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High prevalence of the 437G mutation associated with sulfadoxine resistance among *Plasmodium falciparum* clinical isolates from Iran, three years after the introduction of sulfadoxine–pyrimethamine

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SUMMARY

Objective: The objective of this study was to determine the frequency of *dhfr* and *dhps* resistance-associated haplotypes in *Plasmodium falciparum* isolates, three years after the introduction of sulfadoxine–pyrimethamine (SP) as the first-line antimalarial treatment in Iran.
Methods: Blood samples ($N = 182$) were collected from patients presenting with falciparum malaria from southeastern Iran, and analyzed by nested-PCR/restriction fragment length polymorphism, followed by sequencing analysis.
Results: In *pfdhfr*, double mutation at positions 59R and 108N was a predominant allele with a prevalence of 95.7%. The pure double mutations of *pfdhfr* ($I_{51}N_{108}$) were detected, and showed an increase from 0.7% to 4.3% after the introduction of SP as first-line drug. Furthermore, a significant decrease in double mutations/wild-type of *pfdhfr/pfdhps* ($R_{59}N_{108}/A_{437}$) was observed from 2004 (83.5%) to 2008 (44%) after changes in treatment policy. With regards to *pfdhps*, the results showed a rapid increase in frequency of the single pure form of *pfdhps* at position 437G (54.4%) and that of triple *pfdhfr/pfdhps* ($R_{59}N_{108}/G_{437}$) mutant haplotype (51.7%) after three years.
Conclusions: The absence of quintuple mutations in the examined isolates supports the continued use of SP as the treatment of choice for uncomplicated malaria as a partner drug to artemisinin combination therapy in Iran. However, the increase in the triple *pfdhfr/pfdhps* ($R_{59}N_{108}/G_{437}$) mutant haplotypes indicates that the *P. falciparum* parasite populations have the potential to evolve into *dhfr/dhps* quintuple mutants in the near future. Therefore, monitoring the status of *dhps* alleles as a predictor of the development of clinical resistance to sulfadoxine should be a high priority in this region.

1. Introduction

Attempts to control malaria have been hampered because of resistance in *Plasmodium falciparum* to the most commonly used drugs, such as chloroquine (CQ). Increasing CQ resistance has resulted in sulfadoxine–pyrimethamine (SP) becoming the first-line drug for the treatment of uncomplicated malaria in several countries. However, SP has a short useful therapeutic life,[1–3] and when SP is widely used, resistance develops relatively quickly. Therefore, the World Health Organization (WHO) recommends the use of combination therapies of two or more drugs that target different pathways to overcome resistance, in particular artemisinin combination therapies (ACTs).[4]

Molecular markers of SP resistance are based on mutations in the parasite's dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes.[3,5] SP acts as a synergistic inhibitor of folate biosynthesis, which is an obligatory requirement for the production of nucleotides, and hence DNA synthesis, in malaria parasites. A *dhfr* codon, Asn108 with Ile51 and Arg59, confers resistance to pyrimethamine and may predict SP treatment failure in some malaria areas. For *dhps*, the Gly437 mutant is associated with sulfonamide resistance, while additional mutations in the Ala436, Glu540, Gly581 and Ser613 codons appear to increase the degree of resistance. These mutations cause alterations in key amino acid residues in the active sites of these enzymes, which reduce the affinity of the enzyme for the drug.[6–13] Increased numbers of mutations in *pfdhfr* and *pfdhps* are associated with increased levels of resistance to SP.

