Plasmodium vivax Drug Resistance Genes; Pvmdr1 and Pvcrt-o Polymorphisms in Relation to Chloroquine Sensitivity from a Malaria Endemic Area of Thailand

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Abstract: The aim of the study was to explore the possible molecular markers of chloroquine resistance in Plasmodium vivax isolates in Thailand. A total of 30 P. vivax isolates were collected from a malaria endemic area along the Thai-Myanmar border in Mae Sot district of Thailand. Dried blood spot samples were collected for analysis of Pvmdr1 and Pvcrt-o polymorphisms. Blood samples (100 μl) were collected by finger-prick for in vitro chloroquine susceptibility testing by schizont maturation inhibition assay. Based on the cut-off IC50 of 100 nM, 19 (63.3%) isolates were classified as chloroquine resistant P. vivax isolates. Seven non-synonymous mutations and 2 synonymous were identified in Pvmdr1 gene. Y976F and F1076L mutations were detected in 7 (23.3%) and 16 isolates (53.3%), respectively. Analysis of Pvcrt-o gene revealed that all isolates were wild-type. Our results suggest that chloroquine resistance gene is now spreading in this area. Monitoring of chloroquine resistant molecular markers provide a useful tool for future control of P. vivax malaria.

Key words: Plasmodium vivax, Pvmdr1, Pvcrt-o, drug resistance, chloroquine

INTRODUCTION

Chloroquine has been used as the first-line treatment for Plasmodium vivax since 1946 [1]. Treatment failure of chloroquine in P. vivax has, however, been increasing in some malaria endemic countries. The resistance was first reported in Papua New Guinea [2], and was subsequently reported in several countries in Asia [3-8]. In Thailand, reduced in vitro parasite’s susceptibility to chloroquine, as well as mefloquine, amodiaquine, and artesunate has been demonstrated in the western border of Thailand [9]. Nevertheless, the drug remains to be the standard treatment for P. vivax in the country due to its clinical effectiveness and affordability. 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 protein (Pvcrt-o), 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 [10-12]. Analysis of the complete sequences of the Pvmdr1 and Pvcrt-o in P. vivax isolates from Brazilian Amazon region demonstrated that Pvmdr1 gene contained 24 single nucleotide polymorphisms (SNPs), whereas Pvcrt-o gene contained 5 SNPs and lysine insertion at the amino acid position 10 [13]. The analysis of Pvmdr1 SNPs at homologous positions of Pfmdr1 did not reveal any polymorphism as that found in P. falciparum [11,14,15]. It is possible that the mechanisms of chloroquine resistance in P. vivax may differ from that in P. falciparum. The aim of the present study was to investigate whether chloroquine resistance genes of P. vivax (Pvmdr1 and Pvcrt-o) have already spread in malaria endemic areas of Thailand, and whether their chloroquine resistance phenotypes were associated with in vitro drug susceptibility.
MATERIALS AND METHODS

Sample collection
The study was conducted at malaria clinic, Mae Sot, Tak Province, Thailand, during April to August 2010. This malaria endemic area is located along the Thai-Myanmar border, which is well-documented as an area of multidrug resistance P. falciparum [16,17]. The study protocol was reviewed and approved by the Ethics Review Committee for Research Involving Human Research Subject, Health Science Group, Chulalongkorn University, Thailand. Written informed consent for study participation was obtained from each patient prior to participation. Inclusion criteria included P. vivax parasitemia of 1,000-100,000 parasites/µl blood, absence of signs of severe diseases, and no previous antimalarial treatment during the preceding 4 weeks. Blood films were stained with Giemsa and examined by a light microscope. Asexual stages of P. vivax were counted against 1,000 erythrocytes in thin blood films or against 200 white blood cells in thick films and 70% of dominant ring stage were selected for in vitro assay. Two hundred micro-liters of blood samples were spotted onto a filter paper and stored in small plastic zip lock bags prior to the extraction of parasite DNA for identification of Pvmdr1 and Pvcrt-o polymorphisms. Blood samples (1 μl each) were collected into sodium heparinized tube prior to treatment with a 3-day chloroquine phosphate (Chloroquine phosphate: RPMI 1640 medium to obtain the desired drug concentrations. A stock solution of each drug was prepared in 1% dimethyl sulfoxide (DMSO), and was subsequently diluted in RPMI 1640 medium. The number of normal schizonts (containing > 8 nuclei) per 200 asexual stage parasites of control well and each drug containing well was counted and expressed as the percentage of schizont maturation inhibition. The dose-response curve was analyzed using CalcuSyn™ software (BioSoft, Cambridge, UK) to obtain the IC50 values (the concentrations that inhibit schizont maturation by 50% compared with the control).

