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RESEARCH ARTICLE

Prevalence of antifolate drug resistance markers in *Plasmodium vivax* in China

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Abstract **The dihydrofolate reductase (*dhfr*) and dihydropteroate synthetase (*dhps*) genes of *Plasmodium vivax*, as antifolate resistance-associated genes were used for drug resistance surveillance. A total of 375 *P. vivax* isolates collected from different geographical locations in China in 2009–2019 were used to sequence *Pvdhfr* and *Pvdhps*. The majority of the isolates harbored a mutant type allele for *Pvdhfr* (94.5%) and *Pvdhps* (68.2%). The most predominant point mutations were S117T/N (77.7%) in *Pvdhfr* and A383G (66.8%) in *Pvdhps*. Amino acid changes were identified at nine residues in *Pvdhfr*. A quadruple-mutant haplotype at 57, 58, 61, and 117 was the most frequent (57.4%) among 16 distinct *Pvdhfr* haplotypes. Mutations in *Pvdhps* were detected at six codons, and the double-mutant A383G/A553G was the most prevalent (39.3%). *Pvdhfr* exhibited a higher mutation prevalence and greater diversity than *Pvdhps* in China. Most isolates from Yunnan carried multiple mutant haplotypes, while the majority of samples from temperate regions and Hainan Island harbored the wild type or single mutant type. This study indicated that the antifolate resistance levels of *P. vivax* parasites were different across China and molecular markers could be used to rapidly monitor drug resistance. Results provided evidence for updating national drug policy and treatment guidelines.**

Keywords drug resistance; antifolates; molecular markers; *Plasmodium vivax*; China

Introduction

Plasmodium vivax is the most geographically widespread among the five *Plasmodium* species that cause human malaria [1]. According to the latest world malaria report, about 229 million malaria cases were reported in 2019 in 87 malaria-endemic countries and approximately a quarter of the cases were due to *P. vivax* [2], which was mainly distributed in Southeast Asia. In China, *P. vivax* has been the major *Plasmodium* spp. for several decades. In particular, an epidemic re-emergence and outbreak of *P. vivax* occurred in 2006 on the Huanghuai Plain in Central China, where *Anopheles sinensis* mosquitoes were prevalent [3,4]. Malaria cases have declined to a low level due to targeted strategies and measures, including timely diagnosis and standard treatment; integrated measures consisting of medication for risk groups, vector control, and health education were implemented from 2010 to 2015 [5].

Antifolate drugs comprise compounds that bind to enzymes involved in parasite folate biosynthesis, and the most widely used drug combination within this class is sulfadoxine–pyrimethamine (SP) [6]. To date, SP has been mainly used as an intermittent preventive treatment for infants and pregnant women in Africa [7,8] and has been rarely used for *P. vivax* treatment. However, SP remains in use to treat patients with uncomplicated *P. falciparum* infections in few counties where *P. vivax* is co-endemic with *P. falciparum* [9]. For example, SP plus artesunate serves as first-line treatment for uncomplicated chloroquine-resistant falciparum in most areas in India as well as in Iran, Saudi Arabia, Yemen, and Sudan [10]; as such, the *P. vivax* parasite remains under SP drug selection pressure. In China, pyrimethamine was used for prophylaxis [11] and was combined with primaquine (PQ) for radical treatment of *P. vivax* [12]. According to the latest national

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antimalarial drug policy in China [13], chloroquine (CQ) plus PQ is used to treat *P. vivax*; piperaquine (PPQ), pyronaridine, or artemisinin combination therapies (ACTs) plus PQ are used to treat patients infected with CQ-resistant *P. vivax*.

Clinical, epidemiological, molecular, and biochemical studies have shown the genetic basis of SP resistance, which is caused by mutations in two genes, namely, the dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes. Point mutations associated with pyrimethamine and sulfadoxine resistance have been identified in *P. vivax dhfr* (*Pvdhfr*) at codons 51, 58, 117, and 173 and in *P. vivax dhps* (*Pvdhps*) at codons 382, 383, and 553 [14,15].

A high prevalence of mutations in *Pvdhfr* and *Pvdhps* has been reported in Asia [16–21], Africa [22], and Oceania [23]. Studies involving molecular surveillance of *P. vivax* resistance are limited in China. The present study investigated the prevalence of *Pvdhfr* and *Pvdhps* in field isolates of *P. vivax* from Liaoning, Jiangsu, Tibet, Yunnan, and Hainan provinces in China. Results provide evidence of the level of SP resistance in *P. vivax* in China.

Materials and methods

Study site

Blood samples containing *P. vivax* were collected from Dandong, Suining, Nyingchi, Yingjiang, Tengchong, and Hainan. Dandong City in Liaoning Province, which is located in the northeastern part of China, ranges in latitude from 39°43′ to 41°09′ N and in longitude from 123°22′ to 125°41′ E. Dandong City is the largest border city in China, facing DPR Korea across the Yalu River. Suining County in Jiangsu Province is located in the Huanghuai Plain of Central China at 33°56′27.6″ N and 117°51′10.8″ E and has a typical temperate continental climate. Nyingchi, known as Linzhi, is a prefecture-level city in the southeastern part of Tibet at coordinates of 29°38′ 55.68″ N and 94°21′41.04″ E and has climate defined as subtropical highland. Yingjiang and Tengchong counties in Yunnan Province are located in the southwestern part of China, which border Kachin State, Myanmar. These two counties are mountainous with several alluvial plains and have various climate types. The border has no barrier, and a large number of immigrants cross the border frequently. Hainan is an island province in China and the nation's southernmost point; the climate in Hainan varies from subtropical to tropical.

Sample collection and DNA extraction

Blood was collected from *P. vivax*-infected patients from different geographical locations in China in 2009–2019 prior to antimalarial drug treatment. The blood samples were spotted on filter paper (Whatman™ 903, GE Healthcare, USA) and dried. Microscopic examination of Giemsa-stained thick smears was used to diagnose malaria. Parasite DNA was extracted using a QIAamp DNA Mini Kit (Valencia, CA, USA) as described by the manufacturer. A nested polymerase chain reaction (PCR) for amplifying the small-subunit rRNA gene of *Plasmodium* spp. [24] was performed to confirm the positive samples and identify the species before sequencing *Pvdhfr* and *Pvdhps* genes.

Nested PCR and sequencing

The primers and cycling conditions for nested PCR and the sizes of the PCR products are shown in Table S1. The PCR products were purified using filter plates (Edge Biosystems, Gaithersburg, MD, USA), directly sequenced, and analyzed on an ABI 3730XL automatic sequencer. The amplification products were analyzed by 1.5% agarose gel electrophoresis before sequencing. Bidirectional sequencing was performed, and all independently amplified PCR products were sequenced twice.

