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Assessment of the Origins and Spread of Putative Resistance-Conferring Mutations in *Plasmodium vivax* Dihydropteroate Synthase

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Abstract. Infection with *Plasmodium vivax* is usually treated with chloroquine, but parasites are often exposed inadvertently to sulfadoxine-pyrimethamine. To infer patterns of selection and spread of resistant parasites in natural populations, we determined haplotypes of *P. vivax* dihydropteroate synthase (*dhps*) alleles that could confer resistance to sulfadoxine. We amplified the *P. vivax* pyrophosphokinase (*pppk*)–*dhps* region and its flanking intergenic regions from 92 contemporary global isolates. Introns and exons of *pppk-dhps* were highly polymorphic, as were the flanking intergenic regions. Eighteen haplotypes were associated with wild-type alleles, but several different putatively sulfadoxine-resistant alleles have arisen in areas of intensive sulfadoxine-pyrimethamine use. Even when they encoded changes to the same amino acid, these mutant alleles were associated with multiple different haplotypes. Two main conclusions can be drawn from these data. First, *dhps* alleles resistant to sulfadoxine have arisen multiple times under drug pressure. Second, there has been convergent evolution of a variety of alleles that could confer resistance to sulfa drugs.

INTRODUCTION

Infections with *Plasmodium vivax* are generally treated with chloroquine, but incorrect species diagnosis, presumptive treatment of infections with *P. falciparum*, and mixed species infection frequently expose *P. vivax* populations to the antifolate sulfadoxine-pyrimethamine. Early studies suggested that *P. vivax* may be less susceptible to sulfa drugs than is *P. falciparum*, although sample sizes were generally extremely small and the sulfa drug was often not paired with an inhibitor of dihydrofolate reductase (DHFR).[1] Reports of chloroquine resistance in some *P. vivax* populations have increased interest in alternative drug treatments for persons with *P. vivax* malaria, and several recent studies have shown that sulfadoxine-pyrimethamine is effective against *P. vivax* at least in some regions.[2] Notably, a recent randomized clinical trial found that sulfadoxine-pyrimethamine was as effective as chloroquine against *P. vivax* in Pakistan and Afghanistan.[3] These observations increase the potential importance of understanding the mechanisms and patterns of resistance to sulfadoxine-pyrimethamine in *P. vivax* populations.

The mechanisms of sulfadoxine-pyrimethamine have been intensively studied in *P. falciparum* and *P. vivax*. In both species, point mutations in the gene that encodes DHFR confer resistance to pyrimethamine.[2,4–6] In *P. falciparum*, non-synonymous single nucleotide polymorphisms (SNPs) that alter the amino acids within the active site of dihydropteroate synthase (DHPS) have been demonstrated to confer resistance to sulfadoxine.[7–9] There is no system to directly test whether non-synonymous SNPs in the *P. vivax dhps* gene also confer resistance to sulfa drugs. However, Korsinczky and others[10] noted that in every *P. vivax* isolate assessed to date there is a valine at amino acid 585,[10–16] and suggested that this residue may be responsible for steric hindrance and concomitant reduction of sulfadoxine sensitivity. Based on a homology model, Korsinczky and others predicted additional amino acids in *P. vivax* DHPS that when mutated may play a role in sulfadoxine resistance.[10] It was proposed that polymorphisms in *P. vivax* codons 382, 383, 512, 553, and 585, homologous to *P. falciparum* codons 436, 437, 540, 581, and 613, respectively, may be associated with sulfadoxine resistance in *P. vivax*.

Polymorphisms in the *P. vivax dhps* gene have been assessed from a global sampling of isolates,[10–15] and mutations in codons 382, 383, 512, and 553 are more prevalent in regions with high versus low use of sulfadoxine-pyrimethamine.[15] These codons are homologous to those associated with sulfa drug resistance in *P. falciparum* and they have been observed exclusively in isolates carrying pyrimethamine-resistant *dhfr* alleles.[10,11,14,15] These observations suggest that although valine at residue 585 may reduce the sulfadoxine sensitivity of *P. vivax* parasites relative to that of *P. falciparum*, mutations in additional *P. vivax dhps* residues further compromise sulfadoxine efficacy.

By assessing the emergence of putative sulfadoxine resistance-conferring mutations in the *P. vivax dhps* gene, it is possible to infer the mechanisms by which parasites resistant to sulfadoxine-pyrimethamine are selected and spread in natural populations.[17] This information is crucial to predict how resistance to other drugs, for which molecular markers have not yet been identified, may spread. Several groups have investigated the relatedness of resistance-conferring alleles of the *P. falciparum dhps* gene.[18–20] Roper and others assessed three microsatellites flanking the *P. falciparum dhps* gene and found that several haplotypes were associated with the single-mutant and double-mutant *dhps* alleles in Africa.[20] A more recent study has shown that there were five apparent origins of these sulfadoxine-resistant lineages in Africa.[21] A study from the Amazon region of South America found that the *P. falciparum dhps* triple mutant was associated with a single haplotype,[18] and a more recent study in Venezuela corroborated this observation.[19]

To study this question in *P. vivax*, we determined the sequence of the *P. vivax* pyrophosphokinase (*ppk*)–*dhps* region and the flanking intergenic regions from 92 contemporary isolates from four regions: Colombia, Indonesia, Sri Lanka, and Thailand. We then constructed haplotypes based on the sequence data, and inferred the relatedness of the alleles

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that were identified. We have demonstrated that there have been multiple origins of *P. vivax dhps* alleles associated with sulfa drug resistance, and that there has been strong convergent evolution of these alleles that does focus on the codons predicted to confer sulfa drug resistance.

