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Original Article

Molecular Characterization of Dihydrofolate Reductase-Thymidylate Synthase Gene Concerning Antifolate Resistance of *Plasmodium vivax*

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Abstract

Background: The recently reported resistance to antimalarials contributes to making the control of malaria more difficult. There is a need to evaluate the current antimalaria regimens to prevent this emerging problem. The aim of this study was to determine dihydrofolate reductase-thymidylate synthase gene mutation (*pvdhfr*) regarding antifolate resistance in *Plasmodium vivax*.

Methods: From 2007 to 2009, 117 *P. vivax* infected blood samples collected from two regions of Hormozgan Province, south of Iran were analyzed using PCR, semi-nested-PCR and RFLP methods.

Results: Eighty four isolates (71.8 %) showed no mutation in *pvdhfr* gene of *P. vivax* known as wild type and 33 (28.2%) of the samples revealed nine single (7.7%), twenty two double (18.8%) and two (1.7%) triple mutations.

Conclusion: Genetic diversity was observed by molecular methods in *pvdhfr* gene of *p. vivax* in Hormozgan Province suggests that the antifolate falciparum malaria drug (fansidar) is proportionally affecting *P. vivax dhfr* mutation. Therefore, more studies to evaluate antimalarial drugs that should preferably be effective against both *P. vivax* and *P. falciparum* are recommended.

Keywords: Dihydrofolate reductase-thymidylate synthase, PCR-RFLP, Plasmodium vivax, Mutation, Iran

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Introduction

Plasmodium vivax is the most geographically widespread cause of malaria in the world. Among 2.6 billion people at risk of *vivax* malaria infection, annual estimates of *P. vivax* cases range from 130 to 435 million (1, 2). Malaria is an important infectious disease in Iran and *P. vivax* is the most common cause of malaria (3). The two current frontline therapies for *P. vivax*, chloroquine (CQ) and primaquine (PQ), have been in use since 1946 and 1950, respectively (2). Chloroquine resistant *P. vivax* was not reported until 1989, when it appeared in Papua New Guinea (4). Since then, cases of resistance have been reported from other places of the world (5-8). Although, chloroquine is still an efficacious drug for the treatment of *vivax* malaria in south and southeast Iran, but an early sign for reduced susceptibility of the parasite to the drug was seen (3). This emphasizes the need to search for alternative treatments for *P. vivax* infection, possibly including drugs of the antifolate class (9, 10). In protozoa, dihydrofolate reductase (*dhfr*) and thymidylate synthase (*ts*) are parts of a bifunctional enzyme encoded by a single gene (11). Pyrimethamine, interacts with the folate synthesis pathway of the parasite where dihydrofolate reductase (*dhfr*) enzyme reduces dihydrofolate to tetrahydrofolate. Pyrimethamine competitively inhibits parasite *dhfr* thereby blocking thymidylate synthesis (12).

There are a considerable number of cases with mixed infections (*P. falciparum* and *P. vivax*) in Iranian blood samples, mainly remain undiagnosed by microscopy (13, 14). Therefore, the use of sulphadoxine/pyrimethamine (SP) to treat *P. falciparum* is creating selection pressure in the *P. vivax* population. In vitro and in vivo studies

on *P. falciparum* Iranian isolates have already shown SP resistance (15-18).

The aim of the present study was to obtain information concerning mutations related to pyrimethamine resistance in the *P. vivax dhfr* gene of Iranian field isolates in Hormozgan Province, south of Iran.

Materials and Methods

From April 2007 through January 2009, a total of 117 blood samples were collected from *P. vivax* malaria patients in two endemic areas of Hormozgan Province (Minab and Bandare Jask), south of Iran (Fig. 1).

One milliliter of obtained blood samples including antiagulant EDTA were transferred to the Department of Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran and were kept frozen until DNA extraction. All samples were collected with the consent of the patients or their parents before the start of treatment. *P. vivax* was diagnosed using microscopic examination of Giemsa stained thin and thick blood smears.