Data analysis

The output sequence data were assembled, edited, aligned using Geneious (version 2021.0.3) software, and compared with the reference sequences of *Pvdhps* (accession number: XM001617159) and *Pvdhfr* (accession number: X98123) from GenBank. The mixed alleles were determined according to the emergence of two chromatogram peaks at one nucleotide site through Mutation Surveyor (Soft Genetics LLC., version 5.1, State College, PA, USA). All the detected single-nucleotide polymorphisms (SNPs) of *Pvdhps* and *Pvdhfr* were combined for principal component analysis (PCA) using the EIGENSOFT program [25]. SAS software (SAS Institute Inc., Version 9.2, Cary, NC, USA) was used for data processing and statistical analysis. Chi-square test and Fisher's exact test were used to evaluate differences among subgroups. A P value < 0.05 was used to identify differences with statistical significance.

Results

Pvdhfr haplotype

With respect to the *Pvdhfr* gene, 291 *P. vivax* isolates were sequenced successfully. Amino acid changes in *Pvdhfr* at residues 13, 15, 57, 58, 61, 76, 99, 117, and 173 were detected in 2.1%, 0.3%, 61.5%, 76.3%, 59.5%, 0.3%, 7.9%, 77.7%, and 1.0% of the sequences, respectively (Table 1). The majority of the isolates carried at least one mutation in *Pvdhfr* (94.5%, 275/291), whereas 16 of the

Table 1 Distribution of point mutations in *Pvdhfr* and *Pvdhps* from *P. vivax* isolates in China

Locations	Sites	*Pvdhfr*										
		n	WT	I13L	A15V	F57L/I[a]	S58R[b]	T61M	R76G	H99S	S117T/N[c]	I173L
Northeastern	Dandong	7			1	2				1	2	
Central	Suining	16	10								6	
Southwestern	Nyingchi	3	1				2				1	
Southern	Hainan	3					3				3	
Southwestern	Yingjiang	58	3			16	28	16		19	28	
Southwestern	Tengchong	204	2	6		161	189	157	1	3	186	3
	Total	291	16	6	1	179	222	173	1	23	226	3

Locations	Sites	*Pvdhps*							
		n	WT	S382C	A383G	M399I	K512M/T/E	A553G[b]	E571Q
Northeastern	Dandong	8	5			3			
Central	Suining	16	16						
Southwestern	Nyingchi	25	23		1	1			
Southern	Hainan	26	24		1	1			
Southwestern	Yingjiang	58	29	1	29		1	9	
Southwestern	Tengchong	216	14	40	202		5	155	1
	Total	349	111	41	233	5	6	164	1

[a]Among 179 mutant isolates at codon 57, 97 isolates carried F57L and 82 isolates carried F57I.
[b]One isolate carried mixed alleles S58S/R of *Pvdhfr* and two carried mixed alleles A553A/G of *Pvdhps*.
[c]Among 226 mutant isolates at codon 117, 174 isolates showed S117T and five isolates showed S117N.

291 isolates were identified as containing wild-type *Pvdhfr*. The most predominant point mutation was S117T/N (77.7%), followed by S58R (76.3%), F57L/I (61.5%), and T61M (59.5%). However, the I13L and I173L mutant alleles were rare and detected in only six and three isolates, respectively. The prevalence of wild-type *Pvdhfr* varied significantly in different geographical sites ($P < 0.001$). Most samples from Suining (10/16) harbored the wild type, while only few isolates harbored the wild type in Yingjiang (3/58) and Tengchong (2/204) counties in Yunnan Province. Additionally, two novel point mutations, namely, A15V and R76G, which have not been reported, were detected.

The haplotype analysis revealed 16 distinct allelic forms from all samples (Table 2). The single-mutant haplotypes S58R and S117N and the double-mutant alleles S58R/S117N and F57L/S58R had relatively low prevalence and were identified in two, one, four, and four isolates, respectively. Another single-mutant haplotype, namely, H99S, which is not associated with pyrimethamine resistance, was observed in 21 *P. vivax* isolates. A quadruple-mutant haplotype, $I_{13}A_{15}L_{57}R_{58}M_{61}R_{76}H_{99}T_{117}I_{173}$ (29.2%) was the most common mutant form, followed by the quintuple-mutant haplotype $I_{13}A_{15}L_{57}R_{58}M_{61}R_{76}S_{99}T_{117}I_{173}$ (28.2%). Triple-mutant haplotypes were not observed in this study.

Tandem repeat variation in *Pvdhfr*

Variations in the central tandem repeat region between the amino acid positions 88 and 103 of *Pvdhfr* were identified (Fig. 1). Three types of tandem repeat variation were observed. Type 1 contained three copies of GGDN repeats, the same as the reference strain (accession number: X98123.1), and was designated as the wild type. Type 2 also had three copies of GGDN repeats but showed a mutant allele at codon 99. Type 3 lacked six amino acids from positions 98 to 103. Approximately 40% (118/291) of samples were Type 1, and 35.4% (103/291) were Type 2. Except for one isolate from Dandong, which carried a single mutant H99S, Types 1 and 2 were detected only in samples from Yunnan Province. By contrast, Type 3 was present in 70 *P. vivax* samples with a deletion of 18 nucleotides from amino acid positions 98 to 103. Interestingly, when combined with other point mutants, Type 3 comprised various mutant alleles. The majority were $I_{13}A_{15}F_{57}R_{58}T_{61}R_{76}\_T_{117}I_{173}$ (51.4%, 36/70), whereas $I_{13}A_{15}F_{57}S_{58}T_{61}R_{76}\_S_{117}I_{173}$, $I_{13}A_{15}F_{57}S_{58}T_{61}R_{76}\_N_{117}I_{173}$, $I_{13}A_{15}F_{57}R_{58}T_{61}R_{76}\_N_{117}L_{173}$, $I_{13}A_{15}L_{57}S_{58}T_{61}R_{76}\_S_{117}I_{173}$, and $I_{13}V_{15}F_{57}S_{58}T_{61}R_{76}\_T_{117}I_{173}$ were found in twenty, eight, three, two, and one isolates, respectively. Most samples (85.6%) from Dandong were Type 3 and differed significantly from the samples from the other regions.

