

Different Allele Prevalence in the Dihydrofolate Reductase and Dihydropteroate Synthase Genes in *Plasmodium vivax* Populations from China

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Abstract. Antifolate resistance in *Plasmodium vivax* is caused by point mutations in genes encoding dihydrofolate reductase (*pvdhfr*) and dihydropteroate synthase (*pvdhps*). In this study, we used direct sequencing to survey *pvdhfr* and *pvdhps* mutations in 122 clinical *P. vivax* isolates from a central and a southern province of China. For *pvdhfr*, 36.9% were wild-type, whereas mutations were detected at four codons (57, 58, 61, and 117). The S117N/T mutation was the most prevalent (48.4%), followed by the T61M mutation (18.9%). Six *pvdhfr* mutant alleles were found, ranging from 37.7% to 0.8%. The dramatically different *pvdhfr* allele frequencies between the two *P. vivax* populations might be caused by different drug histories or intrinsic difference between temperate and subtropical strains. In contrast, except polymorphisms within a repeat region, no resistance-conferring mutations were detected in *pvdhps*. Our result suggests that *P. vivax* populations in China may be relatively susceptible to sulfadoxine-pyrimethamine.

INTRODUCTION

Plasmodium vivax is the most widespread cause of malaria outside Africa and causes 132–391 million clinical infections each year.¹ Recent evidence of severe disease manifestations associated with *P. vivax* infections has dramatically changed the traditional view of *P. vivax* malaria as the benign tertian malaria.^{2,3} The ability of *P. vivax* to cause disease relapses adds significantly to the morbidity. In China, the malaria eradication campaign advocated by the World Health Organization in the mid-1950s was highly successful, and malaria was nearly eliminated from the central provinces. However, the predominant malaria parasite *P. vivax* in central China has demonstrated resilience to eradication and become increasingly prevalent in five central provinces for the past 10 years.^{4–6} With *P. vivax* malaria outbreaks occurring frequently in many counties of the five central provinces in recent years, *P. vivax* has become the predominant parasite species and is responsible for more than 90% of malaria cases in China.⁷

Although artemisinin-combination therapies show promise for treating patients with *P. vivax* malaria, first-line therapies for the radical cure of *vivax* malaria in China are still chloroquine (CQ) and primaquine. This drug combination has been used for more than 60 years, and there is increasing evidence showing the emergence and spread of CQ resistance in *P. vivax*, especially in Southeast Asia.⁸ Although the antifolate drug combination of sulfadoxine-pyrimethamine (SP) has replaced CQ for treating patients with *P. falciparum* malaria in many malaria-endemic regions, SP has not been recommended for treating patients with *P. vivax* malaria because earlier studies showed that *P. vivax* parasites seemed intrinsically resistant to SP.^{9,10} However, there is evidence indicating that SP resistance in *P. vivax* also results from drug selection pressure. Because mixed species infections with *P. falciparum* and *P. vivax* are common in many malarious regions,¹¹ extensive use of SP to treat patients with *P. falciparum* malaria may have inadvertently exposed *P. vivax* to these drugs. Likewise, rapid emergence and spread of antifolate resistance observed

in *P. falciparum* populations may be equally applicable to *P. vivax* populations.^{12,13}

Drug resistance has always been a challenge for malaria control, and surveillance plays an essential role in resistance management. Resistance mechanisms to SP in both parasite species involve point mutations in the genes encoding dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS), which are therapeutic targets of pyrimethamine and sulfadoxine, respectively.^{14,15} Because *P. vivax* cannot be cultured continuously, antifolate resistance studies have relied heavily on molecular genotyping and drug assays in heterologous systems expressing parasite DHFR and DHPS.¹⁶