MATERIALS AND METHODS

Orientation of the *P. vivax dhps* gene. *P. vivax pppk-dhps* resides on chromosome 14. A total of 497 basepairs lie between *P. vivax pppk-dhps* and the nearest upstream open reading frame, and 631 basepairs lie between *P. vivax dhps* and the nearest downstream open reading frame (www.tigr.org). Nucleotide coordinates in this report are based upon the El Salvadorian Sal I isolate, the reference sequence from The Institute for Genomic Research. There are two introns and three exons (Figure 1). The first exon spans nucleotides 1–126 and encodes PPPK; the first intron spans nucleotides 127–372. The second exon spans nucleotides 373–2283; nucleotides 373–1241 encode PPPK, and nucleotides 1242–2283 encode DHPS. Intron two spans nucleotides 2284–2432, and the third exon, encoding the remainder of the DHPS domain, spans nucleotides 2433–2549.

Sample sources and DNA extraction. Genomic DNA was extracted from filter paper blotted with the blood of patients infected with *P. vivax* (Qiagen, Rockville MD). We amplified and sequenced the *P. vivax pppk-dhps* region and the entire flanking intergenic region (497 basepairs upstream/631 basepairs downstream) from 92 contemporary patient isolates (Colombia, n = 9; Indonesia, n = 35; Sri Lanka, n = 16; Thailand, n = 28) and from MR4 samples Panama (1966), Nicaragua (pre-1986), Pakchong (Thailand, 1972), and ONG (Vietnamese refugee who had spent time in Indonesia, collected 1980).[22,23]

The *dhfr* haplotypes of all isolates included in this study have been previously reported.[24] The Colombian samples were collected from Urabá on the western coast of Colombia in 2005. The Indonesian samples are derived from two locations, Papua (n = 22) in 1996–1999 and Java (n = 13) in 2000,

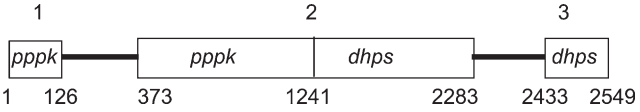

FIGURE 1. Diagram of *Plasmodium vivax* pyrophosphokinase–dihydropteroate synthase (*pppk-dhps*) region. Boxes represent exons and lines represent introns. Numerals above each box are the exon number, and numerals below each box are the nucleotide coordinates of the exon. In the case of exon 2, *pppk* occupies nucleotides 272–1241, and *dhps* occupies nucleotides 1242–2283.

which are separated by approximately 3,200 km. The parent study from which these isolates have been derived is discussed elsewhere.[25] The Thai samples are derived from the Thailand/Cambodia border (n = 6) and from the Thailand/Myanmar border (n = 22) in 2005; the parent study has been described elsewhere.[14,15] The Sri Lankan samples are derived from the north part of the island (n = 12) and the southern part (n = 3) in 2003. In addition, there is one Sri Lankan isolate whose geographic origin is unknown.

All isolates were derived from studies that had been reviewed and approved by the appropriate local Institutional Review Board. These anonymous samples are in the exempt category of the University of Washington Human Subjects Review Board.

Amplification of *P. vivax dhps* and flanking region. A nested polymerase chain reaction (PCR) protocol was used. Primer sequences are shown in Table 1. For the first-round amplification of the *P. vivax dhps* and flanking regions, 0.2 μL of FailSafe enzyme (Epicentre Technologies, Madison WI), 1 μL each of 20 μM S1120 and S1121, 2 μL of genomic DNA, 10 μL of FailSafe buffer E, and water were combined to give a total volume of 20 μL. The PCR protocol used modified hotstart, in which reactions are transferred to the PCR machine only when the machine has reached 94°C. For the first-round and second-round amplifications, the same protocol was used: 94°C for 3 minutes; five cycles at 94°C for 30 seconds, 67°C for 45 seconds, and 72°C for 4 minutes; five cycles at 94°C for

TABLE 1
Primers for amplification and sequencing of *Plasmodium vivax* pyrophosphokinase–dihydropteroate synthase and the flanking intergenic region*