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Malaria is a serious health problem in Iran. In the last decade (1990–1999), the number of annual malaria cases decreased from 96 340 in 1991 to around 16 000 in 2007. Malaria transmission mostly occurs in the southeastern parts of the country, in Sistan and Baluchistan Province, Hormozgan Province, and the tropical areas of Kerman Province. *P. falciparum* is prevalent and responsible for 10–20% of malaria cases. Resistance of *P. falciparum* to CQ has increased since it was first reported in Sistan and Baluchistan Province in 1983,[14] and currently accounts for more than 78.5% of treatment failures in the southeastern provinces of Iran.[15] Resistance to SP has been confirmed in malaria-endemic areas of Iran by *in vitro* and *in vivo* tests.[16] In 2005, as the intensity of CQ resistance increased, the country implemented a change in the first-line antimalarial treatment to a combination of SP/CQ, with artemether–lumefantrine (Coartem®) as the second-line drug.[17] Although SP has remained the treatment of choice for uncomplicated malaria, the high rates of treatment failure seen with CQ,[17] and therefore the inadequate efficacy of treatment with the SP/CQ combination, resulted in a decision by the Center for Disease Management and Control to revise its treatment policy again in 2007, and SP/CQ was replaced with SP/artesunate as the first-line recommendation for falciparum malaria. As a result, there has been an increase in the use of SP in the country, exposing *P. falciparum* isolates to drug pressure, which may result in the rapid emergence of SP resistance due to selection.[18,19] Therefore, molecular surveillance is necessary to detect the emergence of resistance mutations.

The objective of this study was to determine the frequency of *dhfr* and *dhps* resistance-associated haplotypes in *P. falciparum* isolates, three years after the introduction of SP as a partner drug in the first-line treatment of malaria in Iran. This investigation is also a follow-up study to our previous work in the same area.[20]

2. Materials and methods

2.1. Study areas and sample collection

The Chabahar districts of Sistan and Baluchistan Province, in the southeastern part of Iran were selected as the study area. In this region, malaria transmission is year-round with two peaks: the first from May to August, with *Plasmodium vivax* as the predominant species, and the second from October to November, when both *P. falciparum* and *P. vivax* infections are recorded. From October 2005 to October 2008, blood samples ($N = 182$) were collected on day 0 from uncomplicated *P. falciparum*-infected patients of Iranian, Afghani and Pakistani nationality, aged from 1 to 75 years. The inclusion criteria were as follows: presence of fever for the preceding 48 h (axillary temperature $\geq$37.5 °C), mono-infection with *P. falciparum*, no intake of antimalarial drugs in the preceding 4 weeks, no signs of complications, no history of allergic reactions to SP, and informed consent from patients or their parents.

Thin and thick blood films were stained with Giemsa and examined microscopically for the detection of the *P. falciparum* parasite. Approximately 1 ml venous blood was obtained in a tube containing EDTA from the patients who were confirmed to be positive for the presence of *P. falciparum* parasites. All patients were then treated with SP plus either CQ (before 2007, $n = 82$) or artesunate (after 2007, $n = 100$), as the first-line drug and, if needed, with Coartem as the second-line antimalarial drug for the treatment of uncomplicated malaria cases. The patients were given oral SP (Fansidar™, 25 mg/kg sulfadoxine and 1.25 mg/kg pyrimethamine) as a single dose on day 0 under supervision. Treatment was completed with CQ 10 mg/kg on day 0 and day 1 and a 5 mg/kg dose on day 2, or artesunate taken as three doses of tablets (4 mg/kg) given on days 0, 1, and 2. The consumption of each dose was observed by one of the primary healthcare staff (as a member of the study team) and physical complaints were recorded at the time of each visit.

2.2. In vivo assay

In this investigation, 75/182 patients aged 10 to 50 years (median 25 years), presenting to the outpatient clinic with an attack of non-severe malaria, were enrolled for 28 days of follow-up according to the WHO protocol.[21] We excluded subjects meeting the WHO criteria for severe or complicated malaria, pregnant women, children aged less than 5 years, those suffering from malnutrition, those with a severe allergic reaction to the study drugs, those with the onset of a non-malaria febrile illness during follow-up, those with persistent vomiting, those self-medicating with antimalarials during follow-up, and those who withdrew consent. A medical history was taken and clinical examination made for all eligible patients. A finger-prick blood sample was obtained to measure parasite density. Approximately 1 ml venous blood was obtained pre-treatment (day 0) in a tube containing EDTA from patients who were confirmed to be positive for the presence of *P. falciparum* parasites. The patients were then given oral SP (Fansidar, 25 mg/kg sulfadoxine and 1.25 mg/kg pyrimethamine) as a single dose on day 0 under supervision. Treatment was completed with three doses of artesunate (4 mg/kg) on days 0, 1 and 2. The consumption of each dose was observed by one of the primary healthcare staff as a member of the study team. The patients were asked to return on days 7, 14, 21, and 28 following treatment (post-treatment). Parasite clearance was monitored by thick film microscopy each day after treatment. Treatment outcomes were classified according to the WHO guidelines as early treatment failure (ETF), late clinical failure (LCF), late parasitologic failure (LPF), or adequate clinical and parasitological response (ACPR). The study was reviewed by, and received ethical clearance from, the Pasteur Institute of Iran. All blood samples were collected in a tube containing EDTA, stored at 4 °C, and then transported to the main laboratory in Tehran.