In vitro schizont maturation inhibition assay
The schizont maturation assay was performed with all P. vivax isolates according to the modified method of Russell et al. [18]. Briefly, plasma and Buffy coat were separated from blood samples by centrifugation and packed red cells were washed twice with RPMI 1640 medium. The pellets were resuspended in human AB serum to obtain 40% hematocrit, and were then diluted to 4% in McCoy’s 5A medium containing 30% AB serum. Drug plates were prepared fresh to avoid possible degradation. A stock solution of each drug was prepared in 1% dimethyl sulfoxide (DMSO), and was subsequently diluted in RPMI 1640 medium to obtain the desired drug concentrations. Fifty microtiter of the working drug (chloroquine phosphate: Liverpool School of Tropical Medicine, Liverpool, UK) solution in RPMI 1640 medium, at the concentrations ranging from 0-10,000 nM were added into each well of 96 well plates. Well A was free of drug and served as a control, whereas wells B-H contained ascending concentrations of chloroquine, each concentration of which was tested in triplicate. The tested plate was incubated at 37.5°C in a candle jar for 24-36 hr depending on the stages of the parasite before culturing. After incubation, the plate was placed in semi-vertical position for 15 min, and then a thick blood film was prepared from the pellets of each well. The number of normal schizonts (containing > 8 nuclei) per 200 asexual stage parasites of control well and each drug containing well was counted and expressed as the percentage of schizont maturation inhibition.

Table 1. List of PCR primers for amplification of Pvmdr1 and Pvcrt-o genes

| Primer | Sequence 5’ to 3’ | nt position |
|--------|-------------------|-------------|
| Outer primer: | | |
| mdr2F: TGGAACAGAAGGGGACCTT | 82-101 |
| mdr2R: CAACTGCAACTATAGTCAC | 4371-4351 |
| crtR: GTATTGGAAAGAAGCACAAGT | -173 |
|.crtR: GATTGGAAAGAAGCACAAGT | 1853-1834 |
| Nested primer: | | |
| mdr1F: GGGATAAGCTTCTAAATGACCT | 857-866 |
| mdr2F: ATTTAACCTTTCGAAAAAGGTGTT | 783-805 |
| mdr2R: CCACCTCAGAACACTATAGTCAC | 1748-1729 |
| mdr3F: CGTATACAGTGGAGAAAGACT | 1600-1623 |
| mdr3R: AATGTCAGTGCTAGAAAAGC | 1757-1738 |
| mdr4F: CCGTCTACTAGTCTCTATGG | 2600-2820 |
| mdr4R: TGGTCTTGAGAAGCTATCAT | 3531-3510 |
| mdr5F: GGAATCTCATCAGCGAGAGATTG | 2751-2772 |
| mdr5R: CCGATACCCCGGCCAGGTGC | 3354-3335 |
| mdr6F: GGAGTTGTTGCTTCCTAAAGG | 3344-3364 |
| mdr6R: GATCGGACGCTTACGGGAGTTGG | 49-70 |
| crtF: GATCGGACGCTTACGGGAGTTGG | 1772-1751 |
fied by nested or semi-nested PCR using 6 pairs of primers as follows: mdrF-mdr1R, mdr2F-mdr2R, mdr3F-mdr3R, mdr4F-mdr4R, mdr5F- mdr5R, and mdr6F-mdrR. The Pvcrt-o gene was amplified by nested PCR using a pair of primers: crtF-crtR. The nested PCR was carried out in a total volume of 50 µl in the following reaction mixture: 0.2 µM of each primer, 2.5 mM MgCl₂, 100 mM KCl, 20 mM Tris-HCl, pH 8.0, 100 µM deoxy-nucleotides (dNTPs), 1 µl of first PCR product, and 0.5 unit of Taq DNA polymerase. The PCR cycling parameters were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, and then followed by final extension at 72°C for 5 min. The PCR products were fractioned by 1.5% agarose gel electrophoresis, purified with QiAquick PCR purification kit (Qiagen), and sequenced by automated DNA sequencer (ABI system, Singapore). Each fragment was sequenced in both the forward and reverse directions and assembled using Bioedit version 7.1.3. DNA fragments were aligned using the ClustalW multiple sequence alignment. Amino acid sequence alignments of all isolates were compared with the wild-type sequence from GenBank for Pvmdr1 (accession no. AY618622) and Pvcrt-o (accession no. AF314649) using Bioedit version 7.1.3 (Ibis Biosciences, Carlsbad, California, USA).