Molecular genotyping of *pvdhfr* and *pvdhps* has been performed in many *P. vivax*-endemic areas.¹⁶ To date, some 20 mutations have been detected in *pvdhfr*; among them mutations at codons 57, 58, 61, 117, and 173, corresponding to positions 50, 51, 59, 108 and 164 in *pfdhfr*, respectively, are associated with pyrimethamine resistance.^{17–20} A structural study has confirmed that the most common mutations (S58R and S117N) cause structural changes in *P. vivax* DHFR and lead to decreased binding to pyrimethamine.²¹ A limited number of clinical studies have associated *pvdhfr* quadruple mutations with increased risks of SP treatment failure.^{19,22,23} For *pvdhps*, mutations at codons 382, 383, 512, 553, and 585, corresponding to codons 436, 437, 540, 581, and 613 in *pfdhps*, respectively, are predicted to confer increased resistance to sulfadoxine.²⁴ The ubiquitous mutation V585 in global parasite isolates was proposed to be the key amino acid affecting the conformation of the drug binding domain,²⁴ and other mutations may further reduce the sensitivity to sulfadoxine.^{20,24–27} Molecular surveys carried out in *P. vivax*-endemic areas have detected different geographic distributions and prevalences of *pvdhfr* and *pvdhps* mutations; some regions showed near fixation of some mutant alleles, which is correlated with regional difference in the use of SP.^{27–30} The extensive use of SP in malaria treatment in many malarious regions may be responsible to the multiple origins of resistance-conferring mutations in *pvdhfr* and *pvdhps*.^{31,32}

Most of the studies on antifolate resistance in *P. vivax* were performed in regions where *P. vivax* and *P. falciparum* coexist. Because *P. vivax* malaria has not been directly treated with antifolate drugs, the evolution of mutations in the *pvdhfr* and *pvdhps* genes could only be inferred from possible selection

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by treatment of *P. falciparum* malaria with antifolate drugs. So far, mutations in *pvdhfr* and *pvdhps* have been studied from limited *P. vivax* samples from temperate zone countries, where *P. vivax* is the predominant malaria parasite species and has a dramatically different relapse phenotype compared with tropical strains.^{20,33} To study the evolution of antifolate resistance in *P. vivax*, we investigated genetic variations in *pvdhfr* and *pvdhps* genes of 122 malaria samples collected from two temperate zone provinces of central China (Anhui and Guizhou). Our study identified dramatically different patterns of selection for the *pvdhfr* and *pvdhps* genes.

MATERIALS AND METHODS

Sample collection and DNA extraction. A total of 134 blood samples from patients with *P. vivax* malaria were obtained in 2006 and 2008 from symptomatic patients in two provinces of China. One hundred samples were obtained from Wuhe County in Anhui Province in the temperate zone, and 34 samples were obtained from Luodian County in Guizhou Province in the subtropical zone (Figure 1). All patients were diagnosed by microscopic examination of Giemsa-stained thin and thick blood smears. Fingerprick blood samples were collected on filter papers, dried, and stored at -20°C . DNA was extracted from blood spots by using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The study protocol was reviewed and approved by the Ethics Committees of Guangxi and Anhui, China.

Sequencing *pvdhfr* and *pvdhps*. Genomic DNA was used as template for polymerase chain reaction (PCR) with the Advantage 2 polymerase mixture with proof-reading activity (Clontech, Mountain View, CA). The *pvdhfr* gene was amplified using the primer 5'-ATGGAGGACCTTTCAGATGTA TTTGACATT-3' and 5'-CTTGCTGTAAACCAAAAAGTC CAGAGTGGT-3' as described.³⁴ The PCR was performed with the following parameters: 94°C for 5 minutes; followed by 30 cycles at 94°C for 20 seconds, 60°C for 30 seconds, and 72°C for 1 minute; and a final elongation at 72°C 10 minutes. The *pvdhps* gene was amplified by nested PCR with the outer primers (forward 5'-TTGAAACACGCATTATGGTATCG-3' and reverse 5'-AAGCGTAGCGACAGAAGAACG-3') and inner primers (forward 5'-GAGATTACCCTAAGGTTGAT GTATC-3' and reverse 5'-GGTTTATTGTTCGATCCTGT G-3').^{24,27} Another pair of primers (forward 5'-AATGGCAA GTGATGGGGCGAGCGTGATTGA-3' and reverse 5'-CA GTCTGCACTCCCCGATGGCCGCGCCACC-3') was used to amplify an approximately 700-basepair fragment of *pvdhps* in cases of amplification failure with the original primers. For *pvdhps*, PCR was performed using 35 cycles at 94°C for 1 minute, 60°C for 1 minute, and 72°C for 2 minutes. The PCR products were fractionated by electrophoresis on a 1.5% agarose gel, purified by PrepEase Gel Extraction Kit (United States Biochemicals, Cleveland, OH), and sequenced by using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reactions Kit on a 3730 xl Genetic Analyzer (Applied Biosystems, Foster City, CA).