DNA extraction and PCR amplification:

Total DNA was extracted from each 100- μ l positive blood samples by DNG-plus extraction Kit (Cinnagen, Iran) according to the manufacturer's instruction and the extracted DNAs were stored at -20°C until use. A semi-nested PCR protocol was used to amplify the *pvdhfr-ts* gene using two sets of oligonucleotide primers based on the available database gene (Accession no. **X98123**) as previously described (19). For the primary PCR, whole of 1,869bp of the *pvdhfr* gene was amplified using VDT-OF (5'-ATG-GAGGACCTTTCAGATGTATTGA-

CATT-3') and VDT-OR (5'-GGCGGCCATCTCCATGGTTATTTTATC GTG-3') primers. For the second PCR With VDT-OF and VDF-NR (5'-TCA-CACGGGTAGGCGCCGTTGATCCTCGT G-3') primers a fragment of 608bp was amplified (Fig. 2), and for the third PCR with VDT-OF and VDF-NR58 (5'-GGTACCTCTCCCTCTTCCACTTTAGCT TCT-3') primers a fragment of 238bp was obtained (Fig. 3).

The primary PCR mixture contained 1X PCR buffer, 0.8 mM MgCl₂, 0.15 mM dNTP, 1.25 U of AmpliTaq polymerase (RojanAzma, Iran), 2 µl of the genomic DNA and 1 µM each forward and reverse primers in a 30 µl reaction volume. Each of the 30 cycles consisted of 94 °C for 1 min, 64 °C for 1 min, and 72 °C for 2 min after an initial denaturing at 95 °C for 5 min and final extension at 72 °C for 5 min. The second and third PCR mixture were identical except that 1/50 diluted of the primary PCR products were used as DNA template and after an initial denaturing at 95 °C for 3 min, each of the 35 cycles consisted of 94 °C for 35 s, 65 °C for 35 s, and 72 °C for 1 min, ending with 72 °C for 2 min. PCR products were analyzed on 1.5 and 2 % agarose gel and visualized after ethidium bromide staining.

RFLP-PCR analysis:

A sensitive nested-PCR restriction fragment length polymorphism (RFLP) protocol was performed for the study of mutations at codons 33, 57, 58, 117 and 173 of the *pvdhfr* gene (Fig. 2, 3) (19) using the *SacII*, *XmnI*, *AluI*, *PvuII*, and *StyI* enzymes. Ten microliters of the second and third PCR products

were digested for 3 h at 37°C in a total volume of 20µl using 10 U of each restriction enzyme (Fermentas). The PCR products and restricted fragments were electrophoresed on 1.5 and 3% MetaPhor agarose gels respectively.

Results

For the mutations at codons 33, 57, 117 and 173 of the *P. vivax dhfr* gene, the 608 bp PCR product, was digested with the, *SacII*, *XmnI*, *PvuII* and *StyI* enzymes, respectively. The mutation at codon 58 was analyzed by using the 238 bp fragment of the PCR product with *AluI* restriction enzyme. The obtained fragments of wild and mutant types of PCR product with *XmnI*, *AluI* and *PvuII* were represented in Figs. 4, 5 and 6 and no mutations were detected with the *StyI* and *SacII* enzymes in any of the study samples.

A total of 72 *P. vivax* isolates from Bandare Jask and 45 isolates from Minab were analyzed by PCR-RFLP method for mutation detection. The overall frequency of mutant *pvdhfr* gene types was obtained in 33 of the isolates (28.2%). Nine single mutations (7.7%), 22 double mutations (18.8%) and two triple mutations (1.7%) were observed. In Bandare Jask 56 of the 72 isolates (78%) showed wild type alleles. Six single mutations with *PvuII* and ten double mutations with *AluI* and *PvuII* enzymes were observed. In Minab 28 of the 45 isolates (62.2%) showed wild type and three single mutations with *PvuII* or *AluI* enzymes, twelve double mutations with *PvuII* and *AluI* or *XmnI* and *AluI* and two triple mutations with *XmnI/AluI* and *PvuII* were detected.

