Genetic Polymorphisms in *Plasmodium vivax*
Dihydrofolate Reductase and Dihydropteroate Synthase in Isolates from the Philippines, Bangladesh, and Nepal

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Abstract: Genetic polymorphisms of *pvdhfr* and *pvdhps* genes of *Plasmodium vivax* were investigated in 83 blood samples collected from patients in the Philippines, Bangladesh, and Nepal. The SNP-haplotypes of the *pvdhfr* gene at the amino acid positions 13, 33, 57, 58, 61, 117, and 173, and that of the *pvdhps* gene at the positions 383 and 553 were analyzed by nested PCR-RFLP. Results suggest diverse polymorphic patterns of *pvdhfr* alone as well as the combination patterns with *pvdhps* mutant alleles isolated from the 3 endemic countries in Asia. All samples carried mutant combination alleles of *pvdhfr* and *pvdhps*. The most prevalent combination alleles found in samples from the Philippines and Bangladesh were triple mutant *pvdhfr* combined with single mutant *pvdhps* allele and triple mutant *pvdhfr* combined with double wild-type *pvdhps* alleles, respectively. Those collected from Nepal were quadruple mutant *pvdhfr* combined with double wild-type *pvdhps* alleles. New alternative antifolate drugs which are effective against sulfadoxine-pyrimethamine (SP)-resistant *P. vivax* are required.

Key words: Plasmodium vivax, Plasmodium vivax dihydrofolate reductase (*pvdhfr*), Plasmodium vivax dihydropteroate synthase (*pvdhps*), malaria, PCR-RFLP, sulfadoxine-pyrimethamine (SP)

*Plasmodium vivax*, the causative agent of relapsing benign tertian malaria, constitutes the second most common cause of malaria in the world with more than 80 million clinical cases annually [1]. In Nepal, *P. vivax* is the predominant malaria species, with the prevalence rate of about 80% of the total malaria cases [2]. Drug resistance monitoring was initiated in 1978 where both in vivo and in vitro studies were conducted with primary focus on *P. falciparum* resistance in Jhapa and Banke districts [2]. Chloroquine-resistant *P. falciparum* was first reported in the country in 1984 and subsequently resistance was spread to other districts. In 1996-1997, sulfadoxine-pyrimethamine (SP) that replaced chloroquine lost its efficacy. In 2000, the late treatment failure rate of *P. falciparum* cases treated with SP was found to be 57% [2]. For Bangladesh and the Philippines, prevalence rates of *P. vivax* infection were relatively lower (13 and 25%, respectively) [3]. Chloroquine resistance was first detected in 1970 in the bordering districts of Bangladesh, i.e., Mymensingh district and Chakdahpuni Tea Estate in Habigonj district. Subsequently, the prevalence rates (RII + RIII) were increased from 10% in 1979 to 45% in 1987, to 57% in 1992 [4]. The molecular targets of action of sulfadoxine and pyrimethamine are dihydropteroate synthase (dhrs) and dihydrofolate reductase (dfr) enzymes, respectively. SP-resistance is determined by specific point mutations in these 2 parasite genes that cause alterations of key amino acid residues in the active sites of these enzymes and thus reduction in the affinity of the enzyme for the drug [5,6].

In most Asian countries, *P. vivax* infections are rarely treated with SP, but *P. vivax* isolates are exposed to SP during treatment of *P. falciparum* with mixed infections with *P. vivax* [7-10].
This has caused a progressive selection of SP-resistant alleles in *P. vivax* isolates [11]. The prevalence of *P. vivax* dihydrofolate reductase (pvdhfr) and dihydropteroate synthase (pvdhps) mutations have been reported from several malaria endemic countries in Asia including Thailand [12,13], Indonesia [14], Nepal [15], India [16], Afghanistan [17], Iran [18], Papua New Guinea [19], Madagascar [20], and the Philippines [23]. For Nepal, previous reports on pvdhfr mutations were identified at S58R (68%, n = 43) followed by S117N/T (54%, n = 35) and F57L (11%, n = 7). For the Philippines, 1 pattern of pvdhfr combination alleles were found, double (66.7%) mutant allele. The most prevalent pvdhfr and pvdhps combination alleles were double mutant pvdhfr 58R/117N alleles combined with single mutant pvdhps 383G allele (50.0%) and combined with single wild type pvdhps A383 allele (50.0%). In this study, the diversity of pvdhfr and pvdhps mutant alleles in *P. vivax* isolates collected during 2005-2011 from the Philippines, Bangladesh, and Nepal were investigated. The study is the first report of the polymorphic frequency and pattern of pvdhfr and pvdhps in Bangladesh and first report of the polymorphic frequency and pattern of pvdhps in Nepal.

