*, *pvm-dr1*, chloroquine resistance

## ความหลากหลายของ *pvm-dr1* ของเชื้อพลาสโมเดียมไวแวกซ์จากพื้นที่ระบาด ของเชื้อมาลาเรียในจังหวัดภาคใต้ของประเทศไทย

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### บทคัดย่อ

เนื่องจากคลอโรควินถูกใช้เป็นยาหลักในการรักษาการติดเชื้อมาลาเรียชนิดไวแวกซ์  
ดังนั้น การใช้ตัวติดตามการดื้อยาคลอโรควินจึงมีประโยชน์สำหรับการควบคุมเชื้อมาลาเรีย  
วัตถุประสงค์ของการศึกษานี้เพื่อศึกษาความหลากหลายของรูปแบบยีน *pvm-dr1* ในเชื้อ  
พลาสโมเดียมไวแวกซ์ที่เก็บจากภาคใต้ของประเทศไทย จำนวน 70 ตัวอย่าง ด้วยวิธีเก็บจาก  
ปลายนิ้วของผู้ป่วย นำดีเอ็นเอที่เก็บบนกระดาษกรองมาสกัดและวิเคราะห์หาความหลากหลาย  
ของยีน *pvm-dr1* ผลการทดลองพบการกลายพันธุ์ชนิด non-synonymous 7 ตำแหน่งและชนิด  
synonymous 1 ตำแหน่ง โดยยีน *pvm-dr1* 5 รูปแบบที่พบมีความถี่แตกต่างกัน เชื้อมาลาเรีย  
จำนวน 21 (30%) ตัวอย่าง พบการกลายพันธุ์ 4 ตำแหน่ง (515R, 698S, 908L, 958M) โดยพบ  
การกลายพันธุ์ของกรดอะมิโนที่ตำแหน่ง Y976F และ F1076L ซึ่งเป็นตำแหน่งสำคัญที่เกี่ยวข้อง  
กับการดื้อยาคลอโรควินในเชื้อมาลาเรียชนิดไวแวกซ์ จำนวน 17 (24.3%) และ 38 (54.3%)  
ตัวอย่าง ตามลำดับ ความถี่และรูปแบบของ *pvm-dr1* ที่พบในครั้งนี้แสดงให้เห็นว่าการกระจาย  
ของเชื้อดื้อยาคลอโรควินในเชื้อมาลาเรียชนิดไวแวกซ์ในพื้นที่ที่มีการระบาดในภาคใต้ของประเทศไทย  
และการติดตามการดื้อยาคลอโรควินของเชื้อมาลาเรียสามารถใช้เป็นข้อมูลในการเฝ้าระวังและ  
เป็นแนวทางในการใช้ยาต้านมาลาเรีย

**คำสำคัญ:** พลาสโมเดียมไวแวกซ์, *pvm-dr1*, การดื้อยาคลอโรควิน

## Introduction

Combination of chloroquine and mefloquine is the first-line treatment for *Plasmodium vivax* since 1946.<sup>1</sup> Recently, treatment failure of chloroquine in *P. vivax* has been reported in some malaria endemic countries.<sup>2-8</sup> Thailand is highly endemic to *Plasmodium falciparum* but recent reports suggest that malaria due to *P. vivax* is increasingly, especially in Southern peninsula of Thailand. Reduced *in vitro* parasite's susceptibility to chloroquine as well as other antimalarial drugs has been demonstrated in the malaria parasites along the international borders of Thailand.<sup>9</sup> Several studies have been proposed to investigate genetic diversity of *P. vivax*.<sup>10-11</sup> However, this species has been less well studied when compare to *P. falciparum*. Similar approaches have been adopted to investigate *P. vivax* at the molecular level. Although the molecular mechanisms underlying chloroquine resistance in *P. vivax* remain unclear, similar molecular mechanisms of multi-loci genes in *P. falciparum* have been proposed to be involved in *P. vivax* chloroquine resistant phenotype. The *P. vivax* multidrug resistance (*pvmdr*) and putative transporter (*pvcrt-o*) genes, which are orthologous to *pfmdr1* and *pfcr* genes, have been identified as chloroquine resistance markers in *P. vivax*. The mutant alleles of both genes were suggested to be associated with chloroquine resistance in *P. vivax* in Southeast Asia both *in vivo* and *in vitro*.<sup>12-14</sup> Sequence analysis of *pvmdr1* and *pvcrt-o* demonstrated that *pvmdr1* gene contained at least 24 single nucleotide polymorphisms (SNPs), whereas *pvcrt-o* gene contained 5 SNPs.<sup>15</sup> However, sequence analysis of *pvmdr1* SNPs at homologous positions of *pfmdr1* did not reveal any polymorphism as that found in *P. falciparum*<sup>13,16-17</sup>, suggesting the different mechanisms of chloroquine resistance between *P. vivax* and *P. falciparum*. The aim of the present study was to investigate the genetic polymorphisms of *pvmdr1* in *P. vivax* isolates collected from Southern Thailand.