Pvdhps haplotype

Polymorphisms were successfully assessed in the *Pvdhps* gene from 349 *P. vivax* samples. Nonsynonymous mutations of the *Pvdhps* gene were detected at codons 382, 383, 399, 512, 553, and 571; point mutations at

Table 2 Haplotypes of *Pvdhfr* and *Pvdhps* in *P. vivax* isolates in China

	Haplotypes	Total (*n*)	Prevalence (%)	Temperate region			Subtropical region			*P* value
				Dandong	Suining	Nyingchi	Yingjiang	Tengchong	Hainan	
Pvdhfr	Total	291		7	16	3	58	204	3	
	Wild-type	16	5.5							
	$I_{13}A_{15}F_{57}$ S_{58} $T_{61}R_{76}H_{99}S_{117}I_{173}$	16	5.5		10	1	3	2		<0.0001
	Single mutant	24	8.2							
	$I_{13}A_{15}F_{57}$ $R_{58}T_{61}R_{76}H_{99}S_{117}I_{173}$	2	0.7			1		1		0.1727
	$I_{13}A_{15}F_{57}$ $S_{58}T_{61}R_{76}H_{99}N_{117}I_{173}$	1	0.3		1					0.0903
	$I_{13}A_{15}F_{57}$ $S_{58}T_{61}R_{76}S_{99}S_{117}I_{173}$	21	7.2	1			17	3		0.7051
	Double mutant	8	2.7							
	$I_{13}A_{15}F_{57}R_{58}T_{61}R_{76}H_{99}N_{117}I_{173}$	4	1.4			1			3	0.3165
	$I_{13}A_{15}L_{57}R_{58}T_{61}R_{76}H_{99}S_{117}I_{173}$	4	1.4					4		0.6835
	Quadruple mutant	85	29.2							
	$I_{13}A_{15}L_{57}R_{58}M_{61}R_{76}H_{99}T_{117}I_{173}$	85	29.2				5	80		0.0005[a]
	Quintuple mutant	88	30.2							
	$I_{13}A_{15}I_{57}R_{58}M_{61}R_{76}S_{99}T_{117}I_{173}$	82	28.2				11	71		0.0007[a]
	$L_{13}A_{15}L_{57}R_{58}M_{61}R_{76}H_{99}T_{117}I_{173}$	6	2.1					6		0.5639
	Tandem repeat with mutant	70	24.1							
	$I_{13}A_{15}F_{57}$ $S_{58}T_{61}R_{76}$_$S_{117}I_{173}$	20	6.9	2			10	8		0.6989
	$I_{13}A_{15}F_{57}S_{58}T_{61}R_{76}$_$N_{117}I_{173}$	8	2.7	1	5			2		<0.0001
	$I_{13}A_{15}L_{57}S_{58}T_{61}R_{76}$_$S_{117}I_{173}$	2	0.7	2						0.0079
	$I_{13}A_{15}F_{57}R_{58}T_{61}R_{76}$_$N_{117}I_{173}$	36	12.4				12	24		0.0555
	$I_{13}V_{15}F_{57}S_{58}T_{61}R_{76}$_$T_{117}I_{173}$	1	0.3	1						0.0903
	$I_{13}A_{15}F_{57}R_{58}T_{61}R_{76}$_$N_{117}L_{173}$	3	1.0					3		0.7521
Pvdhps	Total	349		8	16	25	58	216	26	
	Wild-type	111	31.8							
	$S_{382}A_{383}M_{399}K_{512}A_{553}E_{571}$	111	31.8	5	16	23	29	14	24	<0.0001[a]
	Single mutant	54	15.5							
	$S_{382}A_{383}I_{399}K_{512}A_{553}E_{571}$	5	1.4	3		1			1	0.0016
	$S_{382}G_{383}M_{399}K_{512}A_{553}E_{571}$	49	14.0			1	20	27	1	0.0091[a]
	Double mutant	157	45.0							
	$C_{382}G_{383}M_{399}K_{512}A_{553}E_{571}$	19	5.4					19		0.0881
	$S_{382}G_{383}M_{399}K_{512}A_{553}Q_{571}$	1	0.3					1		0.8596
	$S_{382}G_{383}M_{399}K_{512}G_{553}E_{571}$	137	39.3				8	129		<0.0001[a]
	Triple mutant	26	7.4							
	$C_{382}G_{383}M_{399}K_{512}G_{553}E_{571}$	21	6.0					21		0.0548
	$S_{382}G_{383}M_{399}M_{512}G_{553}E_{571}$	3	0.9					3		0.6343
	$S_{382}G_{383}M_{399}T_{512}G_{553}E_{571}$	2	0.6					2		0.7386
	Quadruple mutant	1	0.3							
	$C_{382}G_{383}M_{399}E_{512}G_{553}E_{571}$	1	0.3				1			0.8596

[a]Chi-square test was used to evaluate differences among subgroups.

codons 580 and 585 were not observed. Four synonymous mutations (codons 402, 495, 554, and 561) were detected. The wild-type *Pvdhps* was found in all sites, with a total number of 111, the majority of which were found in Suining (16/16), Nyingchi (23/25), Hainan (24/26), and Dandong (5/8) and a few in Tengchong (14/216). Nine different mutant haplotypes were observed, consisting of two single-, three double-, three triple-, and one quadruple-mutant haplotypes (Table 2). In comparison with the wild-type *Pvdhps* (31.8%), the most prevalent haplotype was the double-mutant allele $S_{382}G_{383}M_{399}K_{512}G_{553}E_{571}$ (39.3%, 137/349), which was detected only in Yunnan Province, mostly in Tengchong County (129/137). The single-mutant allele A383G was common in Yingjiang (20/58) but rare in Tengchong, with only one isolate each from Nyingchi and Hainan Island. M399I, a novel mutation in the *Pvdhps* gene detected in this study, was observed in three, one, and one isolates from Dandong, Nyingchi, and Hainan Island,

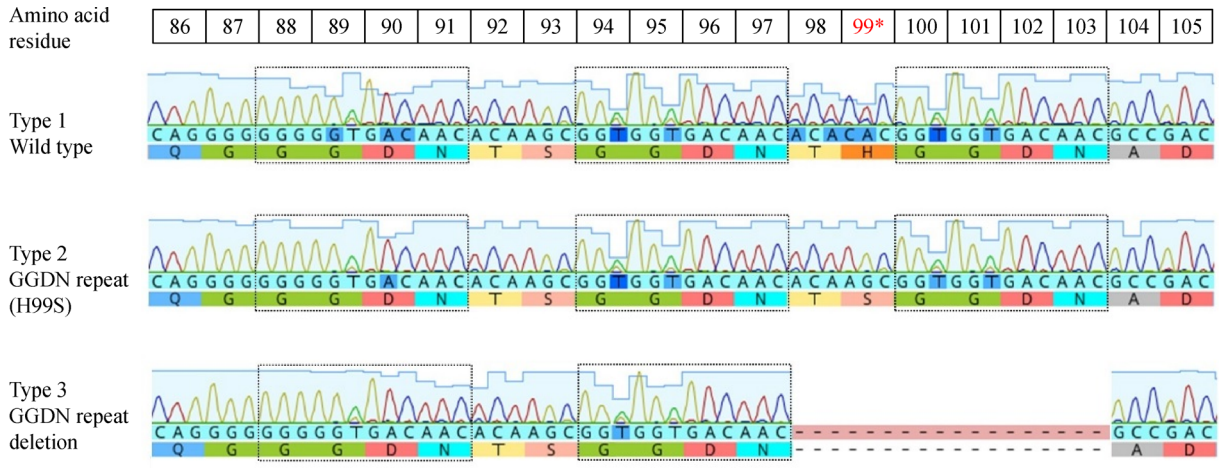

Fig. 1 Tandem repeat variation between amino acid positions 86 and 105 in the *Pvdhfr* gene.