2.3. Parasite DNA extraction

The parasite genomic DNA was extracted from infected red blood cells using phenol/phenol–chloroform followed by ethanol precipitation, as described previously.[22]

2.4. Nested polymerase chain reaction amplification of pfdhfr and pfdhps genes

Nested PCRs were performed for *dhfr* and *dhps* genes, and all reactions were carried out in reaction mixtures of 25 μl containing 1.5–3 mM $MgCl_2$, 200 μM dNTP mixture (Invitrogen, Carlsbad, CA, USA), 1 unit Taq polymerase (Invitrogen, Carlsbad, CA, USA), and a pair of primers (10 pmol each). For both *dhfr* and *dhps*, 1 or 2 μl DNA was used as template in the first reaction, and for the second reaction, 1 μl of first PCR product was used if no band was seen from the first round PCR product. However, in the case of an intense band, 1 μl of a 1/100 dilution of the samples was used as template for secondary PCR. Negative controls (ddH_2O) were used in all PCRs. The PCR primers and the modified conditions for both genes have been described previously.[20,23] Secondary PCR products were resolved by electrophoresis on 1–2% agarose gels and visualized by staining with ethidium bromide.

2.5. Restriction fragment length polymorphism (RFLP) of pfdhfr and pfdhps genes

Mutation-specific restriction endonuclease digestion was used to detect single nucleotide polymorphisms (SNPs) in *dhfr* at

Table 1
The frequency distribution of SNP combinations of *pfdhfr* and *pfdhps* alleles associated with sulfadoxine–pyrimethamine resistance in clinical isolates of *Plasmodium falciparum* in southeastern Iran, three years after changing the first-line antimalarial drug

pfdhfr				pfdhps					2005–2008
N51I	C59R	S108N	I164L	S436A/F	A437G	K540E	A581G	A613S/T	
N	R	N	I	S	A	K	A	A	80 (44%)
N	R	N	I	S	G	K	A	A	94 (51.7%)
I	C	N	I	S	A	K	A	A	3 (1.6%)
I	C	N	I	S	G	K	A	A	5 (2.7%)
I = 4.3%	R = 95.7%	N = 100%	–	–	G = 54.4%	–	–	–	n = 182

SNP, single nucleotide polymorphism.

positions N51I, C59R, S108N/T, and I164L, and in *dhps* at positions A436F/A, A437G, K540E, A581E, and A613S/T.[20,23] A number of restriction enzymes were used for RFLP of PCR products. For *dhfr*, the PCR products were digested with TasI and TaqI to determine the polymorphisms at codons 51 and 59, respectively. Three enzymes, AluI, BsrI, and MvaI, were used to identify wild and mutant *dhfr* allele at codon 108, and DraI was used to detect mutation at position I164L. For *dhps,* the PCR products were digested with HhaI, MnlI and HindIII to determine the polymorphisms at codon 436, AvaII and MwoI at codon 437, FokI, BstUI, and MwoI at codons 540, 580, and 613, respectively. Digestions were done in 20 μl reactions containing 10 μl PCR fragments according to the manufacturer's instructions (New England Biolab, Beverly, MA, USA; Roche, Germany; Invitrogen, Carlsbad, CA, USA). Digested products were subjected to electrophoresis on 1.5–2% agarose or 2–3% Metaphor agarose gels, and visualized by ultraviolet (UV) transillumination.