Primer name	Use	Sequence (5′→3′)
S1120	PCR, 1st nested	TGG TCT TGT GGC AAC TGA AG
S1121	PCR, 1st nested	GAG CAA AGC GAA GAG GAG TC
S1122	PCR, 2nd nested, sequencing	GAA GAA ACA GCT CGC GAA AG
S1123	PCR, 2nd nested, sequencing	AGA GGA AGG AGA ACC AGC AA
S1130	Sequencing	GCA GCA GTA ACG ATA GCG G
S1131	Sequencing	TTC CTA CCG GCT TAT GTA CAT TG
S1133	Sequencing	TTT TAA CTT AAC GGT TGT AGT TAG GAC
S1135	Sequencing	AAG TGT CAT ATT TAA AAG AAA GAA CTC
S1136	Sequencing	TAA GTT GGA GTG TGA GGT TGG C
S1207	Sequencing	TAA TTT GGT GAA AAA AAA AAA CAA C
S1208	Sequencing	GCG CAT TAC AGT GGA TTG AA
S1209	Sequencing	ATA TCC TAT TTA CAG CTT ATT TAT ACC
S1210	Sequencing	CTG AAG GTT TTG TAC CAG TCA TAA AG
S1212	Sequencing	TTA AAC CTA AGT GGT AAA ATT TTC AA
S1216	Sequencing	CAG GAT AAC GTC GGT TAA GGA
S1218	Sequencing	TCC AGG CGA TGC TCT ACG
S1219	Sequencing	AAA GCG AAG AGG AGT CGC AT
S1063	Sequencing	CGG CTA CAT TTT CTC GTT CT
S1162	Sequencing	GAT GAA AGG ATA AAC AAA TGT
S991	Sequencing	GTC TAT TAA GCT GTT GCA GC
S1000	Sequencing	CGC TCA TCA GTC TGC ACT C

* PCR = polymerase chain reaction.

30 seconds, 65°C for 45 seconds, and 72°C for 4 minutes; five cycles at 94°C for 30 seconds, 63°C for 45 seconds, and 72°C for 4 minutes; three cycles at 94°C for 30 seconds, 61°C for 45 seconds, and 72°C for 4 minutes; three cycles at 94°C for 30 seconds, 59°C for 45 seconds, and 72°C for 4 minutes; three cycles at 94°C for 30 seconds, 57°C for 45 seconds, and 72°C for 4 minutes; five cycles at 94°C for 30 seconds, 55°C for 45 seconds, and 72°C for 4 minutes; with a final extension at 72°C for 5 minutes.

For the second-round amplification of *P. vivax dhps* and flanking sequences, 1 μL of FailSafe enzyme (Epicentre Technologies), 5 μL each of 20 μM S1122 and S1123, 10 μL of first-nested product (used without any cleanup), 50 μL of FailSafe buffer E, and water were combined to give a total volume of 100 μL. Products were purified by using the GeneClean kit (Qbiogene, Solon OH) following manufacturer's instructions. Products were quantified by subjecting 5 μL of purified product to electrophoresis on a 0.7% agarose gel stained with ethidium bromide.

Sequencing. Sequencing was performed using an ABI capillary sequencer (Applied Biosystems, Foster City, CA), and double coverage with independent primers was used (Table 1). Sequences were aligned and analyzed with Sequencher (Gene Codes, Ann Arbor, MI). Insertions and deletions were manually verified. Sequences were aligned with the Sal I reference sequence (www.tigr.org).

Data analysis. The freely available program Network was used to generate median joining networks.[26] To aid in generation of Network diagrams, sequences were aligned with the commercially available program DNA Alignment (version 1.1.3.0; Fluxus Engineering, Suffolk, United Kingdom). Briefly, the median joining network enables visualization of the mutational paths that may have led to the observed data. Haplotypes are linked based on the assumption that mutations are more likely to derive from a more frequent haplotype and proceed to a less frequent haplotype. In interpreting network diagrams, circles represent haplotypes; the diameter of the circle is proportional to the number of isolates with that allele. The length of lines linking haplotypes is proportional to the number of mutational steps separating haplotypes. Nodes (black circles) represent hypothetical ancestral haplotypes linking the presently extant haplotypes, or may represent haplotypes that, although presently extant, were not sampled.

The Network diagram is especially helpful when trying to determine whether multiple haplotypes of a given *P. vivax pppk-dhps* allele are the result of independent origins or of mutations accumulating on an already established haplotype. In the case where multiple haplotypes of a given *pppk-dhps* allele are present as a result of independent mutations, the haplotypes will be widely dispersed on the Network diagram, separated by a large number of mutational steps. In the case where multiple haplotypes of a given *P. vivax pppk-dhps* allele are present as a result of mutations accruing on one genetic background (i.e., there is one origin of the allele of interest, with additional polymorphisms accumulating stepwise over time), the haplotypes will cluster and will be separated by a limited number of mutational steps.

