Prevalence of mutation and phenotypic expression associated with sulfadoxine-pyrimethamine resistance in *Plasmodium falciparum* and *Plasmodium vivax*

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**Abstract:** Therapeutic efficacy of sulfadoxine-pyrimethamine (SP), which is commonly used to treat falciparum malaria, was assessed in isolates of *Plasmodium falciparum* (Welch, 1897) and *Plasmodium vivax* (Grassi et Feletti, 1890) of Aligarh, Uttar Pradesh, North India and Taif, Saudi Arabia during 2011–2012. Both the species showed mutations in dihydrofolate reductase (DHFR) enzyme as they have common biochemical drug targets. Mutation rate for pfdhfr was higher compared to pvdhfr because the drug was mainly given to treat falciparum malaria. Since both the species coexist, *P. vivax* was also exposed to SP due to faulty species diagnosis or medication without specific diagnosis. Low level of mutations against SP in *P. falciparum* of Saudi isolates indicates that the SP combination is still effective for the treatment of falciparum malaria. Since SP is used as first-line of treatment because of high level of resistance against chloroquine (CQ), it may result in spread of higher level of mutations resulting in drug resistance and treatment failure in near future. Therefore, to avoid further higher mutations in the parasite, use of better treatment regimens such as artesunate combination therapy must be introduced against SP combination.

**Keywords:** molecular study, malaria, drug resistance, India, Saudi Arabia

Malaria is the most prevalent parasitic disease worldwide, responsible for an estimated 225 million clinical cases each year. It mainly affects children, pregnant women and non-immune adults who frequently die as victims of cerebral manifestations and anaemia (Quintero et al. 2011). It is major cause of death in tropical and sub-tropical countries where it claims approximately 655 thousand lives each year (World Health Organization 2010). This disease is managed by anti-malarial drugs as no effective vaccine is yet available. However, the parasite has developed resistance against most commonly used antimalarial chloroquine (CQ), posing a major problem for malaria control programme (Mitra et al. 2006).

In case of doubtful species diagnosis, sulfadoxine-pyrimethamine (SP) combination is usually prescribed by clinicians to avoid any risk on the life of patient and, thereby, increases SP pressure on parasite population. With the spread of chloroquine resistant malaria in different countries including India and Saudi Arabia, SP alone or in combination with artesunate is used as an alternate drug. This drug combination targets the folate biosynthetic pathway of *Plasmodium* species; blocking the nucleic acid biosynthesis and thus causing parasite death (Peterson et al. 1988, Triglia et al. 1997). Pyrimethamine acts on the dihydrofolate reductase enzyme of *Plasmodium falciparum* (Welch, 1897) (PFDHFR) and inhibits the folate biosynthesis pathway of the parasite (Gregon and Plowe 2005). Point mutation in PFDHFR reduces its capacity to bind with the drug, resulting in the emergence of resistant parasite strains (Mbugi et al. 2006, Gesase et al. 2009). There are mutations at more than three loci in the parasite enzyme in endemic countries (Peterson et al. 1988, Sirawaraporn et al. 1997).

Pyrimethamine resistance develops in a progressive manner and treatment failure occurs. Since both *P. falciparum* and *P. vivax* (Grassi et Feletti, 1890) contain the same target enzymes, the antifolate drug also affects *P. vivax* – see Imwong et al. (2003), Hastings et al. (2004), de Pecoulas et al. (2004), Kaur et al. (2006), Barnadas et al. (2008). Keeping these facts in mind, study on the prevalence of mutations and phenotypic expressions associated with sulfadoxine-pyrimethamine resistance in *P. falciparum* and *P. vivax* is attempted in the samples obtained from India and Saudi Arabia.

**MATERIALS AND METHODS**

Blood samples collected from the patients with fever who attended the Medical College and other malaria clinics of Aligarh, India during 2011–2012, and the Communicable Diseases Control Centre, Taif, Saudi Arabia, from the patients who visited Jazan and Yemen earlier, were included in this study. Thick and thin blood smears of these patients were screened by E-600
An aliquot of the primary amplicon was diluted and subjected to PCR amplification with a 10 min initial denaturation at 94 °C followed by 35 cycles with a 30 sec annealing at 54 °C and a one minute extension at 72 °C as the cycling parameters. Primers PvDA (covering -75 to -55 bp upstream to the pfdhfr-ts start codon), PvDB, PvDF and PvDR were designed at 54 °C and a one minute extension at 72 °C as the cycling parameters. Primers PvDA (covering -75 to -55 bp upstream to the pfdhfr-ts start codon), PvDB, PvDF and PvDR were designed for nesting PCR to amplify the Pvdhfr gene.

