Prevalence of molecular markers associated with drug resistance of *Plasmodium vivax* isolates in Western Yunnan Province, China

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**Abstract**

**Background:** *Plasmodium vivax* is the most widely distributed malaria parasite, and its drug resistance poses unique challenges to malaria elimination. The Greater Mekong Subregion (GMS) is known as the global epicenter of multidrug resistance. Surveillance of molecular markers associated with drug resistance in this area will help to inform drug policy.

**Methods:** Dry blood spots from 58 patients out of 109 with *P. vivax* infection between 2017, December and 2019, March were obtained from Yingjiang County, Yunnan Province, along the China–Myanmar border. *Pvdhfr*, *Pvdhps*, *Pvmdr1* and *Pvcrt-o* were amplified and sequenced to assess gene mutations. The polymorphism and prevalence of these molecular markers were analyzed.

**Results:** Mutations in *Pvdhfr* at codons 57, 58, 61, 99 and 117 were detected in 27.59, 48.28, 27.59, 32.76 and 48.28% of the isolates, respectively. Single mutant haplotype (*I 13F57S58T61 S99S117I173*) was the most frequent (29.31%, 17/58), followed by double mutant haplotype (20.69%, 12/58). Of three types of tandem repeat variations of *Pvdhfr*, deletion type was the most common. *Pvdhps* showed a lower prevalence among mutation genotypes. Single mutant was dominant and accounted for 34.48% (20/58). Prevalence of *Pvmdr1* mutations at codons 958 and 1076 were 100.00% and 84.48%, respectively. The proportion of double and single mutant types was 84.48% (49/58) and 15.52% (9/58), respectively. Eleven samples (18.97%, 11/58) showed K10 "AAG" insertion in chloroquine resistance transporter gene *Pvcrt-o*.

**Conclusions:** There was moderate diversity of molecular patterns of resistance markers of *Pvdhfr*, *Pvdhps*, *Pvmdr1* and *Pvcrt-o* in imported *P. vivax* cases to Yingjiang county in Western Yunnan, along the China–Myanmar border. Prevalence and molecular pattern of candidate drug resistance markers *Pvdhfr*, *Pvdhps*, *Pvmdr1* and *Pvcrt-o* were demonstrated in this current study, which would help to update drug policy.

**Background**

*Plasmodium vivax* is the most widely distributed malaria parasite, and although it causes less significant morbidity and mortality than *Plasmodium falciparum* does, it poses unique challenges in many countries [1]. In 2017, it was estimated to be responsible for 7.5 million cases globally, and nearly 56% in Southeast Asia [2]. Recently, there has been a massive reduction in malaria cases and deaths in the Greater Mekong Subregion (GMS), which comprises Cambodia, Yunnan Province of China, Lao People’s Democratic Republic, Myanmar, Thailand and Vietnam. However, GMS has been the global epicenter of multidrug resistance. Resistance emerged to chloroquine (CQ) in the 1960s, sulfadoxine–pyrimethamine...
(SP) in the 1970s, mefloquine in the late 1990s, and ato- 
imisin in 2008, and then spread progressively through- 
out other malaria-endemic areas [3–6]. This has raised 
concern from the World Health Organization (WHO) 
and local health authorities [2, 7, 8]. Malaria transmis-
sion in international border areas is usually confounded 
by population mobility and distinct chemotherapy pol-
icy strategies and antimalarial strategies. The China–Myan-
mar border, as part of the GMS, included 18 counties of 
Yunnan Province. Although no indigenous cases have 
been identified in Yunnan Province since 2017, P. vivax 
remains a challenge, with increasing evidence of abun-
dant vector species richness and diversity, high malaria 
vulnerability resulting from mobile population, as well as 
drug resistance [9, 10]. In Myanmar, the proportion of 
malaria cases caused by P. vivax has increased steadily 
since 2012 [11].

