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Limited Polymorphism in the Dihydropteroate Synthetase Gene (*dhps*) of *Plasmodium vivax* Isolates from Thailand

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The *dhps* sequences of 55 *Plasmodium vivax* isolates (39 from Thailand and 16 from elsewhere) revealed mutant Pv*dhps* at codons 383 and/or 553 (A → G) in 33 isolates, all from Thailand. Mutations of Pv*dhps* and Pv*dhfr* were correlated. Multiple mutations were associated with high-grade sulfadoxine-pyrimethamine resistance.

After the introduction of the antifols as antimalarial drugs, resistance arose rapidly (10, 12). Pyrimethamine is now usually combined with sulfadoxine (SP) as the two are synergistic. Resistance to pyrimethamine and SP in malaria parasites results from specific point mutations in the parasite genes encoding dihydrofolate reductase and dihydropteroate synthase, respectively. These mutations result in amino acid changes at crucial residues in the active site of these enzymes which reduce drug affinity (2–4, 6–9, 11, 13). Detection of these mutations in field-collected blood samples has proved very valuable in mapping and monitoring resistance and thereby guiding malaria control measures (1, 14).

In this study, we determined the prevalence of mutations in the Pv*dhps* gene from *Plasmodium vivax* isolates from different geographic areas: five from India, two from Iran, two from Madagascar and the Comoros Islands, two reference strains from the Americas, Belem and Sal 1, and Thailand. Thirty-nine isolates were from SP-treated patients whose clinical response to SP was recorded (3). This study was conducted in Thailand, where both *Plasmodium falciparum* and *P. vivax* are often highly resistant to SP. The Pv*dhps* domain was amplified by PCR and sequenced, and PCR-restriction fragment length polymorphism protocols for the sensitive detection of the mutations observed were developed.

Nested PCR amplification strategies were adopted. The oligonucleotides used were designed using a published sequence of the *pppk-dhps* gene of *P. vivax* (GenBank accession no AY 186730) (5). The sequences of primers, Mg^{2+} concentrations, annealing temperatures, numbers of cycles, and sizes of products were individually determined for the different primer pairs (Table 1). Primers for detection of the 553 mutation (VDHPS-553OF) were created by designing primers with mismatches at the 3′ end (coding by the small letter g instead of T), so that polymorphisms not described by natural restriction sites can also be detected to distinguish among all of the polymorphisms in the Pv*dhps* gene identified to date. Digestion of 10 μl of

TABLE 1. Primers and profiles used for amplification of the Pv*dhps* gene

Primer	Sequence[a]	Concn (mM) of Mg^{2+}	Temp (°C)/time (min)			Product size (bp)	Cycles[b]	
			Denaturing	Annealing	Extension		1st	2nd
VDHPS-OF	ATTCCAGAGTATAAGCACAGCACATTTGAG	3	94/2	58/2	72/1	1,354	25	
VDHPS-OR	CTAAGGTTGATGTATCCTTGTGAGCACATC							
VDHPS-NF	AATGGCAAGTGATGGGGCGAGCGTGATTGA	3	94/2	50/2	72/1	705		30
VDHPS-NR	CAGTCTGCACTCCCCGATGGCCGCGCCACC							
VDHPS-553OF	TTCTCTTTGATGTCGGCCTGGGGTTgGCCA	1	94/2	58/2	72/1	171		30
VDHPS-NR	CAGTCTGCACTCCCCGATGGCCGCGCCACC							

[a] Sequences are provided from 5′ to 3′ end.
[b] The two columns indicate conditions for the primary and secondary amplification reactions.

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TABLE 2. Distribution of the Pv*dhfr*/Pv*dhps* haplotypes in relation to clinical outcomes[a]

Early treatment failure (*n*) (total = 11)	Late treatment failure (*n*) (total = 11)	Successful treatment (*n*) (total = 10)
I-F-**R**-T-**N**/**G**-A (4)	I-F-**R**-T-**N**/A-A (1)	I-F-**R**-T-**N**/**G**-A (4)
I-F-**R**-T-**T**/**G***-A (1)	I-F-**R**-T-**N**/**G**-A (3)	I-F-**R**-T-**N**/**G**-**G** (2)
I-**I**-**R**-M-**T**/**G**-**G*** (1)	I-F-**R**-T-**N**/**G**-**G** (2)	I-F-**R**-T-**T**/**G**-**G** (1)
I-**I**-**R**-M-**T**/**G**-**G** (2)	I-F-**R**-T-**N**/**G**-**G*** (1)	I-**L**-**R**-T-**T**/**G**-A (1)
I-**L**-**R**-M-**T**/**G**-**G*** (1)	I-**I**-**R**-M-**T**/A-A (1)	I-**L**-**R**-T-**T**/**G**-**G*** (1)
L-**L**-**R**-M-**T**/**G**-**G** (2)	I-**I**-**R**-M-**T**/**G**-A (1)	I-**I**-**R**-M-**T**/**G**-**G** (1)
	I-**I**-**R**-M-**T**/**G**-**G** (1)	
	I-**I**-**R**-M-**T**/**G**-**G*** (1)	

[a] Haplotypes refer to residues 13, 57, 58, 61, and 117 in Pv*dhfr* and residues 383 and 553 in Pv*dhps*. **G***-A, a mixed A+G genotype was observed in this isolate. **G-G***, a mixed G+A genotype was observed for these residues in one of the isolates. Mutant residues are in bold.