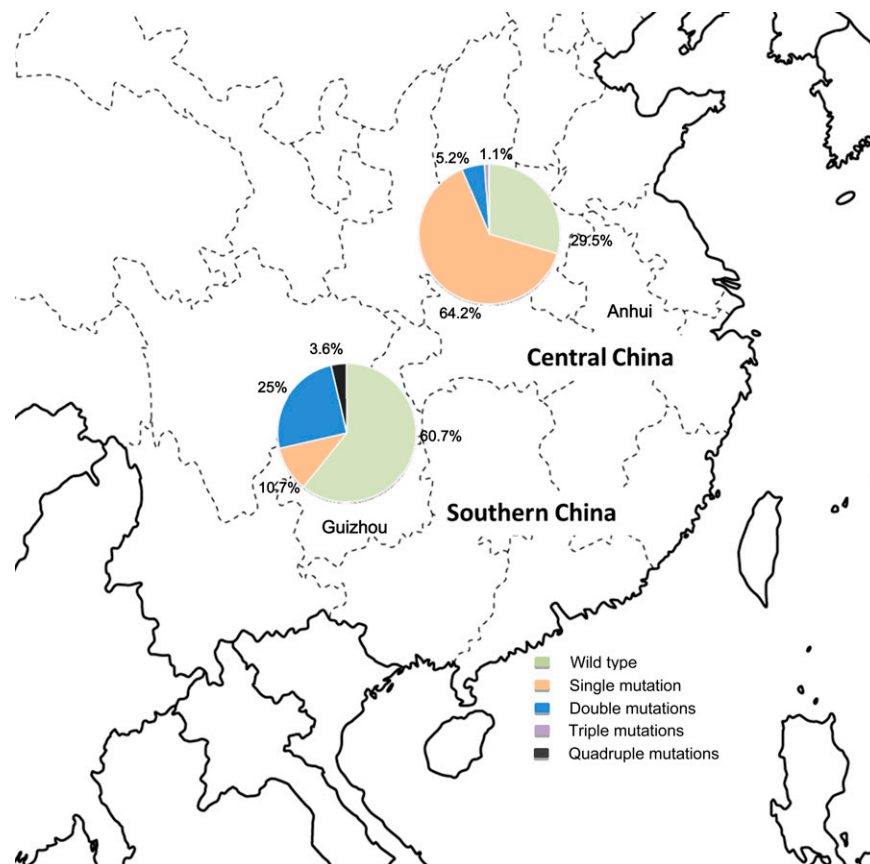


FIGURE 1. Sample collection sites in Anhui Province in central China and Guizhou Province in southern China. The pie charts show the frequency distribution of *Plasmodium vivax* dihydrofolate reductase synthase alleles. This figure appears in color at www.ajtmh.org.

Sequence analysis. Multiple sequence alignment and downstream analysis were carried out by using the CLUSTAL W program³⁵ and the BioEdit package. Amino acid sequences were compared with wild-type sequences (GenBank accession nos. X98123 and AY186730 for *pvdhfr* and *pvdhps*, respectively).

RESULTS

Mutant *pvdhfr* alleles and their frequencies. Sequence polymorphisms were assessed in *pvdhfr* and *pvdhps* genes from 134 *P. vivax* samples obtained from two regions of China. Among these samples, the *pvdhfr* gene was successfully amplified and sequenced in 122 isolates. Compared with the wild-type sequence, *pvdhfr* genes from the 122 clinical samples had point mutations at 10 codons, among which four resulted in amino acid substitutions. The six synonymous point mutations were AAC to AAT (both coding for Asn) at codon 50, and GGT to GGG (both coding for Gly) at codons 89, 94, 95, 100, and 101, respectively. Point mutations at the latter five codons have not been reported¹⁶ and were found in 35 (28.7%) samples. Nonsynonymous mutations were detected at positions 57, 58, 61, and 117, and the point mutation at codon 173 was not observed in our samples (Table 1). Overall, 45 of the 122 samples (36.9%) were identified as containing wild-type *pvdhfr*, and the remaining 63.1% of isolates had at least one amino acid substitution. The most predominant *pvdhfr* S117N mutation was found in almost half of the parasite samples (48.4%), whereas the T61M, S58R, and F57L mutations were found in 23 (18.9%), 12 (9.8%), and 1 (0.8%) of the samples, respectively.