CQ was first produced in 1934 and quickly proved to be 
one of the most successful and important antimalarial 
agents [12]. Nevertheless, the heavy use of CQ throughout 
subsequent decades eventually led to drug resistance. P. 
falciparum developed resistance in various areas since the 
1950s, but drug-resistant P. vivax was not reported until 
the 1980s in Indonesia and Papua New Guinea [12, 13]. 
To date, CQ-resistant P. vivax has been confirmed in 
more than 10 countries, including Myanmar and China 
[14]. There has been a long history of successful applica-
tion of SP in combating malaria due to its safety, good tol-
erance and long-lasting activity [15]. In China, as a 
component of the two combination regimens, pyrimeth-
amine was widely used for malaria prophylaxis between 
the mid-1960s and early 1990s [16]. By now, SP is recom-
manded by WHO as one of the partner drugs for treat-
ment of P. falciparum in the GMS, as well as intermittent 
preventive treatment for infants, children and pregnant 
women [2, 15]. Although SP is rarely used to treat P. vivax 
infection, the parasite is still under SP selection pressure, 
especially in endemic regions where co-infection with P. 
vivax and P. falciparum is common.

Compared with P. falciparum, it is more difficult to de-
termine the underlying mechanisms of antimalarial drug 
resistance of P. vivax because there is no proper in vitro 
cultivation system for P. vivax. This means that the mo-
lecular mechanism of P. vivax resistance remains to be 
established. Several studies suggest that it involves multi-
genic loci, such as CQ resistance marker Pvcrt-o; multi-
drug resistance marker Pvmdr1; and antifolate resistance 
markers Pvdhps and Pvdhfr, which are conferred from 
homologous genes in P. falciparum [11, 17]. Data for mo-
lecular markers associated with drug resistance would be 
beneficial in addressing the resistant parasite. Few studies 
to date have defined the molecular epidemiology of P. 
vivax resistance markers on the China–Myanmar Border 
[18, 19]. Here, we report the prevalence of molecular 
markers of drug resistance in P. vivax to facilitate appro-
priate drug policy in this region.

Methods
Study site
Yingjiang (Longitude 97°31′ ~ 98°16′,Latitude 24°24′ ~ 
25°20′) is one of the 18 counties along the China– 
Myanmar border, located west of Yunnan Province. It 
was selected as the study site due to its long borderline 
with Kachin State, Myanmar and being well documented 
as an epidemic area of resistant P. falciparum [18]. The 
land area of Yingjiang County is 4429 km² and the local 
population was 316, 990 by 2015. It is located in the 
subtropical monsoon climate zones with an average an-
nual temperature of 22.7 °C and annual rainfall of 2.65 
m. Migration, plantation and logging activities are fre-
quently at the border [18]. Anopheles minimus is reported to 
be the dominant species of mosquito [20]. Ninety-
three malaria cases were reported in Yingjiang County in 
2016 and 2017, respectively, and there were 103 cases 
in 2018. P. vivax was the dominant parasite and all the 
cases of malaria were imported after May, 2016.

Sample collection and DNA extraction
Isolates were obtained from 58 out of 109 confirmed P. 
vivax infected patients from December 2017 to March 
2019 in Yingjiang County. All the infections were diag-
nosed and reported by hospitals or clinics in Yingjiang 
County. Yingjiang County Center for Disease Control 
and Prevention carried out epidemiological investigation 
of each patient. They were double-checked for species 
by PCR in Yunnan Institute of Parasitic Diseases. All the 
patients, according to epidemiological history, were 
imported from Laiza, Myanmar, which was along the 
China–Myanmar border. Thick and thin blood smears 
coupled with standard microscopy techniques were used 
to identify parasite species, then PCR was used to double 
check and confirm species. Approximately 200 µL of 
finger- prick blood was obtained from each patient before 
treatment and spotted on Whatman 3 MM filter paper 
(10 cm × 7 cm, Cat. No. 3030–866) and air dried. The 
dried blood spot was about 6 mm for diameter. They 
were stored in small plastic zip lock bags with desiccants 
at ~ 20 °C before parasite genomic DNA extraction. 
QIAamp DNA Mini kit (Qiagen Inc., Hilden, Germany) 
was used to extract genomic DNA following the dried 
blood spot protocol.

DNA amplification and sequencing
Multiple molecular markers, Pvcrt-o, Pvmdr1, Pvdhps 
and Pvdhfr, suspected conferring drug resistance on P. 
vivax, were detected. Pvcrt-o was amplified by regular 
PCR and Pvmdr1, Pvdhps and Pvdhfr by nested PCR, as 
previously described, with some modification [17, 21].
Oligonucleotide primers and cycling conditions are listed in additional file (see Additional file: Table S 1). A final 25-μL reaction volume was performed, of which 1 μL template genomic DNA was used in primary amplification reactions, and 1 μL primary reaction products in the second round of amplification in the case of nested PCRs. Amplification products were sequenced by Sangon Biotech Co. Ltd. (Shanghai, China).