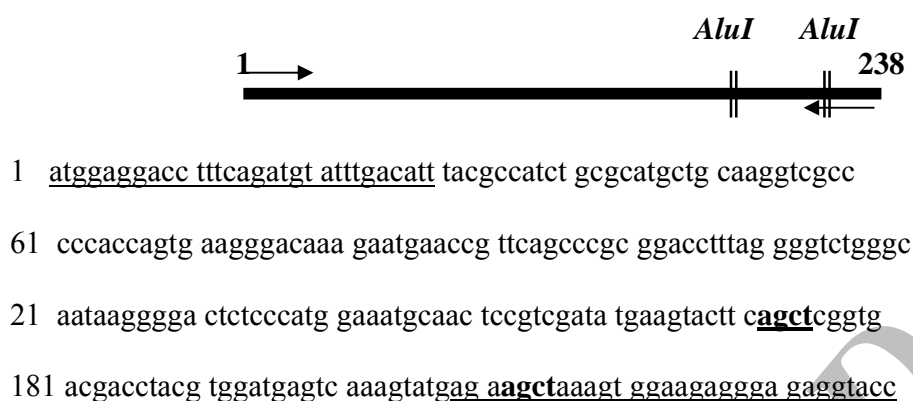


Fig. 3: Schematic representation and nucleotide sequences of the 238bp product of the semi-nested PCR of the *pvdhfr* gene. The primer's VDT-OF and VDF-NR58 annealing sites were underlined and the *AluI* restriction enzyme sites (agct) were bold and underlined

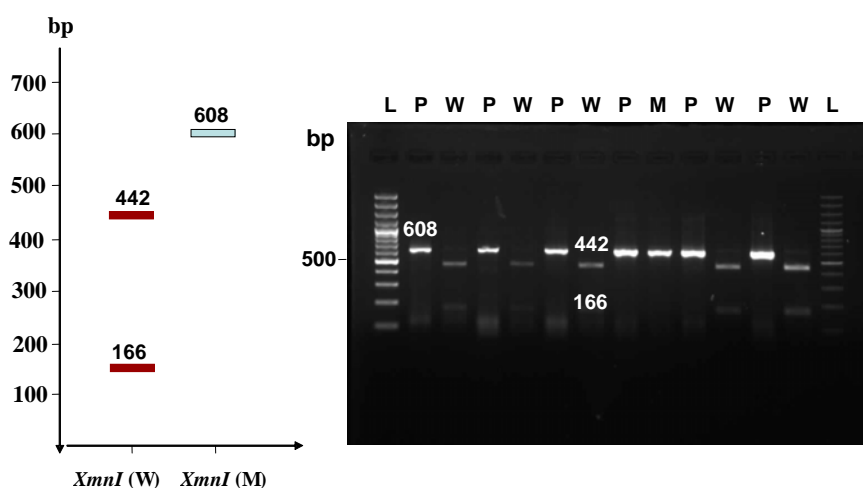


Fig 4 : *XmnI* restricted electrophoresis gel for recognition of mutation at 57 residue of the 608bp PCR product of *pvdhfr* gene with schematic representation of the RFLP analysis.
 L: 100 bp DNA ladder, P: PCR product, W: Wild type (442 + 166bp), M: Mutant type

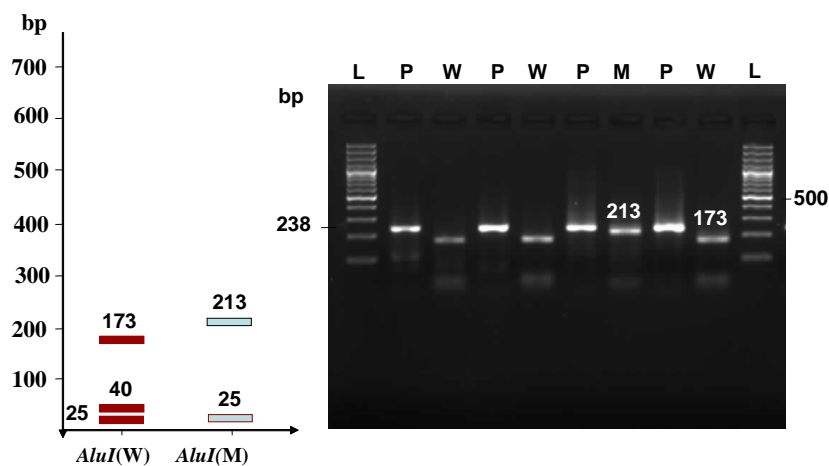


Fig. 5: *AluI* restricted electrophoresis gel for recognition of mutation at 58 residue of the 338bp PCR product of *pvdhfr* gene with schematic representation of the RFLP analysis.
L: 100 bp DNA ladder, P: PCR product, W: Wild type (238bp: 173 + 40 + 25bp), M: Mutant type (238bp: 213 + 25bp)