On the basis of amino acid changes, the *pvdhfr* sequences can be grouped into seven alleles; two alleles (5 and 6) were novel, and the remaining five have been previously identified (1, 2, 3, 4, and 7). Except for the wild-type allele, the most prevalent mutant allele was the one with a single mutation at codon 117 (36.9%). Triple (S58R/T61M/S117N) and quadruple mutations (F57L/S58R/T61M/S117T) were rare and accounted for less than 1% of the parasite isolates. Two types of double mutations (S58R/S117N and T61M/S117N) were found in 8.4% and 1.6% of parasite isolates, respectively. The two *P. vivax* parasite populations in China also differed in *pvdhfr* allele frequencies (Table 1 and Figure 1). The wild-type *pvdhfr* genotype was found in 60.7% of the parasite population from Guizhou and was found in 29.5% of the parasites from central China. Although each site had three mutant *pvdhfr* alleles (single, double, and triple mutations), a quadruple mutant allele (F57L/S58R/T61M/S117T) was only found in the population from southern China.

TABLE 1

Point mutations in dihydrofolate reductase synthase among *Plasmodium vivax* isolates from China*

Allele	Genotype*					Allele frequency: no. (%)		
	57	58	61	117	173	Total	Anhui	Guizhou
1	F	S	T	S	I	45 (36.9)	28 (29.5)	17 (60.7)
2	F	S	T	N	I	45 (36.9)	42 (44.2)	3 (10.7)
3	F	S	M	S	I	19 (15.6)	19 (20.0)	0
4	F	R	T	N	I	10 (8.4)	3 (3.2)	7 (25.0)
5	F	S	M	N	I	2 (1.6)	2 (2.1)	0
6	F	R	M	N	I	1 (0.8)	1 (1.1)	0
7	L	R	M	T	I	1 (0.8)	0	1 (3.6)

* Mutated amino acids are shown in **bold**.

In addition to these point mutations, we observed variations at the central tandem repeat region between amino acid positions 88 and 105 of the *pvdhfr* gene.^{17,28,36} Size polymorphism in this region is resulted from variation in number of copies of the 18-basepair repeat, which is not essential for substrate binding.²¹ Most (77.0%) of the isolates sequenced contain three copies of the repeat, and 23.0% contained two 18-basepair repeats. None had a single copy of the 18-basepair repeat. Short indels and mutations were also found within the repeat unit of the sequence GGDN. A total of 22.1% of the samples showed a pattern in the repeat region similar to that in the wild type. The remaining samples can be divided into three types on the basis of the mutation at residue 99. We identified a deletion mutation, a previously reported H99S mutation,²⁸ and a novel H99D mutation, which accounted for 23.0%, 49.2%, and 5.7% of the parasite samples, respectively.

Mutations in *pvdhps* alleles and their frequencies. The *pvdhps* gene was successfully amplified and sequenced for 87 parasite isolates. In contrast to the *pvdhfr* gene, no single nucleotide polymorphism was present in *pvdhps* except for a novel tandem repeat variation. The amino acids at codons 382, 383, 512, 553, and 585 were all wild type. The 585V mutation has been suggested to be responsible for the intrinsic resistance of *P. vivax* to antifolates.²⁴ We sequenced the repeat region of the *pvdhps* gene (spanning amino acids 603–666) of 63 parasite isolates from central China. Length polymorphism in this region is caused by a variable number of tandem repeat unit G(E/D)(A/G/S)KLTN.

From 63 parasite samples, seven repeat haplotypes (RH1–6, and D) were recognized and only one (type D) was previously reported^{26,32} (Figure 2). The number of repeats in our samples ranged from four to nine. The most common haplotype is RH1, which represented (49.2%) of the analyzed samples and appeared to be different from SalI strain by only two mutational steps. Three other haplotypes (RH2, RH3, and RH4) were present in the samples at similar frequencies (11.1–14.3%). Within the repeat region, seven novel synonymous mutations were observed: position 615 (ACG to ACC) in 47 sequences (74.6%), position 616 (AAT to AAC) in 47 sequences (74.6%), position 631 (GGG to GGT) in 16 sequences (25.4%), position 634 (AAA to AAG) in 7 sequences (11.1%), position 638 (GGG to GGA) in 7 sequences (11.1%), position 662 (AAG to AAA) in 9 sequences (14.3%), and position 664 (ACC to ACT) in 9 sequences (14.3%). Mutation 615 is always linked to mutation 616 and mutation 634 is always linked to the mutation 638. The repeat region is not predicted to directly bind sulfa drugs, and is therefore unlikely involved in resistance to sulfa drugs in *P. vivax*.