2.6. Nucleotide sequencing of pfdhfr and pfdhps

Sequence analysis was used to identify polymorphisms in *pfdhfr and pfdhps* amplified from 10 field samples. The PCR products were gel-purified using the Qiagen DNA purification kit (Qiagen, Germany) following the manufacturer's instructions. DNA sequencing was carried out using the dideoxy chain termination procedure (Chemistry V3.1, Applied Biosystems) and the 3730XL DNA analyzer (Applied Biosystems) by MilliGen sequencing service (Labège, France).

For the whole gene of *dhfr* and *dhps*, the following sequencing primers were used so as to obtain overlapping sequences that span the whole gene: *pfdhfr* (accession number: **J03028**): dhfrF1: ATGATGGAACAAGTCTGCG; dhfrR1: TTGTCATCATTCTTTAAAGGC; dhfrF2: TCCAGAAATAAATGAAAATGAG; dhfrR2: ACCATAAATAGGTCCTAAATCG; dhfrF3: AGAATGTAAGGATATGGGAAG; dhfrR3: AGGTAATTTTGTCATCATTTG. *pfdhps* (accession number: **XM-001349382**): dhpsF1: TGCTTAAATGATATGATACCC; dhpsR1: ATTTCCTCTTTTATGCATTAG; dhpsF2: TAGTGCTTGTACAAATAATCCAG; dhpsR2: ATCACATGTTTGCACTTTCC.

2.7. Statistical analyses

All statistical analyses were performed using SPSS statistical package version 15.0 (SPSS Inc., Chicago, IL, USA). Frequencies of mutations and haplotypes among groups were compared using the Chi-square test. A p-value of <0.05 was considered statistically significant.

3. Results

3.1. In vivo study

In this study, 75 out of 100 *P. falciparum*-infected patients who received SP/artesunate completed the 28 days of follow-up. The median age of the 75 patients was 25 years (range 10–50 years) and parasitemia ranged between 1000 and 35 000 asexual parasites/mm^3. SP/artesunate treatment gave ACPR in 75 (100%) subjects. All follow-up blood samples collected were also negative for *Plasmodium* species by PCR assay.

3.2. pfdhfr and pfdhps genotypes

All 182 *P. falciparum* isolates were successfully genotyped for detection of *dhfr* and *dhps* mutations associated with SP resistance. *P. falciparum* isolates were found to carry the mutant type 108N (100%), and 95.7% of them carried the 59R mutation; however, the 51*I* mutation was present in 4.3% of examined samples (Table 1). The majority of the patients (95.7%) were found to carry both 59R and 108N in pure form (only mutant genotype

Table 2
Distribution of *pfdhfr/pfdhps* polymorphisms based on nested-PCR/RFLP in clinical samples collected from Chabahar districts during 2000–2008.[a]

pfdhfr				pfdhps					Year		
N51I	C59R	S108N	I164L	S436A/F	A437G	K540E	A581G	A613S/T	2000–2002 (n = 101)	2003–2004 (n = 152)	2005–2008 (n = 182)
N	R	N	I	S	A	K	A	A	72 (71.2%)	127 (83.5%)	80 (44%)
N	R	N	I	S	G	K	A	A	1 (1%)	23 (15.1%)	94 (51.7%)
I	C	N	I	S	A	K	A	A	–	1 (0.7%)	3 (1.6%)
I	C	N	I	S	G	K	A	A	–	–	5 (2.7%)
N	C	N	I	S	A	K	A	A	3 (3%)	1 (0.7%)	–
N	C/R	N	I	S	A	K	A	A	2 (2%)	–	–
N	R	S/N	I	S	A	K	A	A	4 (4%)	–	–
N/I	R	N	I	S	A	K	A	A	4 (4%)	–	–
N	R	N	I	S	A/G	K	A	A	14 (13.8%)	–	–
N/I	R	N	I	S	A/G	K	A	A	1 (1%)	–	–

RFLP, restriction fragment length polymorphism.
[a] The double mutations of *pfdhfr* ($I_{51}N_{108}$) were detected and showed an increase from 0.7% to 4.3% after the introduction of sulfadoxine–pyrimethamine as the first-line drug. There was also a rapid increase in frequency of the single pure form of *pfdhps* at position 437*G* and also that of triple *pfdhfr/pfdhps* ($R_{59}N_{108}/G_{437}$) mutant haplotypes three years after the introduction of sulfadoxine–pyrimethamine as the first-line drug.

detected), while retaining a wild-type mutation at position 51 and 164 (Table 1).