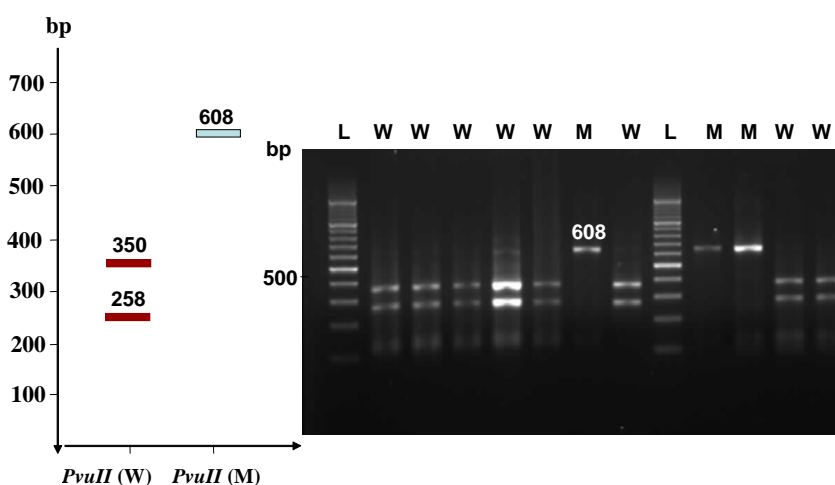


Fig. 6 : *PvuII* restricted electrophoresis gel for recognition of mutation at 117 residue of the 608bp PCR product of *pvdhfr* gene with schematic representation of the RFLP analysis
L: 100 bp DNA Ladder, W: Wild type (350 + 258bp), M: Mutant type (608 bp)

Discussion

The 33 mutant samples (28.2%) in the present study represent the first *pvdhfr* gene mutation in Bandare Jask and Minab cities in Hormozgan Province, Iran. Single and double mutations in *pvdhfr* at codons 57, 58 and 117 have already reported in Chabahar district in Sistan and Bluchistan Province and in Pars Abad in Ardebil Province (20). No mutation was reported by Imwong et al. in Iran in only two *P. vivax* isolates (21). The majority of the mutant isolates harbored double mutations (18.8%) and single mutation (7.7%), respectively. Initial studies revealed that parasites with mutations in codons 58 and 117, which correspond to codons 59 and 108 of the *P. falciparum dhfr* gene, are known to be associated with resistance to pyrimethamine (22). Triple mutation with *XmnI/AluI* and *PvuII* in two samples of the present study is the first triple mutation report in *pvdhfr* gene of Iranian *P. vivax* isolates. The frequency of *pvdhfr* mutations reported from Phillipine, China, Vietnam, India, and Sri Lanka are close to our data (21, 23-26). However, highly mutant alleles were reported from Thailand, Vanuata, Indonesia, and Papua New Guinea (19, 21, 23). The lack of mutations at codones 33 and 173 with *SacII* and *StyI* enzymes are similar with previously reported data of Kaur (26) and Zakeri (20).

Antifolate drugs are commonly used for the treatment of *falciparum* malaria and in Iran; the combination of chloroquine-primaquine is still the first-line treatment against *P. vivax* malaria (27). Existence of mutant alleles in *pvdhfr* gene among isolates of Hormozgan Province may be due to: mixed infection, inaccurate diagnosis of the parasite species and/or prescribing the antimalarial drugs based on clinical findings. Treatment of asymptomatic *P. vivax* patients with trime-

thoprim probably common, causing the exposure of parasites to this drug (28).

In conclusion, appearance of these SP resistance genotypes in *pvdhfr* gene of *P. vivax* in Hormozgan Province despite a low level of SP drug pressure suggests that the antifolate treatment *falciparum* malaria (fansidar) is proportionally affecting *P. vivax dhfr* mutation. Therefore, more studies to evaluate antimalarial drugs that should preferably be effective against both *P. vivax* and *P. falciparum* are recommended.

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