## Materials and Methods

### Sample collection

*P. vivax* field isolates in this study were collected from malaria patients attending malaria clinics in Southern part of Thailand along Thailand-Myanmar and Thailand Malaysia borders. The study protocol was reviewed and approved by the Ethics Review Committee for Research Involving Human Research Subject, Health Science Group, Chulalongkorn University. Written informed consent for study participation was obtained from each patient prior to participation. Blood films were stained with Giemsa stain and examined by light microscope for confirmation of *P. vivax* infection. Approximately 100-200 µL of blood samples from finger-prick were spotted onto filter paper and stored in small plastic zip lock bags prior to extraction of parasite DNA for identification of *pvmdr1* polymorphisms.

### Identification of *pvmdr1* polymorphisms

Genomic DNA was individually extracted from dried blood spots on filter paper using a QIAamp DNA extraction mini-kit (Qiagen, Germany) and used as a template for amplification by polymerase chain reaction (PCR). The *pvmdr1* gene

was amplified by nested PCR using specific primers as previously described.<sup>13, 17</sup> The outer PCR was carried out in the following reaction mixture with a total volume of 20  $\mu$ L: 0.2  $\mu$ M of each primer, 2.5 mM  $MgCl_2$ , 100 mM KCl, 20 mM Tris-HCl (pH 8.0), 100  $\mu$ M deoxynucleotide triphosphates (dNTPs), 10-20  $\mu$ g of genomic DNA, and 0.5 unit of *Taq* DNA polymerase. The nested PCR was carried out in a PCR mixture similar to that used for the outer PCR. One  $\mu$ L of outer PCR product was used as DNA template. The outer and nested PCR cycling parameters were as follows: initial denaturation at 95°C for 5 minutes, followed by 35 cycles at 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and then followed by final extension at 72°C for 5 minutes. The PCR products were fractionated by 1.5% agarose gel electrophoresis, purified with QIAquick PCR purification kit (Qiagen, Germany), and sequenced by automated DNA sequencer (ABI system, Singapore).

#### **Data and sequence analysis**

Each fragment was sequenced in both the forward and reverse directions and assembled using Bioedit version 7.1.3. (Ibis BioSciences, Carsbad, California, USA). DNA fragments were aligned using the ClustalW multiple sequence alignment (freely available from <http://www.genome.jp/tools/clustalw>). Nucleotide sequence alignments of all isolates were compared with the wild-type sequence from GenBank for *pvmdr1* (Accession no. AY618622) using Bioedit version 7.1.3.

#### **Results**

A total of 78 *P. vivax* isolates were collected for analysis of *pvmdr1* genetic polymorphisms. The *pvmdr1* gene was successfully sequenced in 70 *P. vivax* isolates. Among all of the studied mutations of *pvmdr1*, no N91Y, Y189F, S510T, I636T, A829V, K997R, L1022L, S1071C, N1079D and D1291Y mutations were found. Numbers of *pvmdr1* mutant alleles at amino acid residual at codon indicated were identified as shown in Table 1. Seven non-synonymous and one synonymous mutations were identified in *pvmdr1* gene. All isolates contained at least 4 to 6 mutations. Among these, 5 haplotypes of *pvmdr1* were observed, of which the majority of the isolates (21/70) carried 4 mutations (515R, 698S, 908L, 958M). Nineteen isolates (19/70) carried 5 mutations (515R, 698S, 908L, 958M, 1076L) and 17 isolates (17/70) carried 6 mutations (515R, 698S, 908L, 958M, 976F, 1076L). Eleven isolates (11/70) carried 5 mutations of 513R, 515R, 698S, 908L, 958M and 2 isolates (2/70) carried 6 mutations of 513R, 515R, 698S, 908L, 958M, 1076L. Isolates carrying 1076L were observed in 38 isolates (54.3%), whereas isolates carrying both 976F and 1076L were observed in 17 isolates (24.3%) (Table 2).