DISCUSSION

Chloroquine-primaquine is still the first-line treatment for *P. vivax* malaria. With the emergence of CQ-resistant *P. vivax* strains in many malarious regions, it is also becoming a high priority to monitor drug resistance and develop drugs for future treatment of *P. vivax* malaria. Although antifolate drugs have not been recommended for treating *P. vivax* malaria, recent studies suggest that this family of drugs may find a role in future treatment of *P. vivax* malaria in certain regions.^{16,37} In this study, we have analyzed *pvdhfr* and *pvdhps* mutations in two *P. vivax* populations from both temperate zone and subtropical provinces of China. Our study showed

	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13	N (%)
Sal I	GEAKLTNGEGKLTNGEAKLTN	-----	-----	-----	GEGKLTN	-----	-----	GDAKLTN	-----	GDSKLTN	-----	GEAKLTN	-----	
RH1	GEAKLTNGEGKLTN	-----	-----	-----	GEGKLTN	-----	-----	GDAKLTN	-----	GDSKLTN	-----	GEAKLTN	-----	31 (49.2%)
RH2	GEAKLTNGEGKLTN	-----	-----	-----	GEGKLTN	-----	-----	GDAKLTN	-----	GDSKLTN	-----	GEAKLTN	-----	9 (14.3%)
RH3	GEAKLTNGEGKLTN	-----	-----	-----	GEGKLTN	-----	-----	GDAKLTN	-----	GDSKLTN	-----	GEAKLTN	-----	8 (12.7%)
RH4	GEAKLTNGEGKLTN	-----	-----	-----	GEGKLTN	-----	-----	-----	-----	-----	-----	-----	-----	7 (11.1%)
RH5	GEAKLTNGEGKLTN	-----	-----	-----	GEGKLTN	-----	-----	GDAKLTN	-----	GDSKLTN	-----	GDSKLTN	-----	6 (9.5%)
RH6	GEAKLTNGEGKLTN	-----	-----	-----	GEGKLTN	-----	-----	-----	-----	GDSKLTN	-----	GDSKLTN	-----	1 (1.6%)
A*	GEAKLTNGEGKLTN	-----	-----	-----	GEGKLTN	-----	-----	GDAKLTN	-----	GDSKLTN	-----	GEAKLTN	-----	
B*	GEAKLTNGEGKLTN	-----	-----	-----	GEGKLTN	-----	-----	-----	-----	GDSKLTN	-----	GEAKLTN	-----	
C*	GEAKLTNGEGKLTN	-----	-----	-----	GEGKLTN	-----	-----	GDAKLTN	-----	GDSKLTN	-----	GEAKLTN	-----	
D*	GEAKLTNGEGKLTN	-----	-----	-----	GEGKLTN	-----	-----	GDAKLTN	-----	GDSKLTN	-----	GEAKLTN	-----	1 (1.6%)
E*	GEAKLTNGEGKLTN	-----	-----	-----	GEGKLTN	-----	-----	-----	-----	GDSKLTN	-----	GEAKLTN	-----	
F*	GEAKLTNGEGKLTN	-----	-----	-----	GEGKLTN	-----	-----	GDAKLTN	-----	-----	-----	GEAKLTN	-----	
G*	GEAKLTNGEGKLTN	-----	-----	-----	GEGKLTN	-----	-----	GDAKLTN	-----	GDSKLTN	-----	GEAKLTN	-----	
H*	GEAKLTNGEGKLTN	-----	-----	-----	GEGKLTN	-----	-----	GDAKLTN	-----	GDSKLTN	-----	GEAKLTN	-----	
A'	GEAKLTNGEGKLTN	-----	-----	-----	GEGKLTN	-----	-----	-----	-----	-----	-----	-----	-----	
B'	GEAKLTNGEGKLTN	-----	-----	-----	GEGKLTN	-----	-----	-----	-----	-----	-----	-----	-----	
C'	GEAKLTNGEGKLTN	-----	-----	-----	GEGKLTN	-----	-----	-----	-----	-----	-----	-----	-----	
D'	GEAKLTNGEGKLTN	-----	-----	-----	GEGKLTN	-----	-----	-----	-----	-----	-----	-----	-----	

FIGURE 2. Polymorphisms in the repetitive domain of *Plasmodium vivax* dihydropteroate synthase. Deletions are denoted by dashes. RH1–RH6 are from this study. A*–H* correspond to the alleles described by Hawkins and others,³² and A'–D' represent the alleles from Menegons and others.²⁶ Sequences were aligned with the Sal I reference sequence (www.tigr.org), and the repeat units are labeled consecutively as R1 through R13. The frequency of each allele identified in this study is shown as number (N) and percentage (%).

that mutant *pvdhfr* genotypes were present at relatively high levels, but the mutations mostly occurred at single amino acids. In addition, no resistance-conferring mutations were found in *pvdhps*, suggesting that the *P. vivax* parasites in China may be relatively sensitive to SP.