respectively. Multiple-mutation haplotypes were mostly observed in Yunnan Province and comprised double mutants (S382C/A383G, A383G/E571Q, A383G/A553G), triple mutants, and quadruple mutants at codons 382, 383, 512, and 553.

In addition, two isolates with the mixed allele A553A/G were identified, and the mutated alleles at codon 512 showed three different types, K512T/M/E, each of which carried the double-mutation A383G/A553G.

Analysis of allelic combinations of *Pvdhfr* and *Pvdhps*

Allelic combinations of *Pvdhfr* and *Pvdhps* genes are responsible for the biosynthesis of folate and are potentially under similar drug group pressure. A total of 280 *P. vivax* isolates were sequenced successfully for both genes. The combinations of point mutations in *Pvdhfr* and *Pvdhps* were analyzed at codons 57, 58, 61, 117, and 173 and at codons 382, 383, and 553, respectively. Thirty-three haplotypes were identified, whereas 13.2% harbored the wild type. The hexaploidy-mutant alleles $I_{57}R_{58}M_{61}T_{117}$/$G_{383}G_{553}$ and $L_{57}R_{58}M_{61}T_{117}$/$G_{383}G_{553}$ were the two most common haplotypes, with frequencies of 18.2% and 17.5%, respectively. The quintuple-mutant haplotype $L_{57}R_{58}M_{61}T_{117}$/$G_{383}$ and the quadruple-mutant $R_{58}N_{117}$/$G_{383}G_{553}$ were present at a low prevalence rate of 5%. The single-mutant A383G in *Pvdhps*, the single-mutant S117N, and the double-mutant S58R and S117N in *Pvdhfr* and wild-type *Pvdhps* were detected in 6.1% (17/280), 2.5% (7/280), and 4.3% (12/280) of the samples, respectively. Rare haplotypes included single or double mutations at positions 382 and 383 of *Pvdhps* in combination with multiple *Pvdhfr* mutations at positions 57, 58, 61, 117, and 173, which were found in few isolates. One *P. vivax* sample from Yingjiang County showed the haploid-mutant allele $I_{57}R_{58}M_{61}T_{117}$/$C_{382}G_{383}T_{512}G_{553}$.

Geographical distribution of *Pvdhfr* and *Pvdhps* haplotypes

With regard to the different locations of isolates, Dandong, Suining, and Nyingchi, which are located in northeastern, central, and southwestern China, respectively, are classified as temperate regions, whereas Yingjiang and Tengchong in southwest of China and Hainan Island in southern China are subtropical regions. Isolates from temperate regions were dominated by wild-type *Pvdhfr* and *Pvdhps*, whereas multiple-mutation haplotypes, consisting of quadruple and quintuple mutants of *Pvdhfr* as well as double, triple, and quadruple mutants of *Pvdhps*, were all from subtropical regions. The geographical distributions of various haplotypes observed in *Pvdhfr* and *Pvdhps* are shown in Table 2. When the haplotypes of *Pvdhfr* and *Pvdhps* were combined, isolates from Yingjiang and Tengchong counties in Yunnan Province revealed great diversity and 30 distinct mutant alleles were observed. Among them, the hexaploidy-mutant alleles $I_{57}R_{58}M_{61}T_{117}$ /$G_{383}G_{553}$ (8.2%) and $L_{57}R_{58}M_{61}T_{117}$/$G_{383}G_{553}$ (17.5%) were all from the two counties. The single-mutant *Pvdhfr* at positions 57 and 117 combined with the wild-type *Pvdhps* was observed only in isolates from Dandong. Only one isolate from Nyingchi was successfully sequenced and showed the wild type, while three samples from Hainan Island carried double mutations of *Pvdhfr* and wild-type *Pvdhps* $R_{58}N_{117}$/WT (Table 3).

The use of PCA on all the detected SNPs in *Pvdhfr* and *Pvdhps* showed the first PC on the horizontal axis and the second and third PCs on the vertical axis. The samples were colored according to their geographical location. The PCA results indicated a distinction among the isolates. Parasites from Yunnan Province were clustered and separated from the isolates from Hainan Island and from temperate regions, including Liaoning, Jiangsu, and Tibet (Fig. 2).

Table 3 Combination of *Pvdhfr* and *Pvdhps* mutations in *P. vivax* isolates in China