A total of 83 blood samples with mono-infection of *P. vivax* were collected from patients attending healthcare facilities in the Philippines in 2005 (33 samples), Bangladesh in 2010 (31 samples), and Nepal in 2011 (19 samples). Approval of the study protocol was obtained from the Ethics Committees of Ministry of Public Health of the Philippines, Bangladesh, and Nepal. Finger-prick blood (200-300 μl) samples were collected onto a filter paper (Whatman No. 3). The dried filter paper samples were stored in the plastic zip bags until analysis. Giemsa-stained thin and thick blood smears were prepared for qualitative and quantitative examination of *P. vivax* parasitemia under a light microscope. Parasite genomic DNA was extracted from individual dried blood spots on the filter paper using a QIAamp DNA extraction mini-kit (QIAGEN, Valencia, California, USA) and stored at -20°C until use.

The primers and amplification conditions used for genotyping of pvdhfr were according to that previously reported with modifications [15,21]. All primers and restriction enzymes were obtained from Fermentas (Waltham, Massachusetts, USA) and Biolabs (Ipswich, Massachusetts, USA), respectively. *Pvdhfr*-OF and *Pvdhfr*-OR primers were used for amplification of the first reaction of *pvdhfr*. PCR cycling condition was as follows: denaturation at 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 64°C for 30 sec, 72°C for 30 sec, and 72°C for 5 min.

**Amino acid codons 13, 33, 58, and 61**

The amplification of point mutations at amino acid codons 13, 33, 58, and 61 was performed using *pvdhfr*-13F and *pvdhfr*-13R primers. The PCR cycling condition was as follows: denaturation at 95°C for 5 min, followed by 25 cycles of 95°C for 30 sec, 66°C for 30 sec, 72°C for 30 sec, and 72°C for 5 min. The PCR products were digested with restriction enzymes Hae III, Cfr42I (Sac II), Alu I, and Bsp45 I to detect the point mutations I13L, P33L, S58R, and T61M, respectively, and thereafter separated on 3% agarose gel.

**Amino acid codons 57 and 173**

The amplification of point mutations at amino acid codons 57 and 173 was performed using *pvdhfr*-F57 and *pvdhfr*-NR primers. The PCR cycling condition was as follows: denaturation at 95°C for 5 min, followed by 25 cycles of 95°C for 30 sec, 66°C for 30 sec, 72°C for 30 sec, and 72°C for 5 min. The PCR products were digested with restriction enzymes Xmn I and Eco130I (Sty I) to detect the point mutations F57I and I173L, respectively, and thereafter separated on 3% agarose gel.

**Amino acid codons 57 and 117**

The amplification of point mutations at amino acid codons 57 and 117 was performed using *pvdhfr*-OF and *pvdhfr*-NR primers. The PCR cycling condition was as follows: denaturation at 95°C for 5 min, followed by 25 cycles of 95°C for 30 sec, 66°C for 30 sec, 72°C for 30 sec, and 72°C for 5 min. The PCR products were digested with restriction enzymes BsrG I, Pvu II, Bst I, and BstN I to detect the point mutations F57I and S117I/N, respectively, and thereafter separated on 3% agarose gel.

The primers and amplification conditions used for genotyping of pvdhps were according to that previously reported with modifications [15,18,21]. All primers and restriction enzymes were obtained from Fermentas and Biolabs, respectively. The amplification for first reaction of pvdhps was used pvdhps-OF and pvdhps-OR primers. The PCR cycling condition was as follows: denaturation at 95°C for 5 min, followed by 25 cycles of 95°C for 1 min, 58°C for 2 min, 72°C for 2 min, and 72°C for 5 min.