PCR product was performed using 10 U of each restriction enzyme (New England Biolabs Inc., Ipswich, Mass.) for 3 h at 37°C in a total volume of 20 μl. In the majority of cases, sequencing was performed directly on the purified PCR product (around 705 bp), and for a subset of isolates the PCR product was cloned in the pCR2.1 vector (Invitrogen, Groningen, The Netherlands) and sequencing was performed on plasmids purified from positive bacterial colonies. Sequence analysis and alignments were performed using GeneJockey II (Biosoft, Cambridge, United Kingdom).

Mutant Pv*dhps* at codons 383 and/or 553 causing an A-to-G mutation in the amino acid residues was observed in 33 isolates, all from Thailand. Three haplotypes were observed: wild type, single mutation (SGKAV [mutation underlined]), and double mutations (SGKGV). Of the Thai isolates, 32 were collected from *P. vivax*-infected patients who had been treated with SP and observed for 1 month in Bangkok where no reinfections could take place (3). Eleven patients had recurrent vivax parasitemias, but a blood sample was obtained from only five of these patients.

Of the 32 isolates, 9 isolates also had four or more point mutations in *dhfr* (I-L-R-M-T or L-L-R-M-T) together with two point mutations in *dhps* (G-G) (Table 2). Infections with parasites with four or more Pv*dhfr* mutations were 2.5 (95% confidence interval, 1.3 to 4.8) times more likely to have coexisting Pv*dhps* double mutations than the other infections (9/11 versus 7/21; $P = 0.009$). Patients with early treatment failure ($n = 11$), reflecting high-grade resistance, were more frequently infected with parasites with six or more combined mutations of Pv*dhfr* and Pv*dhps* genes compared to the remaining patients (55% versus 14%, $P = 0.016$). The median (range) parasite reduction ratio (PRR) at 48 h after treatment was significantly lower in patients infected with parasites with multiple mutations (range, 0.3 to 29, $P = 0.013$; Table 3). Treatment failure was also associated with multiple mutations in both genes; a higher proportion of patients infected with parasites with multiple mutations of *dhfr* and *dhps* failed treatment: 6/9 versus 5/23; RR (95% confidence interval), 3.04 (1.25 to 7.33); $P = 0.035$ (Table 3). For mutations in the individual genes, these differences were not significant.

No Pv*dhps* point mutations were detected in *P. vivax* from other geographic regions where SP pressure was low and for which the Pv*dhfr* mutation prevalence was also low. Nonsynonymous point mutations at codons 383 (A→G) and 553 (A→G) occurred in the majority of isolates from Thailand. Isolates with double Pv*dhps* mutations had an increased probability of coexisting with multiple mutations (four or more codons) of Pv*dhfr*, which suggests that the selection pressure from widespread use of SP (and also the widely used antibacterial trimethoprim-sulfamethoxazole) applies to both genes and that sulfonamide resistance may contribute to the failure of SP. Parasites harboring the most mutated target genes (with six or more combined mutations of the Pv*dhfr* and Pv*dhps* genes) were cleared more slowly from the blood following SP treatment than less-mutated parasites. Patients with early treatment failures were significantly more likely to be infected with multiple mutants compared to the remaining patients. The linkage of mutations in Pv*dhps* with mutations in Pv*dhfr* and the association of multiple mutations with therapeutic failure suggest that the sulfonamide component contributes to the efficacy of SP. The lack of association of in vivo resistance with mutations in Pv*dhps* alone is consistent with the hypothesis that the main contribution of the sulfonamide component is to provide synergy with pyrimethamine, and this is reduced with these Pv*dhps* mutations. This was a relatively small study and insufficiently powerful to dissect fully the interrelationships between the different mutations and permutations of mutations and resistance. To confirm these suggestions and characterize these relationships, further studies on the purified enzymes and gene transfection experiments are needed in addition to further epidemiological investigations.

TABLE 3. PRR at 48 h and treatment failure rates in relation to multiple mutations in Pv*dhfr* and Pv*dhps*

Gene(s)	Median PRR at 48 h (range)		*P* value[a]	No. of treatment failures/total (%)		*P* value[b]
	≥4 Pv*dhfr* mutations, 2 Pv*dhps* mutations	<4 Pv*dhfr* mutations, <2 Pv*dhps* mutations		≥4 Pv*dhfr* mutations, 2 Pv*dhps* mutations	<4 Pv*dhfr* mutations, <2 Pv*dhps* mutations	
Pv*dhfr*	2.5 (0.3–29)	13 (2–1,500)	0.003	6/11 (55)	5/21 (24)	0.123
Pv*dhps*	8.8 (0.33–1,500)	11.2 (2–1,500)	0.637	6/16 (38)	5/16 (31)	0.710
Both	2.5 (0.3–29)	12.3 (2–1,500)	0.013	6/9 (67)	5/23 (22)	0.035

[a] Determined by Kruskal-Wallis test.
[b] Determined by Fisher's exact test.

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