Data analysis
Nucleotide and amino acid sequences of Pvcrt-o, Pvmdr1, Pvdhps and Pvdhfr were aligned and compared with reference sequences from NCBI database by Mega version 7.0.26 (https://www.megasoftware.net/). Accession numbers for reference sequences were: Pvcrt-o (AF314649), Pvmdr1 (AY618622), Pvdhps (XM001617159) and Pvdhfr (X98123). A database was constructed by Microsoft Excel 2017, and descriptive statistical analysis was performed with SPSS Statistics for Windows version 21.0 (IBM Corp., Armonk, NY, USA). Categorial data were summarized by percentage, quantitative variables were expressed as median.

Results
General information
We collected data from 58 patients (40 males, 68.97%; 28 females, 31.03%) with P. vivax infections between 2017 and 2019, of which, the majority (89.66%, 52/58) were collected in 2018. The median (range) age of the 58 patients was 34.5 (3–69) years. Nine (15.52%) patients had a history of malaria. Most patients lived (4/58, 6.90%), studied (1.72%, 1/58) or worked (planting, 50.00%, 29/58; trading, 6.90%, 4/58) in Myanmar, whereas 20 patients were infected when they visited relatives or friends (27.59%, 16/58), or during business trips (6.90%, 4/58) in Myanmar (Table 1).

Prevalence and patterns of Pvdhfr mutations
Mutations in Pvdhfr at codons 57, 58, 61, 99 and 117 were detected in 27.59, 48.28, 27.59, 32.76 and 48.28% of isolates, respectively. No mutations were found at position 13 or 173 (Table 2). Analysis of Pvdhfr haplotype revealed that prevalence of mutant types was present at high levels (Table 3, Fig. 1). Both single and multiple mutant Pvdhfr (double, quadruple and quintuple) were found. Single mutant haplotype (I 13F57S58T61S99S117I173) was the most frequent (29.31%, 17/58), followed by double mutant haplotype (20.69%, 12/58). Quadruple mutant haplotypes, exhibiting two distinct patterns, were also found in 14 isolates, and the pattern I 13F57R58M61H99T117I173 was more frequent (27.59%, 16/58). Table 1 gives a summary of the general information of P. vivax infections.

Table 1 General information of P. vivax infections

| General information | Number (%) |
|---------------------|------------|
| Year                |            |
| 2017                | 3 (5.17)   |
| 2018                | 52 (89.66) |
| 2019                | 3 (5.17)   |
| Gender              |            |
| Male                | 40 (68.97) |
| Female              | 18 (31.03) |
| Age                 |            |
| Range               | 3 ~ 69 yr  |
| Median              | 34.5 yr    |
| History of malaria infection |       |
| Yes                 | 9 (15.52)  |
| No                  | 49 (84.48) |
| Activities in Myanmar |         |
| Planting            | 29 (50.00) |
| Visiting relatives or friends | 16 (27.59) |
| Business trip       | 4 (6.90)   |
| Trading             | 4 (6.90)   |
| Living              | 4 (6.90)   |
| Studying            | 1 (1.72)   |

Table 2 Prevalence of point mutations at specific positions in Pvcrt-o, Pvmdr1, Pvdhps and Pvdhfr of P. vivax isolates

| Genes   | Mutation at codon | Number (%) |
|---------|-------------------|------------|
| Pvdhfr  | 13                | 0          |
|         | 57                | 16 (27.59) |
|         | 58                | 28 (48.28) |
|         | 61                | 16 (27.59) |
|         | 99*               | 19 (32.76) |
|         | 117               | 28 (48.28) |
|         | 173               | 0          |
| Pvdhps  | 382               | 1 (1.72)   |
|         | 383               | 29 (50.00) |
|         | 512               | 1 (1.72)   |
|         | 553               | 9 (15.52)  |
|         | 580               | 0          |
|         | 585               | 0          |
| Pvmdr1  | 958               | 58 (100.00) |
|         | 976               | 0          |
|         | 997               | 0          |
|         | 1076              | 49 (84.48) |
| Pvcrt-o | K10 insertion     | 11 (18.97) |

* Deletion type was not included
common. Quintuple mutant $I_{13}I_{57}R_{58}M_{61}S_{99}T_{117}I_{173}$ was detected in two isolates. Notably, two genotypes were detected at codons 57 and 117. Specifically, $F_{57}I$ and $F_{57}L$ at position 57, were observed in 11 (18.97%) and 5 (8.62%) isolates, respectively, and the frequency for $S_{117}T$ and $S_{117}N$ was 16 (27.59%) and 12 (20.69%), respectively.

