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 Pvdhps at codons 383 and/or 553 (A → G) in 33 isolates, all from Thailand. Mutations of Pvdhps and Pvdhfr 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 the 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 Pvdhps 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 Pvdhps 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, Mg2+ 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 Pvdhps gene identified to date. Digestion of 10 μl of

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequencea</th>
<th>Conc (mM) of Mg2⁺</th>
<th>Temp (°C)/time (min)</th>
<th>Product size (bp)</th>
<th>Cyclesb</th>
</tr>
</thead>
<tbody>
<tr>
<td>VDHPS-OF</td>
<td>ATCCAGAGTATAAGGCACGACACATTTGAG</td>
<td>3</td>
<td>94/2</td>
<td>1,354</td>
<td>25</td>
</tr>
<tr>
<td>VDHPS-OR</td>
<td>CTAAAGGTGATGATCTCTGGGACATCTC</td>
<td></td>
<td>58/2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VDHPS-NF</td>
<td>AATGGCAAGTGATGCTCTCCGGATGCGCCGCCACC</td>
<td>3</td>
<td>94/2</td>
<td>705</td>
<td>30</td>
</tr>
<tr>
<td>VDHPS-NR</td>
<td>GGCTTGGGTCGAGCTGCAGAGGCAGAGTCGAG</td>
<td></td>
<td>50/2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VDHPS-553OF</td>
<td>TTCTCTTTGATGCTCGCTGGGTTgGCCA</td>
<td>1</td>
<td>94/2</td>
<td>171</td>
<td>30</td>
</tr>
<tr>
<td>VDHPS-NR</td>
<td>CAGTCTGCACTCCGGATGGCCGCCGCCACC</td>
<td></td>
<td>58/2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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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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 Pdhsps 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 (1-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 Pdhsf mutations were 2.5 (95% confidence interval, 1.3 to 4.8) times more likely to have coexisting Pdhsps 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 Pdhsf and Pdhsps 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 Pdhsps point mutations were detected in P. vivax from other geographic regions where SP pressure was low and for which the Pdhsf 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 Pdhsps mutations had an increased probability of coexisting with multiple mutations (four or more codons) of Pdhsf, 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 Pdhsf and Pdhsps 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 Pdhsps with mutations in Pdhsf 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 Pdhsps 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 Pdhsps 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.
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REFERENCES