DNA extraction, PCR amplification and nucleotide sequencing of DHFR gene:

DNA extraction, amplification and nucleotide sequencing were done as described earlier by Alam et al. (2007). DNA was extracted from the blood of patients that were infected with P. falciparum and P. vivax using Accu Prep Genomic DNA extraction kit (Bioneer Corporation of Korea). The DNA was eluted in Tris EDTA buffer (pH 8.0) and an aliquot was used for PCR amplification. A fragment of 1014 bp of P. vivax dhfr-ts gene was amplified using primers PvDA (5'-AccgcAC-CAGTGATTTCTAC-3') (forward) and PvDB (5'-ACCgcAC-CAGTGATTTCTAC-3') (reverse). The cycling parameters were a 10 min initial denaturation at 94 °C followed by 35 cycles with a 30 sec denaturation at 94 °C, one minute annealing at 58 °C, one minute extension at 72 °C and a final 10 min extension at 72 °C. After diluting 10 times, this priming PCR product, was used as a DNA template for nesting PCR to amplify the 784-bp region, which covered the entire DHFR domain.

Primers PvDF (5'-ATGGAGCCCTTCCAGATGT-3') (forward) and PvDR (5'-AACGCATTGGATTCTCGA-3') (reverse) were used for nested PCR with a 10 minutes initial denaturation at 94 °C followed by 35 cycles with a 30 sec annealing at 54 °C and a one minute extension at 72 °C as the cycling parameters. Primers PvDA (covering -75 to -55 bp upstream to the Pvdhfr-ts start codon), PvDB, PvDF and PvDR were designed from the known Pvdhfr-ts sequence of pyrimethamine-sensitive P. vivax isolate ARI/Pakistan (98123). DNA was extracted and subjected to PCR amplification of a 720-bp fragment to the pfdhfr gene using the method described by Ahmad et al. (2004). An aliquot of the primary amplicon was diluted and subjected to seminested PCR using primer AMP1 (Plowe et al. 1997) and DHFR2 (5'-ACAGAAATATTGATCTCA-3'). Only 30 cycles were carried out for seminested PCR under the same conditions as has been used for primary PCR. The PCR products were purified and subjected to sequencing according to Mitra et al. (2006), using an ABI Big dye terminator Ready Reaction Kit (version 3.1), and the ABI Prism 310 genetic analyser (PE Applied Biosystems, Foster City, California, USA).

Individual bands of each PCR product were excised from the agarose gel and purified using an Accu Prep gel purification kit (Bioneer Corporation, Korea) according to manufacturer’s instructions. The entire pfdhfr gene was sequenced using primer PvdM (5'-GTTAGGCTCTTGGCTTGGAAAGCAC-3') and PvdR, whereas primers for pfdhfr gene sequencing were same as described by Jalal et al. (2005). The nucleotide sequences were translated into amino acids using the edit sequence program (Lasergene 1999, version 5.1, DNASTAR Inc., Madison, WI, USA). The amino acid sequences were aligned using gen Doc Multiple Sequence Alignment Editor and Shading Utility (Version 2.6.002).

Statistical analysis:
The chi-square test was applied to assess the trends of DHFR mutations in both Indian and Saudi isolates. Among Indian isolates, the pfdhfr mutations were observed at codons S108N, C59R and N51I. Maximum number of isolates (92%) showed mutations at codon 108 (S108N) (P < 0.05) followed by mutations at codon 59 (C59R, 55%) and least of 4% at codon 51 (N51I). Majority of the Indian isolates were found to contain double pfdhfr mutations (Figs. 1, 2; Table 1–3). The double mutation C59R plus S108N was much more common and observed in 28 isolates (55%; n = 51) with genotype A16, N51, R59, N108, I164 than the N51I plus S108N mutations in two isolates (4%; n = 51) with genotype A16, I16, C59, N108, I164. Single mutation at codon 108 (S108N) was detected in 17 isolates (33%; n = 51) having genotypes A16, N51, C59, N108, I164. Only 4 isolates (8%; n = 51) showed wild type amino acids with genotype A16, N51, C59, S108, I164. Out of 14 isolates of Plasmodium falciparum from Saudi Arabia, only two (14%; n = 14) showed wild type amino acids with genotype A16, N51, C59, S108, I164.

RESULTS

Sequencing of pfdhfr and pvdhfr gene showed mutations in both Indian and Saudi isolates. Among Indian isolates, the pfdhfr mutations were observed at codons S108N, C59R and N51I. Maximum number of isolates (92%) showed mutations at codon 108 (S108N) (P < 0.05) followed by mutations at codon 59 (C59R, 55%) and least of 4% at codon 51 (N51I). Majority of the Indian isolates were found to contain double pfdhfr mutations (Figs. 1, 2; Table 1–3). The double mutation C59R plus S108N was much more common and observed in 28 isolates (55%; n = 51) with genotype A16, N51, R59, N108, I164 than the N51I plus S108N mutations in two isolates (4%; n = 51) with genotype A16, I16, C59, N108, I164. Single mutation at codon 108 (S108N) was detected in 17 isolates (33%; n = 51) having genotypes A16, N51, C59, N108, I164. Only 4 isolates (8%; n = 51) showed wild type amino acids with genotype A16, N51, C59, S108, I164. Out of 14 isolates of Plasmodium falciparum from Saudi Arabia, only two (14%; n = 14) showed wild type amino acids with genotype A16, N51, C59, S108, I164.
showed mutations at codon 59 (C59R) with genotype A166N, R59S, S108I. Remaining 12 (86%; n = 14) showed wild amino acids A166, N59, C59, S108, I164.

