

ORIGINAL ARTICLE

MOLECULAR SURVEILLANCE OF *Plasmodium vivax* AND *Plasmodium falciparum* DHFR MUTATIONS IN ISOLATES FROM SOUTHERN IRAN

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SUMMARY

In Iran, both *Plasmodium vivax* and *P. falciparum* malaria have been detected, but *P. vivax* is the predominant species. Point mutations in dihydrofolate reductase (*dhfr*) gene in both *Plasmodia* are the major mechanisms of pyrimethamine resistance. From April 2007 to June 2009, a total of 134 blood samples in two endemic areas of southern Iran were collected from patients infected with *P. vivax* and *P. falciparum*. The isolates were analyzed for *P. vivax* dihydrofolate reductase (*pvdhfr*) and *P. falciparum* dihydrofolate reductase (*pfdhfr*) point mutations using various PCR-based methods. The majority of the isolates (72.9%) had wild type amino acids at five codons of *pvdhfr*. Amongst mutant isolates, the most common *pvdhfr* alleles were double mutant in 58 and 117 amino acids (58R-117N). Triple mutation in 57, 58, and 117 amino acids (57L/58R/117N) was identified for the first time in the *pvdhfr* gene of Iranian *P. vivax* isolates. All the *P. falciparum* samples analyzed (n = 16) possessed a double mutant *pfdhfr* allele (59R/108N) and retained a wild-type mutation at position 51. This may be attributed to the fact that the *falciparum* malaria patients were treated using sulfadoxine–pyrimethamine (SP) in Iran. The presence of mutant haplotypes in *P. vivax* is worrying, but has not yet reached an alarming threshold regarding drugs such as SP. The results of this study reinforce the importance of performing a molecular surveillance by means of a continuous chemoresistance assessment.

KEYWORDS: *Plasmodium vivax*; *Plasmodium falciparum*; Pyrimethamine; Point mutations and drug resistance.

INTRODUCTION

Antifolates, most notably sulfadoxine–pyrimethamine (SP), are important in antimalarial therapy. They are inexpensive, relatively safe and treatment requires only a single dose¹. The enzymes of SP, involved in the folate biosynthesis pathway of the parasite, are commonly used for *Plasmodium falciparum* malaria treatment². Point mutations in the dihydrofolate reductase (*dhfr*) gene in both *P. falciparum* and *P. vivax* are the major mechanisms of pyrimethamine resistance³. The molecular mechanisms associated with two (2) key *dhfr* mutations, S58R and S117N, which are equivalent to the C59R and S108N mutations of the *P. falciparum* dihydrofolate reductase (*pfdhfr*) gene involved in the development of SP resistance in *P. falciparum* and *P. vivax*, are most likely similar. They are known to be associated with pyrimethamine resistance⁴. Substitution of serine to asparagine at codon 117, increased the IC₅₀ value more than 80-fold. The addition of a second mutation, resulting in the substitution of serine to arginine at codon 58, produced an enzyme that was more than 400-fold resistant to pyrimethamine⁵. The distribution of mutant alleles of these genes in different geographical regions varied⁶. In Iran, national malaria control programs initiated in the 1950s led to the eradication of malaria in the northern Caspian region in 1977, and to a substantial reduction of transmission in the south-eastern provinces of Sistan and

Baluchistan, Hormozgan and tropical regions of Kerman⁷⁻⁹. In this area, both *P. vivax* and *P. falciparum* malaria have been detected, but *P. vivax* is the predominant species that causes about 90-95% of the total malaria cases^{8,10}. The standard treatment for this parasitic infection has been chloroquine (CQ) and primaquine for eradication of both asexual stages and hypnozoites¹¹. A major challenge for the control and elimination of *P. vivax* is the emergence of drug-resistant strains. Chloroquine resistance (CQR) in *P. vivax* was first reported in 1989 from Papua New Guinea (PNG) and Indonesia in 1991^{12,13}. Since then, cases of resistance have been reported from several areas of the *vivax* endemic world¹²⁻¹⁵. Chloroquine resistance has not been reported in Iran, but a declining trend of the parasite's *in vivo* susceptibility to the drug has been noted¹⁰. This emphasizes the urgent need to search for alternative treatments for *P. vivax* infection, possibly including drugs of the antifolate class¹⁶. Although, SP has not been used for the treatment of *P. vivax* parasites in any *vivax* malaria endemic region so far, SP resistance in *P. vivax* has been reported¹⁷. These infections could be misdiagnosed or undiagnosed, and as a result, exposure of *P. vivax* to SP may lead to selection of SP-resistant strains.