Data analysis
The IC₅₀ are presented as median (range) values. The association between Pvmdr1 polymorphisms and in vitro susceptibilities of the parasite isolates to chloroquine was assessed by Mann-Whitney U test and chi-square test. Statistical significance level was set at P = 0.05 for all tests (SPSS version 12).

RESULTS
In vitro susceptibilities of parasite isolates
A total of 30 P. vivax isolates were successfully evaluated for their susceptibilities to chloroquine by schizont maturation inhibition assay. The median (range) IC₅₀ was 134.7 (1.1-264.9) nM. Based on the cut-off IC₅₀ of 100 nM, 19 isolates (63.3%) were classified as chloroquine resistant isolates.

Analysis of Pvmdr1 gene polymorphisms
The Pvmdr1 gene was successfully sequenced in 30 P. vivax isolates. Analysis of Pvmdr1 sequence showed absence of mutations at the amino acid residues 91, 189, 1,071, 1,079, and 1,291, corresponding to the amino acid residues 86, 184, 1,034, 1,042, and 1,246 of the Pfmdr1 gene, respectively. Seven non-synonymous mutations were identified, i.e., S513R (agt/aga) (23.3%), S515R (agc/agg) (100%), G698S (ggc/agc) (100%), M908L (atg/ctg) (100%), T958M (acg/atg) (100%), Y976F (tac/ttc) (23.3%), and F1076L (ttt/ctt) (53.3%). Two synonymous mutations at the amino acid residues T529 (acg=90%) or T529 (aca=10%), and L1022 (cta) (100%) were also identified (Table 2). Five haplotypes of Pvmdr1 were observed, of which the majority of the isolates (14/30) carried 5 mutations (513R+515R+698S+908L+958M and 515R+698S+908L+958M+1,076L). Isolates carrying 4 mutations (515R+698S+908L+958M) and 6 mutations (513R+515R+698S+908L+958M+1,076L and 515R+698S+908L+958M+976F+1,076L) were found in equal

| Table 2. Frequency distribution of mutation at each codon and haplotype in Pvmdr1 gene of 30 P. vivax isolates |
|----------------------------------------------------------|
| **Amino acid residue at the codon indicated** | **No. (%) of isolates with Pvmdr1 mutant alleles** |
| S513R (agt/aga) | 7 (23.3) |
| S515R (agc/agg) | 30 (100) |
| T529 (aca/acg) | T529 (aca)= 3 (10.0), T529 (acg)= 27 (90.0) |
| G698S (ggc/agc) | 30 (100) |
| M908L (atg/ctg) | 30 (100) |
| T958M (acg/atg) | 30 (100) |
| Y976F (tac/ttc) | 7 (23.3) |
| L1022 (cta/ta) | L1022 (cta)= 30 (100) |
| F1076L (ttt/ctt) | 16 (53.3) |

**Frequency distribution of the 5 haplotypes in Pvmdr1**
- 513R+ 698S+ 908L+ 958M (4 mutations) 8 (26.7)
- 513R+ 515R+ 698S+ 908L+ 958M (6 mutations) 6 (20.0)
- 513R+ 608S+ 908L+ 958M+ 1,076L (5 mutations) 8 (26.7)
- 513R+ 515R+ 698S+ 908L+ 958M+1,076L (6 mutations) 1 (3.3)
- 515R+ 698S+ 908L+ 958M+ 976F+ 1,076L (6 mutations) 7 (23.3)
The polymorphisms in Pvmdr1 gene at the 4 highly polymorphic loci (S513R, Y976F, F1076L, and T529) including the other 5 loci and IC50 values of 15 selected P. vivax isolates with wild-type and mutant genotypes