Pvdhfr					*Pvdhps*				Haplotype of *Pvdhfr*/*Pvdhps*	Dandong *n* = 7	Suining *n* = 16	Nyingchi *n* = 1	Hainan *n* = 3	Yingjiang *n* = 58	Tengchong *n* = 195	Total (*n*)	Prevalence (%)
57	58	61	117	173	382	383	512	553									
F	S	T	S	I	S	A	K	A	WT/WT	3	10	1		13	10	37	13.2
F	S	T	N	I	S	A	K	A	N_{117}/WT	1	6					7	2.5
F	S	T	T	I	S	A	K	A	T_{117}/WT	1						1	0.4
L	S	T	S	I	S	A	K	A	L_{57}/WT	2						2	0.7
F	R	T	N	I	S	A	K	A	$R_{58}N_{117}$/WT				3	9		12	4.3
I	R	M	T	I	S	A	K	A	$I_{57}R_{58}M_{61}T_{117}$/WT					5	1	6	2.1
L	R	M	T	I	S	A	K	A	$L_{57}R_{58}M_{61}T_{117}$/WT					2	2	4	1.4
F	S	T	S	I	S	G	K	A	WT/G_{383}					17		17	6.1
F	R	T	N	I	S	G	K	A	$R_{58}N_{117}$/G_{383}					1	5	6	2.1
I	R	M	T	I	S	G	K	A	$I_{57}R_{58}M_{61}T_{117}$/$G_{383}$					1	5	6	2.1
L	R	M	T	I	S	G	K	A	$L_{57}R_{58}M_{61}T_{117}$/$G_{383}$					1	13	14	5.0
L	R	T	S	I	S	G	K	A	$L_{57}R_{58}$/G_{383}						3	3	1.1
F	S	T	N	I	S	G	K	G	N_{117}/G_{383}						1	1	0.4
F	R	T	N	I	S	G	K	G	$R_{58}N_{117}$/$G_{383}G_{553}$					2	12	14	5.0
F	S/R	T	S	I	S	G	K	G	S/R_{58}/$G_{383}G_{553}$						1	1	0.4
F	R	T	N	L	S	G	K	G	$R_{58}N_{117}L_{173}$/$G_{383}G_{553}$						3	3	1.1
I	R	M	T	I	S	G	K	G	$I_{57}R_{58}M_{61}T_{117}$/$G_{383}G_{553}$					5	46	51	18.2
I	R	M	T	I	S	G	K	A/G							1	1	0.4
L	R	M	T	I	S	G	K	G	$L_{57}R_{58}M_{61}T_{117}$/$G_{383}G_{553}$					1	48	49	17.5
L	R	M	T	I	S	G	K	A/G							1	1	0.4
F	R	T	N	I	C	G	K	A	$R_{58}N_{117}$/$C_{382}G_{383}$						1	1	0.4
I	R	M	T	I	C	G	K	A	$I_{57}R_{58}M_{61}T_{117}$/$C_{382}G_{383}$						6	6	2.1
L	R	M	T	I	C	G	K	A	$L_{57}R_{58}M_{61}T_{117}$/$C_{382}G_{383}$						10	10	3.6
F	S	T	S	I	C	G	K	G	WT/$C_{382}G_{383}G_{553}$						1	1	0.4
F	R	T	N	I	C	G	K	G	$R_{58}N_{117}$/$C_{382}G_{383}G_{553}$						1	1	0.4
L	R	T	S	I	C	G	K	G	$L_{57}R_{58}$/$C_{382}G_{383}G_{553}$						1	1	0.4
L	R	M	T	I	C	G	K	G	$L_{57}R_{58}M_{61}T_{117}$/$C_{382}G_{383}G_{553}$						8	8	2.9
I	R	M	T	I	C	G	K	G	$I_{57}R_{58}M_{61}T_{117}$/$C_{382}G_{383}G_{553}$						10	10	3.6
F	R	T	N	I	S	G	M	G	$R_{58}N_{117}$/$G_{383}M_{512}G_{553}$						2	2	0.7
F	S	T	N	I	S	G	M	G	N_{117}/$G_{383}M_{512}G_{553}$						1	1	0.4
L	R	M	T	I	S	G	T	G	$I_{57}R_{58}M_{61}T_{117}$/$C_{382}G_{383}G_{553}$						1	1	0.4
I	R	M	T	I	S	G	T	G	$L_{57}R_{58}M_{61}T_{117}$/$G_{383}T_{512}G_{553}$						1	1	0.4
L	R	M	T	I	C	G	E	G	$I_{57}R_{58}M_{61}T_{117}$/$C_{382}G_{383}T_{512}G_{553}$					1		1	0.4

WT, wild type.

Discussion

The combination SP is the most commonly used antimalarial treatment in almost all malaria-endemic areas between the 1960s and the 1980s [26] and remains in use to treat patients with *P. falciparum* infections in few counties [10] and as an intermittent prophylactic treatment for infants and pregnant women in Africa [7,8]. Prior to the introduction of SP combination therapy, pyrimethamine resistance in 1953 and sulfadoxine resistance in 1957 had already been reported [27]. By the early 1990s, SP resistance-related mutations in *dhps* and *dhfr* were documented [28] and point mutations in *Pvdhps* and *Pvdhfr*, which are associated with SP resistance, were well known [14,18,19,29]. Molecular markers for drug resistance have been recommended for antimalarial drug resistance surveillance by the WHO [30].

Soft and hard selective sweeps have occurred with striking differences in the *Pvdhfr* haplotype among *P. vivax* isolates [31]. Six point mutations in *Pvdhfr* associated with pyrimethamine resistance were identified, namely, P33L, F57L/I, S58R, T61M, S117N/T, and I173F/L, corresponding to residues A16V, C50R, N51I, C59R, S108N, and I164L in *Pfdhfr* [32]. Additionally, mutations in *Pvdhfr* at codons 57 and 61 in combination with those at codons 58 and 117 have been reported to be correlated with SP treatment failure [29]. Indeed, we found that the frequencies of the single-mutant haplotype at S58R or S117N and the double-mutant S58R/S117N were relatively low in all sites, whereas the quadruple-mutant haplotype $L_{57}R_{58}M_{61}T_{117}$ (29.2%) and quintuple-mutant haplotype $L_{57}R_{58}M_{61}R_{76}S_{99}T_{117}$ (28.2%) associated with SP treatment failure were highly prevalent in two sites in Yunnan Province. Moreover, most samples from the three sites in temperate regions were revealed as wild-type or single-mutant haplotypes. The only three isolates from Hainan Island all carried the double-mutation S58R/S117N, indicating high resistance, moderate resistance, and sensitivity to pyrimethamine in Yunnan, Hainan and temperate regions, respectively. When compared with reports from other countries, the prevalence of double or quadruple mutations in China is lower than that in Myanmar [33], Thailand [16], and Cambodia [18]. This difference is related to the duration and dosage of pyrimethamine usage in different countries. Pyrimethamine was widely used for malaria prophylaxis in all malaria-endemic areas in China in the late 1950s and the early 1960s [34] and was added to salt for prophylaxis in the 1980s. Pyrimethamine combined with PQ was used for radical treatment of *P. vivax* in Central China 40 years ago [11]. Tandem repeat variants were identified in the *Pvdhfr* gene. The majority of the samples (221/291) had three copies of tandem repeats (Types 1 and 2) (Table 2), which commonly occurred with quadruple-mutant *Pvdhfr* alleles. Type 3 with a deletion of six amino acids usually coincided with the double-mutation S58R/S117N. However, the mechanism of the tandem repeat variants in pyrimethamine resistance remains unclear [17,35] because these variants are not present in the *Pfdhfr* gene in *P. falciparum* [36,37].