Three types of tandem repeat variations were found in $Pvdhfr$. Type I was the same as the reference strain (accession number X98123), whereas type II showed mutant allele H99S, and type III exhibited a deletion of 18 nucleotides (ACACACGGTGTTGACAC, translated into THGGDN) between amino acid positions 98 and 103 (Fig. 2). Type III was the most common, accounting for 37.93% (22/58), followed by Type II which was observed in 19 (32.76%) isolates. In addition, more than half of Type III isolates (12/22, 54.55%) also carried S58R and S117N mutations.

Prevalence and patterns of $Pvdhps$ mutations
All 58 samples were successfully amplified for $Pvdhps$. Compared with $Pvdhfr$, $Pvdhps$ showed a relatively lower prevalence of mutation genotypes. Minority of isolates carried mutations at codons 382 (1.72%, 1/58), 512 (1.72%, 1/58) and 553 (15.52%, 9/58) (Fig. 2). Mutation at position 383 was detected in half of the isolates. Among the mutant types, single mutant was dominant and accounted for 34.48% (20/58). Double mutant $S_{382}G_{383}K_{512}G_{553}R_{580}V_{585}$ was less frequent (13.79%, 8/58). Quadruple mutant $C_{382}G_{383}E_{512}G_{553}R_{580}V_{585}$ was only found in one $P. vivax$ isolate. $S_{382}C$ and $K_{512}E$ were rarely observed in previous studies.

Prevalence and patterns of $Pvmdr1$ and $Pvcrt-o$ mutations
Prevalence of mutations at codons $Pvmdr1$ 958 and 1076 was 100.00 and 84.48%, respectively. No single nucleotide polymorphism was present at either codon 976 or 997. Analysis of $Pvmdr1$ haplotype prevalence showed that all the isolates were mutant type. In particular, double mutant predominated (84.48%, 49/58). Single mutant was found in nine isolates (15.52%, 9/58). Eleven samples (18.97%, 11/58) showed K10 “AAG” insertion in CQ resistance transporter gene $Pvcrt-o$ (Fig. 2). A combined analysis of all mutations in 58 samples revealed 25 different haplotypes (see Additional file: Table S2).

Discussion
Drug resistance is of great concern for malaria control and prevention, especially in GMS, necessitating monitoring resistance to antimalarial agents. However, since its first report in 1989, the burden of drug-resistant $P.$
vivax is still unclear and its underlying mechanism, epidemiology and drug efficacy have not been well characterized [17]. Four main methods, in vivo therapeutic efficacy studies, in vitro assay, drug concentration measurement, as well as molecular markers analysis, are used to monitor antimalarial drug efficacy and resistance. Among these, molecular markers are widely preferred due to their practical advantages over in vivo and in vitro tests. Molecular markers allow population-level screening, and samples on filter paper are easily obtained, transported and stored, thus avoiding host confounding factors [22]. The epidemiology of drug resistance of P. vivax varies across the GMS, hence molecular marker surveillance is encouraged to inform local drug policy.

In the present study, the prevalence of Pvdhfr mutation type, including point mutation and mutant tandem repeat, was high (94.83%, 55/58), which was similar to the reports in southern Thailand and western Myanmar. However, one study in Xishuangbanna Prefecture of Yunnan Province (southern Yunnan, bordering Myanmar in the west and Laos and Vietnam in the south) between 2009 and
found in our study, while quadruple mutation was the most common in Myanmar, Thailand and southern Yunnan [16, 17, 23, 25]. Previous studies identified that mutations at residues 117 and 58 arose first under drug pressure, so they were more highly mutated than others [26]. These results confirmed that the mutation types at codons 117 and 58 were the most frequent. Triple and quadruple mutations were more associated with high level of SP resistance than double or single mutations were. Our study indicated that *P. vivax* in western Yunnan might be under stronger drug pressure than those in western Myanmar and southern Thailand [27].