| Median (range) Chloroquine IC50 (nM) | S513R | S | 132.56 (39.99-243.17) |
|--------------------------------------|--------|---|------------------------|
|                                     | R      | 141.99 (81.86-248.86)  |
|                                     | S      | 137.04 (39.99-248.86)  |
|                                     | T (aca) | 43.24  |
| G698S (n= 15)                        | T (acg) | 139.52 (39.99-248.86) |
| M908L (n= 15)                        |        | 137.04 (39.99-248.86) |
| T958M (n= 15)                        |        | 137.04 (39.99-248.86) |
| Y976F (n= 13)                        |        | 137.04 (39.99-248.86) |
|                                     | F (n= 2) | 165.19 (87.21-243.17) |
| L1022 (n= 15)                        |        | 137.04 (39.99-248.86) |
| F1076L (n= 6)                        |        | 119.41 (39.99-248.86) |
|                                     | L (n= 9) | 137.04 (43.24-243.17)  |

Data are presented as number of isolates (n) and median (range) IC50 values.

Table 3. The polymorphisms in Pvmdr1 gene at the 4 highly polymorphic loci (S513R, Y976F, F1076L, and T529) including the other 5 loci and IC50 values of 15 selected P. vivax isolates with wild-type and mutant genotypes

Association between gene polymorphisms and chloroquine susceptibility

Fifteen P. vivax isolates were randomly selected for the analysis of in vitro susceptibility to chloroquine together with Pvmdr1 and Pvcrt-o polymorphisms (Table 3). There was no significant correlation between polymorphism of Pvmdr1 and chloroquine IC50 values (Mann-Whitney U test, \( P > 0.05 \)). Based on the cut-off IC50 of 100 nM, the frequencies of Pvmdr1 polymorphisms of all P. vivax isolates classified according to in vitro susceptibility to chloroquine are summarized in Table 4. No association between polymorphism of Pvmdr1 and chloroquine susceptibility was found.

### DISCUSSION

Chloroquine resistance in P. vivax has been reported since 1989 in Indonesia and further sporadic cases were subsequently observed in other Asian countries. 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 [19]. There has been, however, no evidence of clinical resistance of P. vivax to chloroquine until the recent report of first clinically and laboratory confirmed case of high grade chloroquine resistant P. vivax in a pregnant woman from the western border of Thailand [20]. Monitoring of the efficacy of chloroquine in P. vivax is essential for earlier warning system and expedites the appropriate drug policy. Various in vitro assay systems with different endpoint criteria have been applied for monitoring of sensitivity of P. vivax isolates to antimalarial drugs. Although the monitoring of drug susceptibility based on the in vitro schizont maturation assay is more complicated in P. vivax compared with P. falciparum due to the lack of a continuous cultivation system and the asynchronous blood stage of the parasite, the assay remains the gold standard method for assessing the antimalarial drug efficacy. The assay has been applied for assessing P. vivax sensitivity to antimalarial drugs with relatively low success rate compared with P. falciparum. The in vitro cut-off IC50 of 100 nM for P. falciparum was used to define chloroquine resistance, but this cut-off has never been clearly defined for P. vivax. Based on this defined cut-off value, a total of 19 P. vivax isolates (63.3%) included in our current study were classified as chloroquine resistance. These results indicate an increasing prevalence of chloroquine resistant isolates in the western border of Thailand. In a previous study, Suwanarusk et al. [10] defined the cut-off IC50 of 220 nM based on the 35th percentile of the clinical failure rate of 65% isolates.
observed in Indonesian and Thai patients (from Mae Sot District) with *P. vivax* malaria. The sensitivity of *P. vivax* isolates collected from Thailand were found to be significantly lower than that of the Indonesian isolates (geometric mean IC₅₀ of 46.8 nM in Thai isolates compared to 312 nM in Indonesian isolates). Eleven out of 81 (13.6%) Thai isolates exhibited IC₅₀ of chloroquine over 220 nM [10]. Using this defined criteria, about 20% (6 isolates) of *P. vivax* collected in the present study exhibited IC₅₀ values over this threshold.

Unlike chloroquine resistant *P. falciparum*, the mechanisms 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*. Association between the in vitro sensitivity and the polymorphisms of *Pmdr1* and *Pcrt-o* as markers of chloroquine resistance were investigated in the present study. The *Pmdr1* gene was found to be more polymorphic than *Pcrt*, of which 7 non-synonymous and 2 synonymous mutations were observed. *Pmdr1* Y976F mutation which has been identified as a possible genetic marker for chloroquine resistance in *P. vivax* was detected only in 7 out of 30 (23.3%) *P. vivax* isolates. This observed frequency of mutation is in concordance with the results from in vitro drug susceptibility test revealing that 6 isolates (20%) were chloroquine resistant based on the cut-off IC₅₀ of 220 nM. In a previous study, the mutations at Y976F and F1076L were detected in 17% and 21%, respectively in *P. vivax* Thai isolates [11].