Mutations in the *dhfr* gene correlated with sulfadoxine resistance include S382A, A383G, K512E, A553G, and V585A in *P. vivax* and the corresponding mutations S436A/F, A437G, K540E, A581G, and A613S/T in *P. falciparum* [38]. In the present study, wild-type *Pvdhps* was predominant in temperate regions and in Hainan Island, with frequencies of 62.5% (5/8), 100% (16/16), 92.0% (23/25) and 92.3% (24/26) in Dandong, Suining, Nyingchi, and Hainan Island, respectively. Nevertheless,

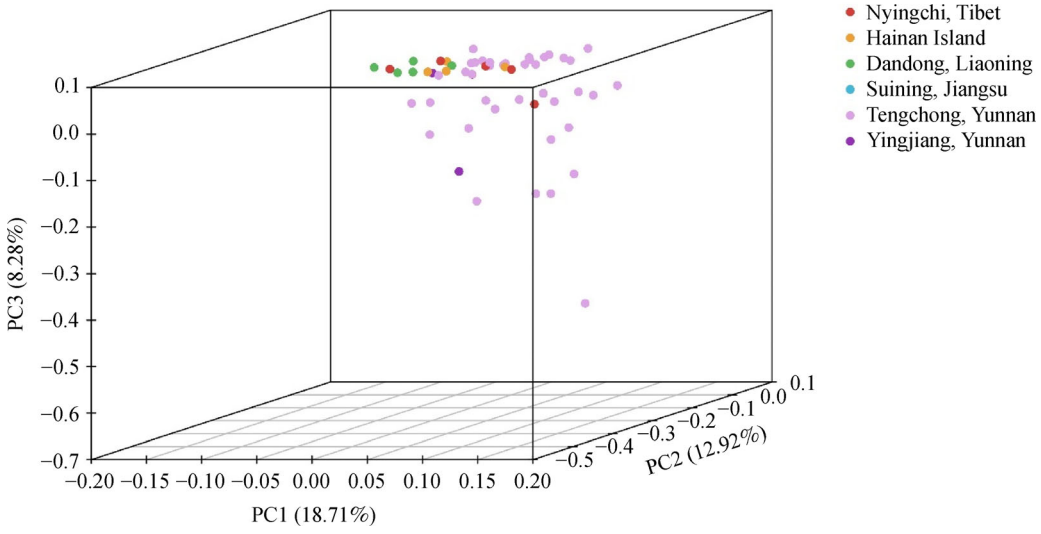

Fig. 2 Principal component analysis of combined SNPs in the *Pvdhfr* and *Pvdhps* genes of *P. vivax* isolates from different sites.

half of the samples (29/58) from Yingjiang and few isolates (14/216) from Tengchong showed wild-type *Pvdhps*. A large proportion of samples (233/349) carried the A383G mutation, followed by A553G (164/349). A383G and A553G are the two key mutations in *Pvdhps* and are strongly linked to sulfadoxine resistance. With respect to *Pvdhps* haplotypes, *P. vivax* isolates from temperate regions and Hainan Island showed only wild-type or single-mutant alleles, whereas the majority of the isolates from subtropical regions carried multiple mutations. The double-mutant A383G/A553G (39.3%, 137/349) was the most frequent haplotype; all mutant samples were detected in Yingjiang and Tengchong counties in Yunnan Province. These findings are in agreement with previous studies [11,39], which indicated that the level of pyrimethamine resistance was significantly different between temperate and subtropical regions in China. Compared with *Pvdhfr*, *Pvdhps* mutations exhibited less diversity and lower prevalence in China mainly due to the relatively limited use of sulfadoxine in China. According to published and unpublished data, sulfadoxine was not introduced in Central China [40,41], while the SP combination was widely used in Yunnan Province and some areas in Hainan Island [42]. This finding was fully verified by the present results. Some studies have shown that mutations in the *Pvdhps* gene were more than twice as likely to emerge in isolates with multiple mutations in the *Pvdhfr* gene [43]. In the present work, the double-mutation A383G/A553G with S58R/T61M/S117T was dominant among the combined mutations in *Pvdhfr* and *Pvdhps* [44,45]. These double mutations cause the disruption in the sulfadoxine binding site in *P. vivax*, similar to that in *P. falciparum* [38].

The prevalence and patterns of SP resistance varied among different regions in China, which may have been caused by the difference in genomic populations in different geographical areas [46–49] or drug pressure of sulfadoxine and pyrimethamine [39,50,51]. The PCA revealed that parasites from Yunnan Province were separated from those in the other sites, indicating limited barriers to gene flow in China. Moreover, behavior or gene changes in *Anopheles* strains or species could favor the subpopulation of *P. vivax* parasites and lead to changes in the parasite population structure [4,52], which should be considered. SP was used in Yunnan Province for over 20 years, starting in the early 1960s [42]. By contrast, pyrimethamine alone was used as prophylaxis in Central China during the same period [40,41] and sulfadoxine was not introduced in this region. In the 1980s, pyrimethamine is also added to salt for prophylaxis in China. In Hainan Island, SP in combination with artemisinin or PQ is used to treat infections caused by *P. falciparum* and CQ-resistant *P. falciparum* [51]. This wide usage of SP may have placed *P. vivax* under drug selection pressure, especially in subtropical regions (Yunnan Province and Hainan Island), where *P. vivax* and *P. falciparum* mixed-species infections are common. Therefore, the history of usage of the antifolate drugs sulfadoxine and pyrimethamine in different regions may be related to differences in drug pressure and resistance levels.

This study has limitations. First, a certain number of samples were not sequenced successfully because of DNA degradation, which caused the sample size to be small at some sites. Second, information or published data on the history of SP usage in China are very limited. Although SP was used widely in the last century in China, many data and results were not published or available, and the development of pyrimethamine resistance in *P. vivax* under natural field conditions in China may not be well described.

Antimalarial drug resistance has been the primary obstacle to global malaria elimination [53]. This study determined the molecular prevalence and patterns of *Pvdhfr* and *Pvdhps* mutations in different geographical regions and provided essential information on the level of resistance to SP in *P. vivax* in China. Another point of concern is that, although SP is not used in China, many malaria cases are imported from Africa and Southeast Asia, where SP is still used. Molecular markers for antimalarial drug resistance could be used to rapidly monitor the emergence and spread of drug resistance in imported malaria cases in China. In addition, this study provides evidence of antifolate drug resistance to inform national drug policy in China and update treatment guidelines.

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Compliance with ethics guidelines

Fang Huang, Yanwen Cui, He Yan, Hui Liu, Xiangrui Guo, Guangze Wang, Shuisen Zhou, and Zhigui Xia declare that they have no conflicts of interest. This study was approved by the Ethical Review Committee of National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the *Helsinki Declaration* of 1975, as revised in 2000. Informed consent was obtained from all patients.