For the presented Network diagram, sequences of all 96 isolates described in the Materials and Methods were included (92 patient isolates and 4 MR4 isolates). A multi-nucleotide in-frame repeat region, as first described by Menegon and others, was identified within the *dhps* coding region; a repeat region in this position has not been observed in *P. falciparum*.[13] There were 8 repeat types, as described in Tables 2 and 3. The Network program is designed to assess the relatedness of alleles that differ by SNPs rather than indels. Therefore the repeats were recoded such that each repeat type was represented as only one SNP removed from any other repeat type. This factor is important because repeats are likely to be the result of the appearance or disappearance of large segments of DNA as a unit, rather than an addition or subtraction of one nucleotide at a time. By entering sequence data with repeats coded as variation of a single nucleotide relative to the wild type sequence, the Network software more accurately portrays the relationships between alleles.

RESULTS

Putative resistance-conferring mutations in the *P. vivax dhps* gene. We first examined the *dhps* coding region, specifically focusing on codons linked to putative sulfadoxine resistance. Second, we examined the extended haplotype on which mutant *dhps* alleles reside to assess whether these putative resistance-conferring alleles have arisen once or many times.

We sequenced both the entire *P. vivax pppk-dhps* gene and the flanking intergenic region (497 basepairs upstream to 631 basepairs downstream) from this geographically diverse set of isolates. All of the isolates in our dataset carried a valine at *P. vivax dhps* codon 585. By homology to codon 613 in *P. falciparum*, this change has been postulated to confer significant sulfa drug resistance to all *P. vivax* isolates. If this change alone were sufficient to confer clinically significant resistance, then selection of alleles with further mutations might not be observed in response to sulfa drug pressure.

To assess this idea, we focused on *P. vivax dhps* codons 382, 383, 512, and 553, homologous to *P. falciparum dhps* codons 436, 437, 540, and 581,[12] because polymorphisms in these *P. falciparum dhps* codons have been implicated in sulfa drug resistance. Our dataset did not include any polymorphisms in *P. vivax dhps* codon 512, although a polymorphism in this codon has been observed in Thailand.[15]

Information about polymorphisms within the *dhps* coding region, the *pppk* region, the introns, and the flanking intergenic regions is shown in Table 2. Note that within exons of *pppk-dhps*, polymorphisms are displayed as amino acid changes. Within introns and flanking intergenic regions, polymorphisms are displayed as nucleotide changes. Because some polymorphisms within the exons of *pppk-dhps* encoded synonymous changes, some columns in Table 2 list the same amino acid as wild type and mutant. Cells describing these synonymous changes are colored blue, but also contain a one-letter amino acid abbreviation. For simplicity, note that in Table 2 and throughout the remainder of this report *dhps* alleles are referred to with the single letter amino acid abbreviation of codons 382, 383, and 553. For example, wild-type alleles at these three codons (carrying 382S/383A/553A) are referred to as SAA.

Of the 96 isolates examined, 40 isolates carried a *dhps* allele wild type at codons 382, 383, and 553. Among these isolates, the *pppk-dhps* gene contained synonymous and non-synonymous mutations and distinct repeat types within the *dhps* gene. The repeats are abbreviated as A through H in Table 2 and described in detail in Table 3.

TABLE 2

Plasmodium vivax pyrophosphokinase–dihydropteroate synthase (*pppk-dhps*) and flanking haplotypes of contemporary global isolates (n = 92) and MR4 samples (n = 4)*

	Upstream	intron 1			pppk			dhps											intron 2			Downstream		
	-540	141	159	262	132	205	292	*dhps*	repeat	459	584	605	617	618	619	622	623	654	665	2327	2389	2410	+212	+258	+301
TIGR	A	G	A	A	E	M	E	SAA		D	I	A	G	E	A	T	N	P	M	G	G	C	G	C	G
1 Nic MR4								SAA																	
2 Pan MR4								SAA																	
3 Viet MR4						I		SAA	B															T	T
4 Thai MR4		T				I		SAA																	
5 Thai (2)						I		SAA										P							
6 Thai (1)						I		SAA	H																
7 Indo (1)								SAA																	
8 Indo (1)							D	SAA																	
9 Indo (1)			G					SAA																	
10 Indo (1)								SAA														T			
11 Indo (8)								SAA	A																
12 Indo (1)								SAA	A													T			
13 Indo (1)								SAA	B																
14 Indo (1)								SAA	C														A		
15 Indo (1)								SAA	A				G	D								T			
16 Indo (1)								SAA	A				G	D											
17 SL (6)								SAA																	
18 SL (1)								SAA	A																
19 SL (1)								SAA	E																
20 SL (1)								SAA	F																
21 SL (1)								SAA		A															
22 SL (6)								SAA	F	A															
23 Col (9)								SGA																	
24 Thai (4)		T				I		SGA														T			
25 Thai (1)						I		SGA												A					
26 Indo (1)								SGA	A				G	D											
27 Indo (3)			G					SGA	D																
28 Indo (1)			G					SGA				A							I						
29 Indo (8)			G					SGA																T	T
30 Indo (1)								SGA																	
31 Indo (1)	T							SGA																T	T
32 Indo (2)								SGA																T	T
33 Thai (1)						I		AGA	G																
34 Thai (8)				T		I		SGG												A		T			
35 Thai (3)				T		I		SGG													T	T			
36 Indo (1)					G			SGG	E																
37 Thai (2)				T		I		CGG												A		T			
38 Thai (4)				T		I		AGG												A		T			
39 Thai (1)				T		I		AGG			V									A		T			
40 Thai (1)						I		AGG	G						G	T	N								