Mutant tandem repeats are also suggested to be associated with *P. vivax* antifolate resistance, and the frequency of Type II (H99S type) and Type III (deletion type) was 32.75 and 37.93%, respectively. This was consistent with a previous studies that reported that most isolates in India and Cambodia were deletion type [24, 28]. Nevertheless, the highest frequency of tandem repeat variants was for wild type in southern Thailand and Xishuangbannna Prefecture, Yunnan [16, 23]. In Anhui Province (Central China), Type II (H99S type) was the most common [16]. Similar to *Pvdhfr*, the frequency of mutant *Pvdhps*, especially the highly mutant types (triple or quadruple types), was less than that in southern Thailand and southern Myanmar [21, 23]. In addition, compared with another border region, Xishuangbannna of Yunnan, *Pvdhps* in our sampling region was more conserved, with higher proportions of wild type and fewer highly mutated types, although the isolates from Xishuangbannna were collected nearly 10 years ago [16]. Considering the similar drug policy in this study area and Xishuangbannna, it is still unclear whether the disagreement resulted from spatial heterogeneity or drug susceptibility to sulfadoxine and as such, further study is required.

Several studies have provided evidence that *Pvmdr1* mutations are associated with reduced susceptibility to CQ [17, 29, 30]. Therefore, *Pvmdr1* is considered to be a strong candidate marker of drug resistance [23]. The prevalence of *Pvmdr1* T958M and F1076L mutations in our study was consistent with previous studies, showing that T958F was harbored in all the isolates and F1076L was in most of them [13, 21]. However, no Y976F was found in our study, while it was frequently reported with considerable prevalence in different endemic areas, including Indonesia, Thailand, Cambodia, India, Papua New Guinea and Ethiopia [29–35]. This is not surprising as 98.51% of patients were categorized as having an adequate clinical and parasitological response to CQ by an in vivo therapeutic efficacy study in Yingjiang and Tengchong, Yunnan [36]. Our study indicated that *Pvmdr1* at codon 976 was conserved in this area, although this needs to be confirmed.

The possible role of *Pvcrt-o* in CQ resistance is controversial. Several studies have found a negative link between K10 insertion and reduced CQ IC50, while others have shown that *Pvcrt-o* expression decreased susceptibility to CQ by 2.2-fold [30, 37]. The K10 *Pvcrt-o* gene insertion was found in 18.97% isolates in our study, which was less than in previous studies in Myanmar that reported 46.15% in Yangon in 1999, 72.73% in Shwegyin, 66.67% in Kawthaung and 48.33% in Buthidaung between 2009 and 2016 [17, 35]. Conversely, K10 insertion was rarely observed in Thailand from 2012 to 2018, or from the Thailand–Myanmar border or Thailand–Cambodia border in 2008 or 2014 [21, 23]. Given the geographical genetic differences among parasite populations from the GMS, the prevalence of K10 insertion in *Pvcrt-o* in the current and previous studies showed significant temporal and spatial heterogeneity [38]. The discrepancy may have resulted from differences in study sites or sample size.

**Conclusions**

In conclusion, the present study demonstrated the prevalence and molecular pattern of candidate drug resistance markers *Pvdhfr*, *Pvdhps*, *Pvmdr1* and *Pvcrt-o* of imported *P. vivax* cases to Yingjiang county in Western Yunnan, along the China–Myanmar border. Diversity of molecular patterns of resistance markers *Pvdhfr*, *Pvdhps*, *Pvmdr1* and *Pvcrt-o* was found. This study helped to provide evidence for drug policy update.

**Supplementary information**

Supplementary information accompanies this paper at https://doi.org/10.1186/s12879-020-05032-4.

**Additional file 1** Table S1 Primers and cycling conditions for *Pvcrt-o*, *Pvmdr1*, *Pvdhps* and *Pvdhfr* genotyping assay. Table S2 Combined analysis of all mutations from *P. vivax* isolates.

**Abbreviations**

GMS: Greater Mekong subregion; WHO: World Health Organization; CQ: Chloroquine; IC50: Inhibitory concentration 50

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Authors’ contributions
XXW, XYF and HY carried out the molecular studies. SSZ and FH conceived the study. XXW and WR analyzed the data and drafted the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets analyzed in this study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
The datasets analyzed in this study are available from the corresponding author.

Consent for publication
XXW, XYF and HY carried out the molecular studies. SSZ and FH conceived the study. XXW and WR analyzed the data and drafted the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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