A combination of CQ and primaquine had been used as a first line antimalarial drug for the treatment of uncomplicated *P. falciparum* in Iran up to 2005. Due to the prevalence of CQ-resistant parasites in this

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region, SP plus CQ was officially introduced as the first line treatment for confirmed *P. falciparum* cases in 2006¹⁸. Due to the inefficacy of treatment with the SP/CQ combination, the Center for Diseases Management and Control (CDMC) decided to revise the treatment policy in 2007, in which SP/CQ was replaced with SP/artesunate as the first-line recommendation for *falciparum* malaria¹⁹. However, SP resistance has been consistently reported among Afghan refugee settlements along the western border of Pakistan, a region from which human migration has recently increased substantially. This raises the concern that SP-resistant malaria parasites are now invading the nearby regions of Iran²⁰. The widespread SP resistance in various endemic regions has been explained by the invasion and subsequent selection of resistant parasites in the presence of SP pressure²¹. Resistance to SP has been confirmed in malaria endemic areas of Iran by means of *in vitro* and *in vivo* tests^{22,23}. A *dhfr* codon, Asn108 with Ile51 and Arg59, confers resistance to pyrimethamine and may predict SP treatment failure in some malaria endemic areas²⁴. Moreover, understanding the epidemiology of drug resistance is necessary for an effective drug policy. In fact, routine monitoring makes it possible to detect drug resistance at early stages of development in order to avoid further selection and spread of multidrug resistance¹⁸. Therefore, in this study we report for the first time the frequencies of relevant mutations in

pfdhfr and *pvdhfr*, among isolates obtained from two different endemic areas (Bandare Jask and Minab) of southern Iran.

MATERIALS AND METHODS

Site

In this study, a total of 134 blood samples were collected from *P. vivax* and *P. falciparum* malaria patients in two endemic areas of Hormozgan Province (Bandare Jask and Minab), located in the southern part of Iran, from April 2007 to June 2009. Hormozgan Province is one of the 31 provinces of Iran and is located in the northern coast of the Persian Gulf and the Gulf of Oman (Fig. 1); its area is 70,697 km² (27° 11' 18.24" N, 56° 16' 36.48" E), where the weather is warm and humid enough for *Anopheles* sp. to be active throughout the year. As such, this environmental condition makes *An. stephensi* the main vector responsible for the transmission of malaria to humans in southern Iran²⁵. The average temperature affected by humidity is moderate and rarely gets higher than 45 °C in summers. The annual rainfall is less than 250 mm and the relative humidity is more than 80%²⁶. In this area, malaria is seasonal and transmission is year-round with two peaks, the first from May to August with *P. vivax* as the predominant species and the second



Fig. 1 - Map of Iran and Hormozgan province (<http://geology.com/world/iran-satellite-image.shtml>, <http://www.operationworld.org/iran>, http://www.irantravel.biz/iran_maps/hormozgan_province.gif).

Table 1
Plasmodium vivax dihydrofolate reductase (*pvdhfr*) variants classified according to the number of point mutations in two areas of Hormozgan province, Iran

Areas (No.)	None (%)	Single mutant (%)	Double Mutant (%)	Triple mutant (%)
Minab (46/118)	29 (63)	1 (2.2)	13 (28.3)	3 (6.5)
Bandare Jask (72/118)	57 (79.1)	3 (4.2)	12 (16.7)	0 (0)
Total	86 (72.9)	4 (3.4)	25 (21.2)	3 (2.5)

peak from October to November when both *P. falciparum* and *P. vivax* infections were recorded²⁷.

DNA extraction and PCR amplification

DNA was extracted using DNG-plus extraction Kit (Cinnagen, Iran), according to the manufacturer’s manual. The DNA pellet was dissolved in 50 µl of sterile distilled water, incubated in a water bath at 65 °C for 5 min, and stored at -20 °C until use.

Two single PCR techniques were employed to amplify a partial DNA sequence containing some single nucleotide polymorphisms (SNPs) for target genes including: SNPs N51I, C59R, and S108N/T for *pfdhfr* and SNPs P33L, F57L/I, S58R, S117T/N and I173F/L for *pvdhfr*.

***Pvdhfr* gene amplification:** Oligonucleotide primers were used to amplify a fragment of 1,869 bp from *pvdhfr* gene using *P. vivaxdhfr-ts* gene (GenBank accession no. X98123). PCR, seminested-PCR and PCR-RFLP were performed as previously described²⁸.

***Pfdhfr* gene amplification:** Oligonucleotide primers were used to amplify a fragment of 514 bp from *pfdhfr* gene using *P. falciparum dhfr-ts* gene (GenBank accession no. M22159). PCR was performed as previously described²⁹.