Legend: WT; 383G; 382A/383G; 383G/553G; 382C/383G/553G; 382A/383G/553G

*The *pppk-dhps* and flanking haplotypes of contemporary global isolates (n = 92) and MR4 samples (n = 4), with each row indicating one distinct haplotype. Only polymorphic positions are included; empty grey cells (print version) or blue cells (online version) indicate identity with the TIGR reference. The table is divided vertically into sixths: the region upstream of *P. vivax pppk-dhps*, the first intron, *pppk, dhps*, the second intron, and the region downstream. Numerals in the first row indicate the position of the polymorphic nucleotide relative to *pppk-dhps* (- refers to positions upstream of *pppk-dhps*, + refers to positions downstream of *pppk-dhps*). Within the *pppk-dhps* coding region, the 3 letters within a single column refer to the amino acids at positions 382, 383 and 553; in the online version, the color coding distinguishes different allelic combinations. Within exons of *pppk-dhps*, polymorphisms are displayed as amino acid changes while in introns and flanking intergenic regions, polymorphisms are displayed as nucleotide changes. Because some polymorphisms within the exons of *pppk-dhps* encoded synonymous changes, some columns of the table list the same amino acid as both the wildtype and mutant. Cells describing these synonymous changes are colored grey (print) or blue (on line), but contain a one-letter amino acid abbreviation. For the repeat within the *dhps* coding region, refer to Table 3.A, insertion of 42 bp after bp 2136 (ggg gag gcc aaa ctg acc aac ggg gag ggt aaa ctg acg aat); B, deletion of bp [2100-2151], C: insertion of 21 bp after bp 2199 (ggg gag gcc aag ctg acc aac); D, insertion of 21 bp after bp 2151 (acg aat ggt gac gcc aaa ctg); E, deletion of bp [2098-2139]; F, deletion of bp [2158-2178]; G, insertion of 21 bp after bp 2121 (gcc aaa ctg acc aac ggg gag); H, insertion of 21 bp after bp 2199 (ggg gag gcc aag ctg acc aac) and insertion of 21 bp after bp 2121 (gcc aaa ctg acc aac ggg gag). Countries are abbreviated as follows: Colombia (Col), Indonesia (Indo), Nicaragua (Nic), Panama (Pan), Sri Lanka (SL), Thailand (Thai), Vietnam (Viet). This table appears in color at www.ajtmh.org.

Thirty-one isolates carried the SGA allele, with various polymorphisms seen within the *pppk-dhps* region in addition to the change at codon 383. Two different double-mutant alleles were identified: the AGA allele was carried by 1 (3.6%) of 28 Thai isolate, and the SGG allele was carried by 12 isolates, 1 (2.9%) of 35 from Indonesia and 11 (39%) of 28 from Thailand. Two triple-mutant alleles were observed, all carried by Thai isolates. The CGG allele was carried by 2 (7.1%) of 28 isolates, and the AGG allele was carried by 6 (2.1%) of 28 isolates.

Arrangement of these alleles according to their geographic origin is shown in Table 4. Significant clustering is obvious. Alleles with mutations in all three codons of interest were found only from at Thailand/Myanmar border, an area under historically high sulfadoxine-pyrimethamine pressure. In contrast, isolates from the Thailand/Cambodia border, Sri Lanka, Indonesia, and Colombia were either wild type at the three codons of interest or carried a single non-synonymous SNP at codon 383. These patterns reflect the sulfadoxine-pyrimethamine use in these regions[14–16] and support the idea that sulfadoxine does exert significant selection on *P. vivax dhps* and that mutations in these three codons augment resistance to sulfadoxine.

Relatedness of putative sulfa drug resistance-conferring alleles. Our second goal was to infer the relatedness of these putatively sulfa drug–resistant alleles. The most straightfor-

TABLE 3

Plasmodium vivax dihydropteroate synthase repeat motif written as amino acid sequence*

Name	Repeat 1	Repeat 2	Repeat 3	Repeat 4	Repeat 5	Repeat 6	Repeat 7	End sequence				No. repeats
A	GEAKLTN	GEGKLTN	GEAKLTN	–	GEGKLTN	GEAKLTN	GEGKLTN	GDAKLTN	–	GDSKLTN	GEAKLTN	9
Tigr†	GEAKLTN	GEGKLTN	GEAKLTN	–	GEGKLTN	–	–	GDAKLTN	–	GDSKLTN	GEAKLTN	7
B	GEAKLTN	GEGKLTN	GEAKLTN	–	-	–	–	–	–	GDSKLTN	GEAKLTN	5
C	GEAKLTN	GEGKLTN	GEAKLTN	–	GEGKLTN	–	–	GDAKLTN	–	GDSKLTN	GEAKLTN	8
D	GEAKLTN	GEGKLTN	GEAKLTN	–	GEGKLTN	–	–	GDAKLTN	GDAKLTN	GDSKLTN	GEAKLTN	8
E	GEAKLTN	GEGKLTN	–	–	-	–	–	–	–	GDSKLTN	GEAKLTN	4
F	GEAKLTN	GEGKLTN	GEAKLTN	–	GEGKLTN	–	–	GDAKLTN	–	–	GEAKLTN	6
G	GEAKLTN	GEGKLTN	GEAKLTN	GEAKLTN	GEGKLTN	–	–	GDAKLTN	–	GDSKLTN	GEAKLTN	8
H	GEAKLTN	GEGKLTN	GEAKLTN	GEAKLTN	GEGKLTN	–	–	GDAKLTN	–	GDSKLTN	GEAKLTN	9