**Amino acid codons 383 and 553**

The amplification of point mutations at amino acid codons 383 and 553 was performed using *pvdhps*-NF and *pvdhps*-NR primers, and pvdhps-553OF and pvdhps-NR primers, respectively. The PCR cycling condition was as follows: denaturation at 95°C for 5 min, followed by 25 cycles of 95°C for 1 min, 50°C...
for 2 min, 72°C for 2 min, and 72°C for 5 min. The PCR products were digested with restriction enzymesMsp I andMsc I to detect the point mutations at positions A383G and A553G, respectively, and separated on 3% agarose gel.

Statistical analysis was performed using the SPSS statistical package (version 11.5 SPSS Inc, Chicago, Illinois, USA). The chi-square test was used to determine the difference in prevalence rates of pvdhfr and pvdhps mutant allele(s) of each SNP in P. vivax isolates collected from the 3 countries in Asia. Statistical significance level was set at P < 0.01; chi-square test).

The diversity of mutations in pvdhfr and pvdhps genes of 83 P. vivax isolates collected during 2005-2011 from the Philippines, Bangladesh, and Nepal were investigated in this study. Results from the present study suggest diverse polymorphic patterns of pvdhfr alone as well as the combination patterns with pvdhps mutant alleles in P. vivax isolates collected from the 3 endemic countries in Asia. The polymorphic patterns of pvdhfr and pvdhps genes were highly diverse (Table 1). For pvdhfr, the wild-type I13 and I173 alleles were detected in all samples from the Philippines, Bangladesh, and Nepal. Three mutant alleles, i.e., 58R, 61M, and 117N were observed in samples collected from the Philippines with frequencies ranging from 81.8 to 100%.

Eight mutant alleles at 5 amino acid residues, i.e., 33L, 57L, 58R, 58SR, 61M, 117N, 117T, and 117NT were detected in samples from Bangladesh at varying frequencies (32.3-87.1%). Five mutant alleles at 4 amino acid residues, i.e., 33L, 58R, 61M, 117N, and 117NT were observed in samples collected from Nepal with frequencies ranging from 47.4-100%. Significant differences in frequencies of the 4 pvdhfr muta
tions (33L, 57L, 58R, and 117N) were observed in samples from the 3 countries carried wild-type A383 allele. The mutation at 553G allele was found in all samples collected from the Philippines, but not in samples from Bangladesh and Nepal.

In the previous study published in 2011 [15], double mutant pvdhfr allele was commonly detected in P. vivax isolates in Nepal, but the triple and quadruple pvdhfr mutant haplotypes which are associated with a high level of in vivo pyrimethamine resistance were not found. In this study, in addition to single (21.0%), double (31.6%), triple (10.6%), and quadruple (36.8%, 33L/58R/61M/117N) mutant alleles, pvdhfr mutant alleles were found in the 2011 Nepal isolates, and all isolates possessed the mutant 61M allele. No isolate carried any mutant allele of pvdhps, suggesting an increased level of pyrimethamine resistance.

Table 1. The frequencies and patterns of pvdhfr and pvdhps single nucleotide polymorphisms in 83 P. vivax isolates (bold letters indicate mutant amino acids)

| Gene | Amino acid position | SNPs | No. of isolates (%) Philippines (n=33) | No. of isolates (%) Bangladesh (n=31) | No. of isolates (%) Nepal (n=19) |
|------|---------------------|------|----------------------------------------|--------------------------------------|---------------------------------|
| Pvdhfr | 13 | I (wild-type) | 33 (100.0) | 31 (100.0) | 19 (100.0) |
|       | 33 | P (wild-type) | 33 (100.0) | 19 (61.3) | 6 (31.6) |
|       | 57 | F (wild-type) | 33 (100.0) | 21 (67.7) | 19 (100.0) |
|       | 58 | S (wild-type) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
|       | 61 | T (wild-type) | 6 (18.2) | 5 (16.1) | 0 (0.0) |
|       | 117 | M (mutant) | 27 (81.8) | 26 (83.9) | 19 (100.0) |
|       | 173 | L (mutant) | 33 (100.0) | 31 (100.0) | 19 (100.0) |
| Pvdhps | 383 | A (wild-type) | 33 (100.0) | 31 (100.0) | 19 (100.0) |
|       | 553 | G (mutant) | 0 (0.0) | 0 (0.0) | 0 (0.0) |

*Statistically significant difference from samples collected from the other 2 countries (P < 0.01; chi-square test).