PCR products were analyzed using ethidium bromide-stained agarose-gel (2%) electrophoresis.

Sequence analysis of *pvdhfr* and *pfdhfr* genes

To verify the results obtained by RFLP, and also search for new SNPs in *pvdhfr* gene, which covers the region that contains drug-resistance related point mutations, mutant isolates *pvdhfr* from five residues and some wild types isolates were confirmed by sequencing the PCR products in both directions using appropriate primers. Furthermore, *pfdhfr* gene polymorphisms of *P. falciparum* were evaluated by sequencing, and for this purpose, the PCR samples were treated with a pre-sequencing kit (USB Corporation, Cleveland, Ohio) then sequenced using an Applied Biosystems (ABI) terminator cycle sequencing ready reaction kit (BigDyeI Terminator V3.1 Cycle SequencingKit) on an ABI 3130 genetic analyzer. The sequences obtained were manually edited and aligned using the Gene Runner software (version 3.05). PCR and sequencing analysis of the isolates with mutations were repeated several times, to rule out any possible errors.

Ethical approval

The study was approved by the Ethics Committee of the Infectious Diseases and Tropical Medicine Research Center, Shahid Beheshti

University of Medical Sciences. All samples were collected with the consent of the patients or their relatives before the beginning of treatment, and stored at -20 °C until DNA extraction. *P. vivax* and *P. falciparum* were diagnosed using microscopic examination of the thin and thick blood smears, stained by Giemsa.

RESULTS

In this study, a sensitive PCR–RFLP and sequencing methods were used to analyze the frequency of mutations in defined residues of the *Pvdhfr* and *pfdhfr* gene related to pyrimethamine resistance in 79 (67%) males and 39 (33%) females, constituting 118 *P. vivax*, and 13 (81%) males and 3 (19%) females, constituting 16 *P. falciparum* isolates in the Hormozgan Province. Participants in this study were aged between 4 to 70 years.

Distribution of mutations in *pvdhfr*: Forty six *P. vivax* isolates from Minab and 72 from Bandare Jask were analyzed for mutations at codons 33, 57, 58, 117 and 173 of the *P. vivax dhfr* gene. Eighty-six of the isolates (72.9%) showed the wild type amino acids at the aforementioned five codons of *pvdhfr* (Table 1 and Fig. 2). Seventeen samples (37%) from Minab and 15 (20.8%) from Bandare Jask showed single, double or triple mutations (Table 1). Mutations at residues 173 (I→L) and 33 (P→L) were not detected in any of the analyzed samples and no mutation was seen in codon 57, in Bandare Jask isolates. Amongst mutant isolates, the most common *pvdhfr* alleles were double mutant (n: 25), with combination of two mutations, S58R-S117N or F57L-S58R and the remaining 7 mutant isolates were single mutation for S117N (n: 4) or triple (n: 3) at F57L -S58R-S117N (Table 2). To verify the results obtained by RFLP, the 32 mutants and 12 wild type alleles were sequenced. Sequencing confirmed the RFLP results with no new point mutation in the wild or mutant samples.

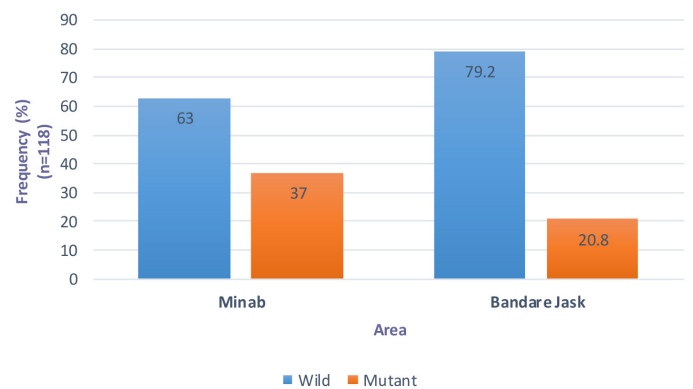


Fig. 2 - The frequency of mutant isolates in *Plasmodium vivax* dihydrofolate reductase (*pvdhfr*) gene of two areas of Hormozgan province, Iran.