The observation of high frequency of F1076L (53.3%) mutation in our present study was similar to that reported in a previous study in isolates from Papua New Guinea. The frequency of Y976F *Pmdr1* mutation was much lower than the Papua New Guinea isolates (53.3% vs 100%), while none was found in the isolates collected from Korea [12]. The frequency of single mutation at Y976F or F1076L (76.6%) observed in our study was higher than the double (Y976F+F1076L) (23.3%) mutant. In another study on *P. vivax* isolates collected from Thailand, Indonesia, Turkey, Azerbaijan, and French Guiana, this Y976F and F1076L mutation of the *pmdr1* gene were found at a relatively high frequency of 60.8% (14 of 23 *P. vivax* isolates), of which 5 isolates (21.7%) was observed in the isolates collected from Thailand, 4 from Azerbaijan, 3 from Turkey, and 2 from Indonesia. Altogether, results 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 the trend of rapid aggravation of chloroquine resistance *P. vivax* in this region. However, no association between polymorphisms of *Pmdr1* and chloroquine susceptibility was observed in our study. This could be due to the limitation of the number of samples included in the analysis.

In a clinical study in Madagascar, no significant association between the *Pmdr1* Y976F mutation and chloroquine treatment outcome of *P. vivax* was also reported [15]. In another study, however, the sensitivity to chloroquine was shown to be significantly higher (1.7 fold) in *P. vivax* isolates from Thailand and Indonesia which carried the Y976F mutation but not with that increased *Pmdr1* copy number, compared with the wild type isolates [10]. Although other molecular determinants may also be involved with chloroquine resistance in *P. vivax*, Y976F and F1076L mutations could be used as reliable molecular markers for monitoring the occurrence and spread of chloroquine resistance in *P. vivax* in Thailand.

Apart from the mutations at both codons, 2 non-synonymous mutations at codon T529 (aca = 10%, aca = 90%) and L1022 (cta = 100%) were also detected in the *P. vivax* isolates collected in the current study. The commonly found mutations at codon S515R, G698S, M908L, and T958M were also detected in all isolates. Sequence analysis of *Pmdr1* gene has shown no mutation at codons 91, 189, 1,071, 1,079, and 1,291 homologous to *Pfmdr1* at codons 86, 184, 1,034, 1,042, and 1,246, respectively, which are associated with chloroquine resistance in *P. falciparum*. The majority of isolates (46.6%) carried 5 mutations, whereas 26.7% carried 4 and 6 mutations in *Pmdr1* gene, respectively. It is interesting that polymorphism in *Pmdr1* gene was higher than *Pfmdr1* gene which might be a factor that contributed to in vitro resistance parasites.

Unlike *P. falciparum* of which mutation of *Pfcr* gene are closely linked with parasite’s resistance to chloroquine, the role of the polymorphisms of this gene in conferring chloroquine resistance may be limited to *P. falciparum*. Point mutations in *Pfcr* gene especially K76T are strongly associated with chloroquine resistance phenotype in *P. falciparum* [21]. However, chloroquine resistance in *P. vivax* does not seem to involve *Pcrt*10, the *P. vivax* ortholog of *Pfcr* [22]. Only wild-type *Pcrt-o* was detected in our samples, which is similar to that found in the isolates from Madagascar [15]. Identification of difference in the orthologous gene of *P. falciparum* and *P. vivax* is important for comparison of genetic determinants of chloroquine resistance in both malaria species. The mechanism of chloroquine resistance is probably similar in both *P. falciparum* and *P. vivax*, but the development of resistance may be differ-
ent in these 2 malaria species.

ACKNOWLEDGMENTS

The study was supported by Chulalongkorn University Centenary Academic Development Project, and the Office of Commission on Higher Education (RG and NRU Projects), Ministry of Education of Thailand. We thank the patients who participated in this study and the staff of the Mae Sot General Hospital and Section 4, Vector Borne Disease Control, Mae Sot District, Tak Province for their kind assistance during blood sample collection.

CONFLICT OF INTEREST

We have no conflict of interest related to this work.

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