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References

1. Price RN, Commons RJ, Battle KE, Thriemer K, Mendis K. *Plasmodium vivax* in the era of the shrinking *P. falciparum* map. Trends Parasitol 2020; 36(6): 560–570
2. World Health Organization. World malaria report 2020. Geneva: WHO, 2020
3. Zhang HW, Liu Y, Zhang SS, Xu BL, Li WD, Tang JH, Zhou SS, Huang F. Preparation of malaria resurgence in China: case study of vivax malaria re-emergence and outbreak in Huang-Huai Plain in 2006. Adv Parasitol 2014; 86: 205–230
4. Zhang S, Guo S, Feng X, Afelt A, Frutos R, Zhou S, Manguin S. Anopheles vectors in mainland China while approaching malaria elimination. Trends Parasitol 2017; 33(11): 889–900
5. Zhou SS, Huang F, Wang JJ, Zhang SS, Su YP, Tang LH. Geographical, meteorological and vectorial factors related to malaria re-emergence in Huang-Huai River of central China. Malar J 2010; 9(1): 337
6. Nzila A. The past, present and future of antifolates in the treatment of *Plasmodium falciparum* infection. J Antimicrob Chemother 2006; 57(6): 1043–1054
7. World Health Organization. Implementing malaria in pregnancy programs in the context of World Health Organization recommendations on antenatal care for a positive pregnancy experience. Geneva: WHO, 2018
8. World Health Organization. Intermittent preventive treatment for infants using sulfadoxinepyrimethamine (SP-IPTi) for malaria control in Africa: implementation field guide. Geneva: WHO, 2011
9. World Health Organization. World malaria report 2019. Geneva: WHO, 2019
10. Mohamed NS, Abdelbagi H, Osman HA, Ahmed AE, Yousif AM, Edris YB, Osman EY, Elsadig AR, Siddig EE, Mustafa M, Mohammed AA, Ali Y, Osman MM, Ali MS, Omer RA, Ahmed A, Sibley CH. A snapshot of *Plasmodium falciparum* malaria drug resistance markers in Sudan: a pilot study. BMC Res Notes 2020; 13 (1): 512
11. Huang B, Huang S, Su XZ, Tong X, Yan J, Li H, Lu F. Molecular surveillance of *pvdhfr, pvdhps*, and *pvmdr-1* mutations in *Plasmodium vivax* isolates from Yunnan and Anhui provinces of China. Malar J 2014; 13(1): 346
12. Huang F, Zhou S, Zhang S, Li W, Zhang H. Monitoring resistance of *Plasmdium vivax*: point mutations in dihydrofolate reductase gene in isolates from Central China. Parasit Vectors 2011; 4(1): 80
13. National Health Commission of the People's Republic of China. Technical regulations for application of antimalarials (WS/T 485-2016). 2016
14. Imwong M, Pukrittakayamee S, Looareesuwan S, Pasvol G, Poirreiz J, White NJ, Snounou G. Association of genetic mutations in *Plasmodium vivax dhfr* with resistance to sulfadoxine-pyrimethamine: geographical and clinical correlates. Antimicrob Agents Chemother 2001; 45(11): 3122–3127
15. Imwong M, Pukrittayakamee S, Rénia L, Letourneur F, Charlieu JP, Leartsakulpanich U, Looareesuwan S, White NJ, Snounou G. Novel point mutations in the dihydrofolate reductase gene of *Plasmodium vivax*: evidence for sequential selection by drug pressure. Antimicrob Agents Chemother 2003; 47(5): 1514–1521
16. Thongdee P, Kuesap J, Rungsihirunrat K, Tippawangkosol P, Mungthin M, Na-Bangchang K. Distribution of dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) mutant alleles in *Plasmodium vivax* isolates from Thailand. Acta Trop 2013; 128 (1): 137–143
17. Tantiamornkul K, Pumpaibool T, Piriyapongsa J, Culleton R, Lek-Uthai U. The prevalence of molecular markers of drug resistance in *Plasmodium vivax* from the border regions of Thailand in 2008 and 2014. Int J Parasitol Drugs Drug Resist 2018; 8(2): 229–237
18. de Pécoulas PE, Tahar R, Yi P, Thai KH, Basco LK. Genetic variation of the dihydrofolate reductase gene in *Plasmodium vivax* in Snoul, northeastern Cambodia. Acta Trop 2004; 92(1): 1–6
19. Asih PB, Marantina SS, Nababan R, Lobo NF, Rozi IE, Sumarto W, Dewi RM, Tuti S, Taufik AS, Mulyanto, Sauerwein RW, Syafruddin D. Distribution of *Plasmodium vivax pvdhfr* and *pvdhps* alleles and their association with sulfadoxine-pyrimethamine treatment outcomes in Indonesia. Malar J 2015; 14(1): 365
20. Joy S, Ghosh SK, Achur RN, Gowda DC, Surolia N. Presence of novel triple mutations in the *pvdhfr* from *Plasmodium vivax* in Mangaluru city area in the southwestern coastal region of India. Malar J 2018; 17(1): 167
21. Rakmark K, Awab GR, Duanguppama J, Boonyuen U, Dondorp AM, Imwong M. Polymorphisms in *Plasmodium vivax* antifolate resistance markers in Afghanistan between 2007 and 2017. Malar J 2020; 19(1): 251
22. Barnadas C, Tichit M, Bouchier C, Ratsimbasoa A, Randrianasolo L, Raherinjafy R, Jahevitra M, Picot S, Ménard D. Plasmodium vivax dhfr and *dhps* mutations in isolates from Madagascar and therapeutic response to sulphadoxine-pyrimethamine. Malar J 2008; 7(1): 35
23. Barnadas C, Timinao L, Javati S, Iga J, Malau E, Koepfli C, Robinson LJ, Senn N, Kiniboro B, Rare L, Reeder JC, Siba PM, Zimmerman PA, Karunajeewa H, Davis TM, Mueller I. Significant geographical differences in prevalence of mutations associated with *Plasmodium falciparum* and *Plasmod*ium *vivax* drug resistance in two regions from Papua New Guinea. Malar J 2015; 14(1): 399
24. Snounou G, Viriyakosol S, Zhu XP, Jarra W, Pinheiro L, do Rosario VE, Thaithong S, Brown KN. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol Biochem Parasitol 1993; 61(2): 315–320
25. Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, Reich D. Principal components analysis corrects for stratification in genome-wide association studies. Nat Genet 2006; 38(8): 904–909
26. Kremsner PG, Krishna S. Antimalarial combinations. Lancet 2004; 364(9430): 285–294
27. Miller KD, Lobel HO, Pappaioanou M, Patchen LC, Churchill FC. Failures of combined chloroquine and Fansidar prophylaxis in American travelers to East Africa. J Infect Dis 1986; 154(4): 689–691
28. Plowe CV, Cortese JF, Djimde A, Nwanyanwu OC, Watkins WM, Winstanley PA, Estrada-Franco JG, Mollinedo RE, Avila JC, Cespedes JL, Carter D, Doumbo OK. Mutations in *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthase and epidemiologic patterns of pyrimethamine-sulfadoxine use and resistance. J Infect Dis 1997; 176(6): 1590–1596
29. Hastings MD, Porter KM, Maguire JD, Susanti I, Kania W, Bangs MJ, Sibley CH, Baird JK. Dihydrofolate reductase mutations in