Table 2

Single, double and triple *Plasmodium vivax* dihydrofolate reductase (*pvdhfr*) nucleotide polymorphisms as point mutation for 57, 58, and 117 identified from two areas of Hormozgan province, Iran

Genotype (s)	No. in Minab (%)	No. in Bandare Jask (%)	Total
Wild type	29 (63)	57 (79.1)	86 (72.9)
S117N	1 (2.2)	3 (4.2)	4 (3.4)
S58R-S117N	8 (17.4)	12 (16.7)	20 (17)
F57L-S58R	5 (10.9)	0 (0)	5 (4.2)
F57L -S58R-S117N	3 (6.5)	0 (0)	3 (2.5)
	46 (100)	72 (100)	118 (100)

Size polymorphism of the *pvdhfr* at repeat region: A repetition (ACA CAC GGT GGT GAC AAC or ACA CAC GGT GGT GAC AAT) between amino acids 103 and 104 of *pvdhfr* was found in 3/44 and 2/44 of the sequenced samples, respectively. Therefore, two types A and B were found among the isolates. The frequency distribution for types A and B were 88.7% (39/44) and 11.3% (5/44), respectively. Interestingly, all mutant alleles were type A (Table 3).

Distribution of mutations in *Pfdhfr*: All the 16 *P. falciparum* isolates were obtained from Bandare Jask and were successfully genotyped for detection of *dhfr* mutations associated with SP resistance. *P. falciparum* isolates were found to carry the mutant type 108N and 59R (100%) while retaining a wild-type mutation at position 51 (Fig. 3).

All the allelic types of *pvdhfr* (7 types) and *pfdhfr* (1 type) were represented in the GenBank (accession number AB547452 –AB547459).

DISCUSSION

In areas where *P. falciparum* and *P. vivax* co-exist, parasite-specific diagnosis and choice of effective treatment is crucial to prevent the emergence and spread of resistance³⁰. Mutation rate for *pfdhfr* was higher compared to *pvdhfr* because the drug was mainly given to treat *falciparum* malaria. The most common haplotypes of *pvdhfr* were the wild type and double mutants. Quadruple mutants were not detected in any of the examined isolates. The appearance of these SP resistant genotypes in *P. vivax* creates a need for new antimalarial drugs that should preferably be effective against both *P. vivax* and *P. falciparum* species³¹. After determining SP as the first line treatment of *falciparum* malaria in 2001 in Iran⁴, it was expected that the trend of reducing sensitivity of *P. vivax* to the drug would be intensified, as reported in Thailand. Analyses of field isolates from Thailand, where SP has been extensively used in the

past, revealed the predominance (100%) of parasites harboring 3 *Pvdhfr* mutant alleles; S58R and S117N; F57L, S58R, T61M and S117T; and F57I, S58R, T61M and S117T¹⁻⁶. A majority of the isolates carrying mutations showed the presence of double mutations at residues 58R/117N (n. 20 or 17%) and 57L/58R (n. 5 or 4.2%), (Table 2). The prevalence and distribution of the resistant allelic types in Hormozgan Province are similar to those reported in China, East Timor, the Philippines, and Vietnam, where SP has not been widely used until recently¹. A previous study²⁷ in northern and south-eastern Iran reported similar frequencies of mutant alleles in codons 57, 58 and 117 with the exception of triple mutation in three samples (2.5%) of this study. The triple mutation in

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M22159 isolate (Resistant to pyrimethamine)
MMEQVCDVFDIYAICACCKVESKNEGKNEVFNNYTFRGLGNKGVLPWKNSLDMKYFRA
ShH(Pf)IJ-IR
.....
3D7 isolate (Sensitive to pyrimethamine)
.....
.....
M22159
VTYYVNESKYEKLYKRCCKYLNKETVDNVNDMPNSKKLQNVVVMGRNTNWESIPKFKFPLS
ShH(Pf)IJ-IR
.....
3D7
.....
.....
M22159
NRINVLRSRTLKKEDEFDVYIINKVEDLIVLLGKLNLYKCFIIGGSVVYQ
ShH(Pf)IJ-IR
.....
3D7
.....

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Fig. 3 - Comparative of amino acids in mutant and wild type alleles in *P. falciparum* dihydrofolate reductase (*pfdhfr*) in *P. falciparum* isolates of Hormozgan province, Iran. Codon 59 (Wild): → Cysteine (Cys or C); Codon 59 (Mutant): → Arginine (Arg or R); Codon 108 (Wild): → Serine (Ser or S); Codon 108 (Mutant): → Asparagine (Asn or N);₂ShH(Pf)IJ-IR (Accession No: AB547459).