* Repeat one begins at residue 603. Repeat names are as listed in Table 2. – indicates absence of given repeat.
† Reference.

TABLE 4

Polymorphisms in *Plasmodium vivax* dihydropteroate synthase (*Pvdhps*) codons 382, 383, and 553 by country and region*

Pvdhps allele	Col	SL	Java Indo	Papua Indo	Thai/Myan	Thai/Cam
382S/383A/553A		16	4	13		4
382S/**383G**/553A	9		1	16	3	2
382A/383G/553A					1	
382S/**383G/553G**			1		11	
382A/383G/553G					5	
382C/383G/553G					2	

* Mutant condons are in **bold**. Col = Colombia; SL = Sri Lanka; Javo Indo = Java Indonesia; Papua Indo = Papua Indonesia; Thai/Myan = Thailand/Myanmar border; Thai/Cam = Thailand/Cambodian border.

ward approach is to compare the diversity of the extended haplotypes of alleles bearing mutations linked to sulfa drug resistance. Extended haplotypes, including polymorphisms within exons and introns of the *pppk-dhps* region and within flanking intergenic regions, are shown in Table 2. As expected, wild type alleles at codons of interest are associated with many haplotypes; this is true even for those that have similar geographic origins. For example, the Sri Lankan wild type alleles at all codons of interest are associated with six haplotypes. Additionally, there are three repeat regions within the *P. vivax dhps* gene represented among the Sri Lanka isolates. Overall, there were 18 extended haplotypes carried by the 40 isolates that were wild type at *dhps* codons 382, 383, and 553.

Thirty-one isolates carried the single mutant SGA allele. The nine Colombian isolates were all identical, but the five Thai isolates showed two haplotypes and there were seven haplotypes associated with the 17 Indonesian isolates. Eleven Thai isolates carried the double-mutant SGG allele with two haplotypes observed. These two haplotypes were fairly similar, differing only in polymorphisms of the second intron. One Indonesian isolate carried the SGG allele and was associated with a haplotype unlike the Thai SGG haplotypes. One Thai isolate carried the AGA allele (row 33) and was associated with a haplotype similar to that carried by one Thai isolate bearing the triply mutated AGG allele (row 40). These isolates were unique in carrying repeat type G.

All isolates bearing triply mutated *dhps* alleles were from Mae Sot in northwestern Thailand. Two triple-mutant alleles were identified, the CGG allele and the AGG allele. The two isolates with the CGG allele shared a single haplotype. The remaining six isolates carried the AGG allele, with three haplotypes observed; two of these haplotypes were closely related to each other and to the haplotype associated with the CGG allele. However, one AGG allele was quite divergent (row 40) and carried a novel non-synonymous mutation in *dhps* codon 619 and silent mutations in codons 622 and 623. Unlike the other isolates, this AGG allele carried no polymorphisms in the introns. As noted above, this unusual haplotype was similar to the haplotype associated with the Thai AGA double mutant (row 33).

Relationships between putative sulfa drug resistance–conferring alleles. To better visualize relationships among these varied alleles of *P. vivax pppk-dhps*, we analyzed the data set using the Network program described in detail in the Materials and Methods. The diagram in Figure 2 summarizes this analysis. Inspection of the diagram enables several inferences to be drawn. First, as expected, the wild-type alleles and those that carried few mutations in the positions putatively important for resistance are associated with a wide variety of