Plasmodium vivax from Indonesia and therapeutic response to sulfadoxine plus pyrimethamine. J Infect Dis 2004; 189(4): 744–750
30. World Health Organization. Methods for the surveillance of antimalarial drug efficacy. Geneva: WHO, 2009
31. Shaukat A, Ali Q, Connelley T, Khan MAU, Saleem MA, Evans M, Rashid I, Sargison ND, Chaudhry U. Selective sweep and phylogenetic models for the emergence and spread of pyrimethamine resistance mutations in *Plasmodium vivax.* Infect Genet Evol 2019; 68:221–230
32. Leartsakulpanich U, Imwong M, Pukrittayakamee S, White NJ, Snounou G, Sirawaraporn W, Yuthavong Y. Molecular characterization of dihydrofolate reductase in relation to antifolate resistance in *Plasmodium vivax.* Mol Biochem Parasitol 2002; 119(1): 63–73
33. Na BK, Lee HW, Moon SU, In TS, Lin K, Maung M, Chung GT, Lee JK, Kim TS, Kong Y. Genetic variations of the dihydrofolate reductase gene of *Plasmodium vivax* in Mandalay Division, Myanmar. Parasitol Res 2005; 96(5): 321–325
34. Zhou Z. Malaria Control and Research in China. Beijing: People's Medical Publishing House, 1991
35. Yaqoob A, Khattak AA, Nadeem MF, Fatima H, Mbambo G, Ouattara A, Adams M, Zeeshan N, Takala-Harrison S. Prevalence of molecular markers of sulfadoxine-pyrimethamine and artemisinin resistance in *Plasmodium falciparum* from Pakistan. Malar J 2018; 17(1): 471
36. de Pécoulas PE, Tahar R, Ouatas T, Mazabraud A, Basco LK. Sequence variations in the *Plasmodium vivax* dihydrofolate reductase-thymidylate synthase gene and their relationship with pyrimethamine resistance. Mol Biochem Parasitol 1998; 92(2): 265–273
37. Lu F, Lim CS, Nam DH, Kim K, Lin K, Kim TS, Lee HW, Chen JH, Wang Y, Sattabongkot J, Han ET. Mutations in the antifolate-resistance-associated genes dihydrofolate reductase and dihydropteroate synthase in *Plasmodium vivax* isolates from malaria-endemic countries. Am J Trop Med Hyg 2010; 83(3): 474–479
38. Korsinczky M, Fischer K, Chen N, Baker J, Rieckmann K, Cheng Q. Sulfadoxine resistance in *Plasmodium vivax* is associated with a specific amino acid in dihydropteroate synthase at the putative sulfadoxine-binding site. Antimicrob Agents Chemother 2004; 48 (6): 2214–2222
39. Miao M, Yang Z, Cui L, Ahlum J, Huang Y, Cui L. Different allele prevalence in the dihydrofolate reductase and dihydropteroate synthase genes in *Plasmodium vivax* populations from China. Am J Trop Med Hyg 2010; 83(6): 1206–1211
40. Shang LY, Xue CG, Su SZ. Evaluation of the effect of 40 years antimalaria measure in Henan Province. Chin J Parasitol Parasit Dis (Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi) 2000; 18(3): 189 (in Chinese)
41. Liu XZ, Xu BL. Malaria situation and evaluation on the control effect in Henan Province during 1990–2005. Chin J Parasitol Parasit Dis (Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi) 2006; 24(3): 226–229 (in Chinese)
42. Yang HL. Retrospect and prospect of application of antimalarial drugs in Yunnan Province. J Practical Parasitic Dis 1999; 7(4): 174–176
43. Hawkins VN, Joshi H, Rungsihirunrat K, Na-Bangchang K, Sibley CH. Antifolates can have a role in the treatment of *Plasmodium vivax.* Trends Parasitol 2007; 23(5): 213–222
44. Kyabayinze D, Cattamanchi A, Kamya MR, Rosenthal PJ, Dorsey G. Validation of a simplified method for using molecular markers to predict sulfadoxine-pyrimethamine treatment failure in African children with falciparum malaria. Am J Trop Med Hyg 2003; 69(3): 247–252
45. Triglia T, Cowman AF. Primary structure and expression of the dihydropteroate synthetase gene of *Plasmodium falciparum.* Proc Natl Acad Sci USA 1994; 91(15): 7149–7153
46. Brashear AM, Huckaby AC, Fan Q, Dillard LJ, Hu Y, Li Y, Zhao Y, Wang Z, Cao Y, Miao J, Guler JL, Cui L. New *Plasmodium vivax* genomes from the China–Myanmar border. Front Microbiol 2020; 11: 1930
47. Liu Y, Auburn S, Cao J, Trimarsanto H, Zhou H, Gray KA, Clark TG, Price RN, Cheng Q, Huang R, Gao Q. Genetic diversity and population structure of *Plasmodium vivax* in Central China. Malar J 2014; 13(1): 262
48. Li YC, Wang GZ, Meng F, Zeng W, He CH, Hu XM, Wang SQ. Genetic diversity of *Plasmodium vivax* population before elimination of malaria in Hainan Province, China. Malar J 2015; 14(1): 78
49. Huang B, Huang S, Su XZ, Guo H, Xu Y, Xu F, Hu X, Yang Y, Wang S, Lu F. Genetic diversity of *Plasmodium vivax* population in Anhui Province of China. Malar J 2014; 13(1): 13
50. Huang F, Zhou S, Zhang S, Zhang H, Li W. Meteorological factors-based spatio-temporal mapping and predicting malaria in central China. Am J Trop Med Hyg 2011; 85(3): 560–567
51. Ding S, Ye R, Zhang D, Sun X, Zhou H, McCutchan TF, Pan W. Anti-folate combination therapies and their effect on the development of drug resistance in *Plasmodium vivax.* Sci Rep 2013; 3(1): 1008
52. Pan JY, Zhou SS, Zheng X, Huang F, Wang DQ, Shen YZ, Su YP, Zhou GC, Liu F, Jiang JJ. Vector capacity of *Anopheles sinensis* in malaria outbreak areas of central China. Parasit Vectors 2012; 5(1): 136
53. World Health Organization. Artemisinin resistance and artemisinin-based combination therapy efficacy. Geneva: WHO, 2019