Table 3

Polymorphism in *Plasmodium vivax* dihydrofolate reductase (*pvdhfr*) gene related to two different types of tandem repeats

Group	Wild		Mutant		Total	
	No.	%	No.	%		
A	7	18	32	82	39	88.7
B	5	100	0	0	5	11.3
Total	12	27.3	32	72.7	44	100

A: KLKWKRERYLRMEASQGGDN TSGGDN THGGDN ADKLQNVVVMG
 B: KLKWKRERYLRMEASQGGDN TSGGDN THGGDN THGGDN ADKLQNVVVMG

the 57L/58R/117N codons of this study is the first report of the *pvdhfr* gene, in Iranian *P. vivax* isolates. However, no quadruple mutant was identified. In another study in Iran, no mutation at codon 57 and no triple mutant alleles were seen. Furthermore, the mutant alleles of Hormozgan Province were rarely seen⁴ while 6.8% of the examined isolates were mutant at this codon and 27.1% of this study's isolates were mutant. Mutations in *pvdhfr*, including 58R and 117N, have been implicated in *in vivo* pyrimethamine resistance and seem to arise first under drug pressure. Studies have shown that the 58R/117N mutant had a lower affinity for pyrimethamine and cycloguanil than the wild type enzyme³². SP has not been used for the treatment of *P. vivax* parasites in any *vivax* malaria endemic region of Iran. *P. vivax* and *P. falciparum* coexist in some areas, the correct diagnosis of mixed infections is not easy based on microscopic examination of blood smears and the clinical symptoms of the two species cannot be differentiated. As a result, *P. vivax* may often be treated with SP because of mixed infections and inaccurate diagnosis^{27,33}. In addition, other antifolates such as co-trimoxazole that are routinely used against urinary tract infections and chronic bronchitis in the region could add to the overall antifolate pressure in Iran²⁷.

All the isolates in this study were mutant at codons 59 and 108 (100%) of *pfldhfr* and none of them was mutant at codon 51, with no evidence of clinical failure of SP in patients. Studies of genetic transfection of *P. falciparum* confirmed that the amino acid substitution at *pfldhfr* codon 108 (S→N) increases approximately ten times the resistance to pyrimethamine³⁴, and the addition of mutations at codons 51, 59, and 164 progressively increases the level of resistance². In Iran, SP has not been widely used as monotherapy in first-line treatment. It was used as a combination therapy with CQ for only two years and then replaced by SP/artesunate. In fact, the high prevalence of mutations in *dhfr* might be affected by other chemically-related drugs such as co-trimoxazole, which is commonly used in the study areas for treating bacterial infections in the setting of malaria in Iran²⁴. In addition, the high prevalence of these mutations in areas with low clinical failure to SP may be associated with prior primaquine exposure, which could explain the fixed prevalence of this mutation among isolates²³. Furthermore, when SP was widely used, resistance developed relatively quickly. Therefore, the World Health Organization (WHO) recommends the use of combination therapies composed of two or more drugs that target different pathways in order to overcome resistance, in particular artemisinin combination therapies (ACTs)²⁴. Although, the results of this study revealed the high prevalence of these two mutations in Iranian *P. falciparum* isolates, this has not yet reached a level at which SP will completely fail in the treatment of patients. Therefore, 51I mutation might be a good molecular marker for the triple mutant, indicating a failure of pyrimethamine in Iran.

CONCLUSION

This study determined the low molecular prevalence of resistance to SP in *P. vivax* and a high level of SP resistance in *P. falciparum*. In fact, the presence of mutant haplotypes in *P. vivax* is worrying, but has not yet reached an alarming threshold regarding drugs such as sulfadoxine-pyrimethamine, unlike in many parts of the world. This indicates the emergence of drug tolerant/resistant *P. vivax* isolates in Iran in the near future. This study evaluated the implementation of new therapeutic strategies based on combinations that include SP, like the protocol that is now being implemented in Iran (artesunate combined with SP), as the first-line drug for uncomplicated malaria (*P. falciparum*) treatment.

ACKNOWLEDGMENTS

This study was financially supported by Grant No. 766 from the Infectious Diseases and Tropical Medicine Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran. The authors are grateful to F. Tahvildari and S.J. Seyyed-Tabaei of the Department of Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences and A. Keshavarz at the Health and Treatment office of NEZAJA, Tehran, Iran for their invaluable support. In addition, H. Ahmadpour, GH. Mohseni, M. Yerian, H. Rasti, and K. Ameri at the Hormozgan University of Medical Sciences and Health Services, are gratefully acknowledged for providing *P. vivax* and *P. falciparum* blood samples. The authors declare that there are no conflicts of interest.

AUTHORS' CONTRIBUTIONS

K S-S designed the study, contributed in the laboratory work, analyzed the data and wrote the manuscript. NT, BK, LG and EN contributed in the laboratory work and helped with analysis of the data. AH supervised the study and also helped with the writing of the manuscript. All the authors have read and approved the final manuscript.

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Received: 06 January 2014

Accepted: 16 June 2015