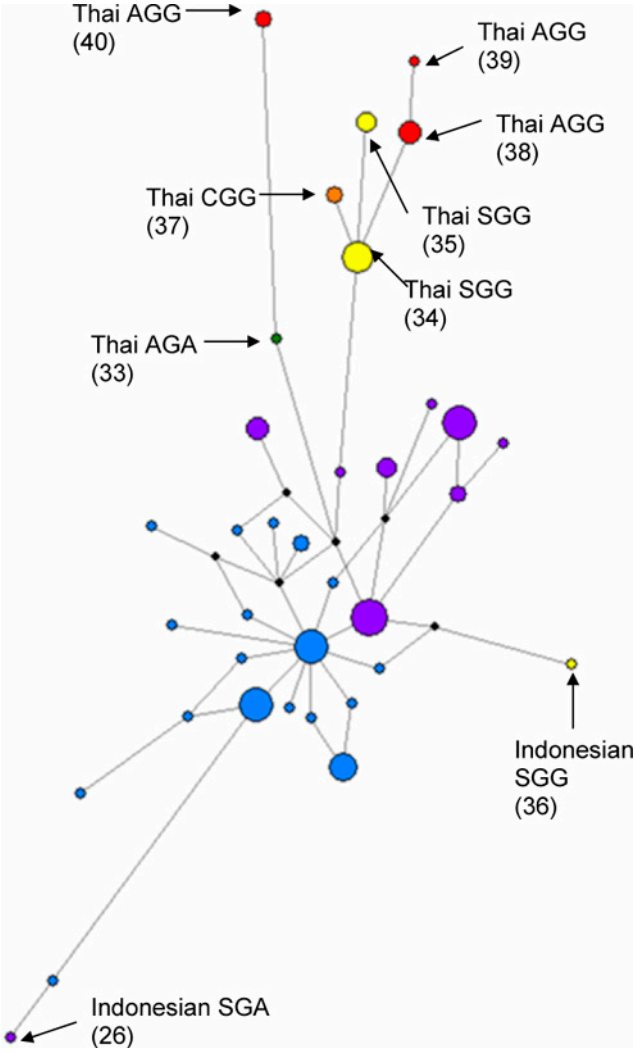

FIGURE 2. Network diagram of *P. vivax dhps* and flanking sequences. Note that only haplotypes with more than one isolate are included in the diagram. In the online version, colored circles represent *dhps* and flanking haplotypes; lines represent mutational steps connecting haplotypes, and black nodes represent hypothetical ancestral haplotypes or haplotypes present in the population but not sampled. Color coding in the online version is as follows: wild type, blue; 383G, purple; 383A/383G, green; 383G/553G, yellow; 382C/383G/553G, orange, 382A/383G/553G, red. The number in parentheses below the country of origin and genotype label refers to the row occupied by the selected haplotype in Table 2. This figure appears in color at www.ajtmh.org.

haplotypes, but most cluster fairly closely. The single mutant SGA allele was found in Colombia, Thailand, and Indonesia, and also is associated with a variety of haplotypes. On the Network diagram, these haplotypes also cluster together for the most part. One isolate from Indonesia (Table 2, row 26) is separated from the other isolates bearing the SGA allele by a large number of mutational steps on the Network diagram. This allele is clearly derived from a different mutational event than the other SGA alleles. However, although most SGA haplotypes cluster together, it is evident from Table 2 that a variety of polymorphisms within the *dhps* coding region, the *pppk* region, the introns, and the flanking regions differentiate these haplotypes. The substantial variation distinguishing the haplotypes with the SGA mutation suggests that these have arisen from at least two different origins.

Second, similar reasoning suggests that there are also at least two distinct origins of the double mutant SGG allele. Isolates bearing this allele were found in Indonesia and Thailand. The Thai isolates bearing the SGG allele (Table 2, rows 34 and 35) are clearly closely related, differing only in the second intron, and cluster in the diagram. In contrast, the lone Indonesian isolate bearing the SGG allele (Table 2, row 36) is associated with a haplotype different from that associated with Thai SGG alleles. The one AGA double mutant (Table 2, row 33) is distinct from the other double-mutant haplotypes, and most closely associated with a Thai isolate carrying the AGG triple mutant allele (Table 2, row 40).

From a practical point of view, it is most important to determine how often clinically relevant, highly mutant alleles have arisen. Are the current alleles in this category independent or are they all related, having spread from a single original origin? In this data set, all of the triple-mutant alleles were isolated from a single location (Mae Sot Thailand). Despite this limited geographic origin, there are two distinct AGG alleles. Five of the six AGG alleles carried a polymorphism at nucleotide 262 within the first intron and polymorphisms within the second intron (Table 2, rows 38 and 39), and the CGG triple-mutant shares this haplotype. In contrast, the haplotype of the sixth AGG isolate is clearly distinct (row 40). The unusual isolate also carried repeat type G within *dhps*, and novel nonsynonymous mutations in *dhps* and *pppk* not observed in any other isolates. Not surprisingly, this unusual isolate clusters far from the other AGG isolates, highlighting the substantial mutational distances between these two sets of alleles. The haplotype of the AGG isolate in row 40 appears to be most closely related to the lone AGA double mutant isolate, also from Thailand (row 33).

Thus, we have identified numerous alleles bearing putative sulfadoxine resistance–conferring polymorphisms in isolates from Colombia, Thailand, and Indonesia. These mutations have arisen independently on a variety of genetic backgrounds. Most notably, the double-mutant SGG has two distinct origins, one in Thailand and one in Indonesia, and the triple-mutant AGG allele has two origins, both in Thailand.