Mutation analysis by sequencing DHFR domain of the pvdhfr-ts from 46 *P. vivax* isolates revealed that the mutations occurred at S58, N51, S93 H and S93 R codons. Frequency of mutation among the isolates from Aligarh, Uttar Pradesh, north India was higher at codon S117 N followed by the codon S93 H. A total of 28 isolates (61%; n = 46) contained the wild type amino acids with genotype at codons F57, S58, T61, S93, S117. Among the remaining isolates, the frequency of double mutations of DHFR was highest and observed in 12 cases (26%; n = 46) where genotype was F57R, R58T, S93N, S117N. Only six isolates (13%; n = 46) showed mutations at single codon S93H/S117N of 6.5% each and the genotypes were F57S, S58T, T61S, H93S, S117S and F57S, S58T, I164S, S93N, S117N.

During the present study, it was observed that rings of *P. falciparum*, which otherwise varied in size and shape, showed little more prominent variations in terms of shape and thickness of the cytoplasm (data not shown). In *P. falciparum* having mutations at two codons, rings were much oblong with the thickened cytoplasm at one end. It remains unclear whether this change in size and shape of rings of *P. falciparum* is a mere coincidence or it is due to the alterations of the amino acid in DHFR enzyme.

**DISCUSSION**

Antifolate drugs are generally used for the treatment of falciparum malaria in developing countries. According to the National Drug Policy of both, India and Saudi Arabia, chloroquine-resistant uncomplicated falciparum malaria cases should be treated with sulfadoxine-pyrimethamine (SP). In regions where resistance due to chloroquine (CQ) has reached a very high level, SP is the first-line of anti-malarial. There are few primary health care centres and medical colleges in India and Saudi Arabia where artesunate-based combined therapy has been introduced as an alternative treatment. Several countries have already abandoned chloroquine and SP monotherapy because of emergence and worsening rise of CQ and SP-resistance (Bosco et al. 1998, Shretta et al. 2000). If *Plasmodium falciparum* and *P. vivax* coexist in any region, both are at risk of being exposed to the antifolate drug (SP) as they have common target enzymes (Tjitra et al. 2002, Imwong et al. 2003, de Pecoulas et al. 2004, Gregon and Plowe 2005, Barradas et al. 2008).

In the present study, a low level of SP resistance with a total of 59% pfdhfr mutations as C59R plus S108N and N51 plus S108N were recorded from Aligarh, Uttar Pradesh of North India where chloroquine-resistant uncomplicated falciparum malaria is lower. Studies with molecular markers have confirmed rapid development of resistance after the drug was used.
at national level in a number of settings. (Sridaran et al. 2010, Zakeri et al. 2010).

We observed low level of mutations in pvdhfr gene in Aligarh, Uttar Pradesh, which is located in northern India, having wild type of amino acids in majority of the cases (61%; n = 46). Double and single mutations were observed in 12 (26%; n = 46) and six isolates (13%; n = 46), respectively. These figures are almost half of pfdhfr in the same region, which might be due to the simple reason that P. falciparum was actually treated with SP. Since P. vivax is the most prevalent species in the study area and have mixed infections too, dhfr of this species showed mutations against SP as it has the same drug target and was exposed to this drug because of wrong diagnosis and/or medications without species diagnosis.

Unfortunately, resistance developed relatively quickly when SP was widely used in endemic countries. Epidemiological and molecular studies on P. falciparum and P. vivax very clearly indicate that the dhfr enzyme is the therapeutic target of SP (Nzila et al. 2000, Tjitra et al. 2002, Imwong et al. 2003, de Pecoula et al. 2004, Hastings 2004). As a result, resistance to the SP is determined by specific point mutations in the parasite dhfr gene. These mutations cause alterations of key amino acid residues in the active sites of these enzymes reducing the affinity of enzymes for the drug (Foote and Cowman 1994).

Plasmodium vivax infections are not often treated with SP but this species got exposed to SP because of mixed infection and caused mutations (Kaur et al. 2006, Khatoon et al. 2009, Zakeri et al. 2010). The continuous use of SP as first-line antimalarial drug in endemic areas, where both P. vivax and P. falciparum species coexist, may increase the risk of mutations in the dhfr gene leading to a higher level of resistance against this combination in the near future. Therefore, continuous surveillance of P. vivax and P. falciparum molecular markers are needed to monitor the development of resistance to SP in endemic foci in poor countries, where this drug is used quite extensively due to its cost-effectiveness and easy availability, and taken as a single dose.

In conclusion, both P. falciparum and P. vivax showed mutations in DHFR enzyme against SP. In India, mutation rate for pfdhfr against SP was higher compared to pvdhfr. Low level of mutation in pfdhfr against this drug in Saudi Arabia indicates that SP is still effective for treatment of falciparum malaria, but its use as first-line of treatment may result in higher level of mutations resulting in drug resistance and treatment failures in near future. Therefore, to avoid further higher mutations in dhfr enzyme of Plasmodium, use of better treatment regimens such as artemisinin combination therapy must be introduced against SP combination.

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