It is well established that in *P. falciparum* accumulation of multiple mutations in *pfdhfr* and *pfdhps* is associated with *in vitro* resistance and clinical treatment failures with SP.^{14,15} A resistance mechanism to antifolates in *P. vivax* has been proposed that is similar to that in *P. falciparum* and linked to mutations at homologous positions in *pvdhfr* and *pvdhps*. For example, mutations at amino acids 58 and 117 of *pvdhfr* correspond to mutations at positions 59 and 108 in *pfdhfr*, respectively, which are associated with pyrimethamine resistance. Results from *in vitro* drug assays with limited numbers of field samples and tests using a yeast expression system are generally agreeable with this assumption.^{18,20,38} Furthermore, limited clinical assessments of the efficacy of SP have associated *pvdhfr* quadruple mutations and increased risks of clinical resistance to SP,^{19,22,23,25} suggesting that molecular genotyping data for *pvdhfr* and *pvdhps* should provide useful information about SP resistance in *P. vivax*.

Molecular epidemiologic studies in different areas have showed dramatically different mutations rates in *pvdhfr* and *pvdhps*.¹⁶ In Thailand where antifolates have been used heavily since the 1970s as a replacement of CQ for treating *P. falciparum* malaria, quadruple *pvdhfr* mutations and double *pvdhps* mutations have reached prevalences of approximately 60% and 72%, respectively, especially in *P. vivax* populations along Thailand–Myanmar border areas.²⁷ The prevalence of highly mutated *pvdhfr* and *pvdhps* is correlated with the poor therapeutic efficacy of SP against *P. vivax* malaria in this region.^{23,39} Similar *pvdhfr* quadruple mutations are also prevalent in certain areas of Indonesia and Papua New Guinea.¹⁸ In comparison, in southern Asia areas such as India, Pakistan, and Sri Lanka, parasites harboring multiple mutations in *pvdhfr* and *pvdhps* are relatively rare.^{28,30,40} In

such regions where *P. falciparum* and *P. vivax* coexist, mutations in *pvdhfr* and *pvdhps* have been attributed to selective pressure of antifolate drugs specific for controlling *P. falciparum*. Accordingly, *pvdhfr* and *pvdhps* mutation rates are expected to be much lower in temperate zone regions where *P. vivax* is the only or predominant malaria parasite. Limited studies on some temperate zone *P. vivax* samples support this prediction.^{33,41} Although an earlier study that used seven *P. vivax* isolates collected in 1994 from China showed that 71% of the parasites were wild-type at the *pvdhfr* locus, the small sample size may not be representative.²⁰ In this study, we found that mutant *pvdhfr* genotypes were present in approximately 63% of the clinical isolates. However, most *pvdhfr* mutations occurred at single amino acids, and triple and quadruple mutations were only found in two parasite isolates. In addition, no additional amino acid changes were found in *pvdhps*.

It has been proposed that point mutations in *pvdhfr*, such as those in *pfdhfr*, are acquired sequentially in response to drug pressure and mutations at residues 117 and 58 arise first.¹⁷ Consistent with this proposal, mutation S117N was the most prevalent point mutation in *pvdhfr* in the two *P. vivax* populations in China. In regions of extensive SP use, two mutant genotypes at residue 117 (S117T and S117N) have been observed; the former has been associated with highly mutated *pvdhfr* and may be a key mutation for subsequent acquisition of additional mutations and development of high resistance to SP.^{19,22} In Thailand, for example, the *pvdhfr* T61M mutation, which has reached a high level of prevalence (> 60%), is mostly linked with the S117T mutation.^{17,27} Consistent with the scarcity of the S117T mutation in China, highly mutated *pvdhfr* (triple or quadruple mutations) was also rare. However, the 61M mutation was relatively frequent (20%), especially in the temperate zone *P. vivax* population. Also noteworthy is that most of the 61M mutations were found as a single mutation in the central China *P. vivax* population, suggesting that this mutation could arise independently.