***P. vivax pppk* protein.** Despite the bifunctional nature of the PPPK-DHPS protein, previous assessments of *P. vivax* have focused primarily on the codons within the *dhps* gene that are implicated in sulfadoxine resistance.[10–15] Because we were interested in complete haplotypes, we sequenced the entire *P. vivax pppk-dhps* gene from this geographically diverse set of isolates (Table 2). To our surprise, we identified a common polymorphism, M205I, that was present in all of the Thai isolates, and two polymorphisms, E132G and E292D, that were observed in single isolates from Indonesia. There was no association of these polymorphisms with putatively sulfadoxine-resistant domains of *dhps*, and the significance of these changes on the PPPK enzyme function is unknown.

DISCUSSION

The selection of resistance to sulfadoxine-pyrimethamine has been studied intensively in *P. falciparum.* In that species, alleles of the *dhfr* gene that confer high levels of resistance to pyrimethamine have evolved remarkably rarely, but have spread widely from their apparent foci in Southeast Asia and South America.[18,20,27–32] We have shown previously that pyrimethamine-resistant alleles of *P. vivax dhfr* appear to have

been selected in numerous locations, and spread only locally, a different pattern from that seen in *P. falciparum dhfr*.[24]

In this study, we addressed two issues. First, has sulfadoxine exerted significant selection pressure on the *P. vivax dhps* gene, its presumed target? Second, if these alleles are selected by drug pressure, do resistant alleles arise in many locations, or in just a few locations and then spread as drug use increases?

Because there is no method for testing directly the sensitivity of *P. vivax dhps* to sulfa drugs or sulfones *in vitro*, Korsinczky and others used computational models to compare the *P. vivax* and *P. falciparum* DHPS enzymes.[10] Based on the structural homology of the two enzymes, they proposed that *P. vivax* may be less susceptible to sulfadoxine than is *P. falciparum* because of valine found ubiquitously at *P. vivax dhps* codon 585. Our data are consistent with this idea, but also demonstrate that mutations in *P. vivax dhps* codons 382, 383, and 553 augment sulfadoxine resistance. Mutations in these *P. vivax dhps* codons correspond to positions that have been shown clearly to contribute to sulfa drug resistance in *P. falciparum*.[8,33,34] Polymorphisms in these key *P. vivax dhps* codons have arisen repeatedly in a variety of global isolates, an observation that lends credibility to the contention that mutations in *P. vivax dhps* confer resistance to sulfadoxine in a manner similar to that observed in *P. falciparum*.

The contribution of these mutations to sulfa drug resistance is further supported by the fact that alleles carrying these *P. vivax dhps* mutations were more common in parasites from regions under high versus low sulfadoxine-pyrimethamine pressure. For example, all 22 *P. vivax dhps* alleles from the Thailand/Myanmar border carried mutations in one or more of codons 382, 383, and 553 (Table 4). This region has been subject to significant sulfadoxine-pyrimethamine pressure in the past, and the quadruple-mutant *P. vivax dhfr* allele is highly prevalent in this region.[14,15] In contrast, sulfadoxine-pyrimethamine has not been heavily used in Sri Lanka, and wild-type *P. vivax dhfr* and *dhps* predominate.[16] In the present study, all Sri Lankan isolates carried *dhps* wild-type alleles at the codons of interest. Finally, in *P. falciparum*, mutant alleles of *dhps* are observed only among isolates that also carry a highly mutant *P. falciparum dhfr*,[6] and this pattern is also observed in *P. vivax*.[10,11,14,15] Taken together, these observations strongly support the idea that, as in *P. falciparum*, mutations in *P. vivax dhps* are associated with drug pressure from sulfadoxine-pyrimethamine use, and that these mutant alleles provide a selective advantage to the parasites bearing them.

If these mutations in *P. vivax dhps* confer resistance to sulfadoxine, then the haplotypes associated with mutant alleles may be compared to infer the relatedness of putative resistance-conferring alleles. The isolates that we studied came principally from Southeast Asia. Within this relatively restricted sample set, we found remarkable variation in the *P. vivax pppk-dhps* gene and in the flanking intergenic regions, and detected several novel non-synonymous polymorphisms in the *pppk* and *dhps* coding regions (Table 2). Most important, we found that double-mutant and triple-mutant alleles of *P. vivax dhps* have arisen on several distinct haplotype backgrounds. The SGG double-mutant allele has arisen twice, once in Thailand and once in Indonesia. The AGG triple-mutant allele has two distinct origins, both in Thailand. This diversity contrasts markedly with the relatively few haplotypes associated with highly mutant *P. falciparum dhps* alleles, even those from geographically distant sites in South America and Africa[18–21] and underscores the need for far more extensive examination of the genetic epidemiology of *P. vivax*.

Further studies will be needed to determine the frequency with which resistance-conferring mutations arise, and to identify the parameters that govern the spread of these mutations through *P. vivax* populations. Although molecular markers of chloroquine resistance have not been identified in *P. vivax,* chloroquine resistance has been selected, at least in Indonesia.[35] It is crucial to understand gene flow among *P. vivax* populations to predict how resistance to chloroquine and to other drugs may spread. Improved understanding of this topic will shed light on the poorly understood biology of *P. vivax*, and may have important implications for malaria control policy.[17]

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