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methamine resistance, but not sulfadoxine in Nepal in 2011.

Only 1 pattern of \( pv dhfr \) allele, i.e., double (66.7%, 58R/117N) mutant allele and 2 patterns of \( pv d h p s \) alleles (50% 383G and 50% A383), was previously reported in the isolates from the Philippines in 2002 [23]. In the current study, 2 patterns of \( pv dhfr \) combination alleles, i.e., triple (81.8%) and double (18.2%) mutant alleles were found in 2005, and all isolates possessed the mutant 58R and 117N/T alleles. The most prevalent \( pv dhfr \) and \( pv d h p s \) combination alleles were triple mutant \( pv dhfr \) 58R/61M/117N allele combined with single mutant \( pv d h p s \) 553G allele (78.8%) (Table 2), suggesting increased level of SP resistant \( P. \) vivax isolates in the Philippines.

Three patterns of \( pv dhfr \) combination alleles were found in samples collected from Bangladesh, i.e., quadruple (32.3%), triple (54.8%, 58R/61M/117N), and double (12.9%) mutant alleles. No mutant \( pv d h p s \) allele was detected, indicating that \( P. \) vivax isolates circulating in Bangladesh possessed high pyrimethamine resistance genotype, but not for the sulfadoxine in 2010.

The mutants \( pv dhfr \) 58R and 117N/T were shown to be the dominant alleles in Southeast Asia, i.e., Thailand [12,13,15,22], Vietnam and the Philippines [23], East Timor [24], Myanmar [25], and Indonesia [14]. The mutants \( pv d h p s \) 383G and 553G alleles were observed at low frequencies in most geographic regions including East Timor [24], Korea [25], Iran [18,21], and Pakistan [17,21], with highest frequency in Thailand [17,21,27]. The diversity of mutations in \( pv dhfr \) and \( pv d h p s \) suggest different intensity of selective pressure resulting from SP uses for treatment of \( P. \) falciparum in 3 countries in Asia. This drug should not therefore be used in these countries for treatment of \( P. \) vivax infection.

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**Table 2.** Distribution of \( pv dhfr \) and \( pv d h p s \) combination alleles in 83 \( P. \) vivax isolates included in the analyses (bold letters indicate mutant amino acids)

| \( Pvdhps \)  | Philippines (33) | Bangladesh (31) | Nepal (19) |
| | A383G | A553G | I13L | P33L | F57/L | S58R | T61M | S117T/N | I173L | A | G | I | P | F | R | T | N | I | 26 | 0 | 0 |
| | A | G | I | P | F | R | M | N | I | 1 | 0 | 0 |
| | A | A | I | P | F | S | M | S | I | 0 | 2 | 0 |
| | A | A | I | P | F | R | M | S | I | 0 | 1 | 0 |
| | A | A | I | P | F | R | M | N | I | 0 | 1 | 0 |
| | A | A | I | P | F | R | M | N | I | 0 | 10 | 1 |
| | A | A | I | P | F | R | M | S| N | I | 0 | 1 | 1 |
| | A | A | I | P | L | R | M | S | I | 0 | 1 | 0 |
| | A | A | I | P | L | R | T | T | I | 0 | 3 | 0 |
| | A | A | I | L | F | S | M | S | I | 0 | 1 | 6 |
| | A | A | I | L | F | S | M | N | I | 0 | 2 | 7 |
| | A | A | I | L | F | R | M | T | I | 0 | 1 | 0 |
| | A | A | I | L | F | R | M | S| N | I | 0 | 1 | 0 |
| | A | A | I | L | F | S,R | M | T | I | 0 | 1 | 0 |
| | A | A | I | L | L | S | M | S | I | 0 | 1 | 0 |
| | A | A | I | L | L | R | M | S | I | 0 | 3 | 0 |
| | A | A | I | L | L | R | T | T | I | 0 | 2 | 0 |

Total 33 31 19
CONFLICT OF INTEREST

We have no conflict of interest related to this work.

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