Although the presence of the *pvdhfr* 57L58R61M117T quadruple mutant associated with clinical resistance to SP was found in an isolate from southern China, it is unknown whether this mutant genotype was generated locally or imported from neighboring subtropical provinces, where *P. falciparum* was also present. It is obvious that the two *P. vivax* populations in China have drastically different *pvdhfr* haplotype frequencies, which may be attributed to different drug selection pressures or the intrinsic differences between the temperate and tropical strains of *P. vivax*. Furthermore, highly polymorphic repeat motifs in *pvdhfr* and *pvdhps* may indicate a lack of SP-selective sweep in these parasite populations. The different resistance-conferring *pvdhfr* mutation haplotypes may have evolved independently in different *P. vivax* populations in China.³¹ Because in malarious regions where *P. falciparum* and *P. vivax* coexist mutant *pvdhfr* and *pvdhps* alleles are presumably the result of exposure to antifolate drugs that are used for treatment of *P. falciparum* malaria, it is thus counterintuitive that the parasites carrying *pvdhfr* mutations are more prevalent in the temperate population from Anhui province, where *P. vivax* is the only malaria parasite species, than in the subtropical population from Guizhou Province. However, in the temperate regions in which *P. vivax* is endemic, antifolate drugs have been used mostly as prophylaxis. In contrast, other antimalarial drugs such as piperazine and artemisinins, have been used to treat *P. falciparum* malaria in the subtropical regions. Therefore, different *pvdhfr* allele prevalences in the two *P. vivax* populations in China might be attributed to different drug histories in these areas.

It appears that in *P. falciparum* SP poses asymmetric selection of *pfdhfr* and *pfdhps* and mutations in *pfdhfr* usually occur first.¹⁴ This selection seems to be similar in *P. vivax* because higher frequencies of *pvdhps* mutations were only observed in regions such as Thailand where highly mutated *pvdhfr* haplotypes were present.^{17,27} This finding has been thought to be caused by the intrinsic difference in mutation rates associated with these two genes, which reflects the relative fitness of the resulting enzymes in the absence of the drug pressure.¹⁴ Similar to *P. vivax* populations from other regions with low antifolate use, the *P. vivax* populations from China contain resistance-conferring mutations in *pvdhfr* but not in *pvdhps*.

When compared with other malaria-endemic regions of world, the antifolate drug use history in China is different. From late 1950s to early 1960s, only pyrimethamine was used heavily for malaria prophylaxis in all malaria-endemic areas of China.⁴² In *P. vivax*-endemic areas, use of pyrimethamine plus primaquine for eight weeks has been a practice for malaria prophylaxis during the transmission season.⁴³ From 1966 to early 1970s, it was replaced with antimalarial drug combination no. 1 (pyrimethamine-dapsone) and later no. 2 (sulfadoxine and pyrimethamine) for prophylaxis. Drug combination no. 2 contains only half the amount of sulfadoxine (250 mg) as Fansidar (500 mg).⁴² Therefore, it is plausible that differential use of pyrimethamine and sulfa drugs in China may be partially responsible for the asymmetric selection of *pvdhfr* and *pvdhps*.

The *pvdhfr* and *pvdhps* mutation rates generally coincide with the extent of SP use and may be a useful indicator for predicting clinical efficacy of SP. In China, extensive use of pyrimethamine for *P. vivax* malaria prophylaxis and treatment may have led to evolution of *pvdhfr* mutations. Nevertheless, the rarity of highly mutated *pvdhfr* and absence of mutations

in *pvdhps* suggest that these parasite populations might be relatively susceptible to SP.²² A recent *in vitro* study on *in vitro* sensitivity of *P. vivax* isolates from this region to a number of antimalarial drugs supported this assumption (Feng L and others, unpublished data). In addition, an earlier clinical study in southern China indicated that eight-week use of pyrimethamine-primaquine prophylaxis during the *P. vivax* transmission season was highly effective in preventing *P. vivax* malaria.⁴⁴ Similarly, a clinical study found pyrimethamine monotherapy was effective in 65.5% of *P. vivax* malaria cases in central China, whereas the 34.5% of the cases displayed grade I and II resistance.⁴⁵ These data indicate that *P. vivax* populations in China may be relatively susceptible to antiparasite drugs.

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