**Table 1.** Prevalence of *pvmdr1* mutant alleles.

Amino acid residue	Number (%) of <i>pvmdr1</i> mutant alleles
N91Y (aac/tac)	N91Y = 0 (0)
Y189F (tac/ttc)	Y189F = 0 (0)
S510T (agc/acc)	S510T = 0 (0)
S513R (agt/aga)	S513R = 13 (18.6)
S515R (agc/agg)	S515R = 70 (100)
T529 (aca/acg)*	T529 (aca) = 6 (8.6), T529 (acg) = 64 (91.4)
I636T (atc/acc)	I636T = 0 (0)
G698S (ggc/age)	G698S = 70 (100)
A829V (gcc/gtc)	A829V = 0 (0)
M908L (atg/ctg)	M908L = 70 (100)
T958M (acg/atg)	T958M = 70 (100)
Y976F (tac/ttc)	Y976F = 17 (24.3)
K997R (aag/agg)	K997R = 0 (0)
L1022 (cta/tta)*	L1022 (cta) = 0 (0)
S1071C (agt/tgt)	S1071C = 0 (0)
F1076L (ttt/ctt)	F1076L = 38 (54.3)
N1079D (aac/gac)	N1079D = 0 (0)
D1291Y (gat/tat)	D1291Y = 0 (0)

\* indicates synonymous mutation

**Table 2.** Haplotype frequencies of *pvmdr1*.

Haplotype	S513R	S515R	G698S	M908L	T958M	Y976F	F1076L	Number (%)
1	S	R	S	L	M	Y	F	21 (30)
2	S	R	S	L	M	Y	L	19 (27.1)
3	S	R	S	L	M	F	L	17 (24.3)
4	R	R	S	L	M	Y	F	11 (15.7)
5	R	R	S	L	M	Y	L	2 (2.9)

## Discussion

*Plasmodium vivax* control is now being problematic due to drug resistance. The first case of chloroquine resistance in *P. vivax* has been reported since 1989 in Indonesia<sup>2</sup> and further sporadic cases were subsequently observed in Oceanian and other Asian countries.<sup>8,18</sup> In Thailand, the proportion of *P. vivax* infection has become increasing and a trend of gradual decline of *in vitro* sensitivity to chloroquine has been reported in some areas.<sup>19</sup> Since the effective *in vitro* culture systems for *P. vivax* are not available and more complicated when compared with *P. falciparum*. Thus, monitoring of genetic polymorphisms associated with drug resistance markers provides an understanding of molecular mechanism underlying chloroquine resistance in *P. vivax* infection.

Unlike chloroquine-resistant *P. falciparum*, the mechanism of chloroquine resistance in *P. vivax* remains unclear. The orthologous *P. falciparum* genes linked to chloroquine resistance have been used to identify chloroquine resistance gene in *P. vivax*.<sup>12-13, 17, 20</sup> The *pvmdr1* gene was found to be more polymorphic than *pvcrt-o*.<sup>21</sup> No wild-type haplotype was found in our study. Seven non-synonymous and one synonymous mutations were observed in *pvmdr1*. Y976F and F1076L mutations, possible genetic markers for chloroquine resistance in *P. vivax*, were detected in 24.3% and 54.3% of *P. vivax* isolates which are lower than those observed in 9 *P. vivax* Thai isolates from Mae Sod (44% and 56%).<sup>13</sup> The observation of high frequency of F1076L mutation (54.3%) in our present study was similar to that reported in a previous study in the isolates from Papua New Guinea. However, the frequency of Y976F *pvmdr1* mutation (24.3%) was much lower than the Papua New Guinea isolates (100%). None of both mutations was found in the isolates collected from Korea.<sup>14</sup>

This finding may suggest that Y976F and F1076L mutations in *P. vivax* were spread and distributed from Southeast Asia and high prevalence of F1076L mutation may indicate a trend of rapid aggravation of chloroquine resistance *P. vivax* in this region. Apart from the mutations at both codons, one non-synonymous mutation at codon T529 (aca = 8.6%, acg = 91.4%) was also detected in our study. The commonly found mutations at codon S515R, G698S, M908L and T958M were also detected in all *P. vivax* isolates. Sequence analysis of *pvmdr1* gene has shown no mutation at codons 91, 189, 1071, 1079, and 1291 homologous to *pfmdr1* at codons 86, 184, 1034, 1042, and 1246, respectively, which are associated with chloroquine resistance in *P. falciparum*. Five haplotypes of *pvmdr1* were observed with different frequencies. However, majority of isolates (30%) carried 4 mutations (515R, 698S, 908L and 958M). It is interesting that polymorphism in *pvmdr1* gene was higher than that in *pfmdr1* gene, which might be one factor that contributed to *in vitro* resistant parasites.<sup>22</sup> Identification of differences in the orthologous gene between *P. falciparum* and *P. vivax* is important for comparing genetic determinants of chloroquine resistance in both malaria species. The mechanism of chloroquine resistance in both *P. falciparum* and *P. vivax* is probably similar, but the development of resistance may be different.

## Conclusion

We have analyzed the polymorphisms in *pvmdr1*, a chloroquine drug resistant gene, in 70 *P. vivax* isolates collected from Southern part of Thailand. Seven non-synonymous and one synonymous mutations were identified. Five haplotypes of *pvmdr1* were observed with different frequency. The finding suggested that monitoring of chloroquine drug resistance marker in *P. vivax* is essential for early warning system and expedites the appropriate drug policy.

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