In the case of the *pfdhps* gene, polymorphisms in different loci of *dhps* (A436F/A, A437G, K540E, A581E and A613S/T) were investigated. All isolates were found to carry wild-type amino acids at positions 436, 540, 581, and 613, while 437G mutation in pure form was detected in 54.4% examined samples collected from Chabahar districts (Table 1). Mutations at codons 59R, 108N of *pfdhfr* with *pfdhps* 437G were detected in 51.7% of examined samples (Table 1).

3.3. Distribution of pfdhfr and pfdhps haplotypes before and after the introduction of sulfadoxine–pyrimethamine

Combinations of *pfdhfr and pfdhps* haplotypes among all 182 samples in this study demonstrated 4 distinct haplotypes (Table 1). The two most prevalent haplotypes were $N_{51}R_{59}N_{108}/A_{437}$ (44%) and $N_{51}R_{59}N_{108}/G_{437}$ (51.7%).

Table 2 and Fig. 1 show a comparison of *pfdhfr/pfdhps* haplotypes among Iranian *P. falciparum* isolates before and after the introduction of SP. The analysis of the samples collected from Chabahar districts during 2000–2002 ($n = 101$),[24] 2003–2004 ($n = 152$),[20] and 2005–2008 ($n = 182$) showed that mutations were detected at positions 51, 59 and 108 of *dhfr* and 437 of *dhps* in all analyzed samples. Most of the mutations were in mixed forms in the years 2000–2002, but in pure form after that time (Table 2). During the years 2000–2002, the most prevalent haplotypes were $N_{51}R_{59}N_{108}/A_{437}$ (71.2%) and $N_{51}R_{59}N_{108}/A_{437}G$ (13.8%); however, $N_{51}R_{59}N_{108}/A_{437}$ (83.5% and 44%) and $N_{51}R_{59}N_{108}/G_{437}$ (15.1% and 51.7%) were the most frequent haplotypes in analyzed samples in the years 2003–2004 and 2005–2008, respectively (Table 2, Fig. 1). A significant increase in the frequency of SNPs at position 437G in *pfdhps* was found after three years (Chi-square test, $p < 0.0001$). In addition, during 2000–2008 the numbers of haplotypes decreased from eight to four because of changes in the mutated positions from mixed to pure forms. Interestingly, when combining the two loci, the frequency of parasites carrying *pfdhfr/pfdhps* triple pure mutations ($R_{59}N_{108}/G_{437}$) increased from 1% in 2000 to 51.7% in 2008 (Chi-square test, $p < 0.0001$) (Fig. 1). The two other haplotypes: $I_{51}C_{59}N_{108}/A_{437}$ (1.6%) and $I_{51}C_{59}N_{108}/G_{437}$ (2.7%) were detected in samples examined in 2008, but were absent in 2000 (Table 2, Fig. 1).

3.4. Sequence analysis

In this study, based on the different haplotypes detected, 10 isolates were sequenced for both *pfdhfr* and *pfdhps* genes. All isolates were wild at codons 16, 51, and 164 and no new mutations were detected in the *pfdhfr* sequence. For the *pfdhps* gene, the only mutation detected was at codon 437, with wild-type at other codons. Similarly, no new mutations were detected in the *pfdhps* sequence. These results confirmed the PCR-RFLP analysis.

4. Discussion

The continuous monitoring of SP resistance and resistance to other antimalarial drugs is of major importance to guide national malaria treatment policies and also to monitor parasite drug susceptibility following changes in malaria treatment policies. This study was designed to assess the *pfdhfr* and *pfdhps* mutations associated with SP resistance, three years after the introduction of SP as first-line treatment in Iran.

All 182 *P. falciparum* isolates examined in this study carried the *pfdhfr* 108N mutation (100%) with no evidence of clinical failure to SP in patients. The results showed 95.7% *P. falciparum* isolates with double mutations at codons 59 and 108, indicating the development of resistance against antifolate in this region. The double

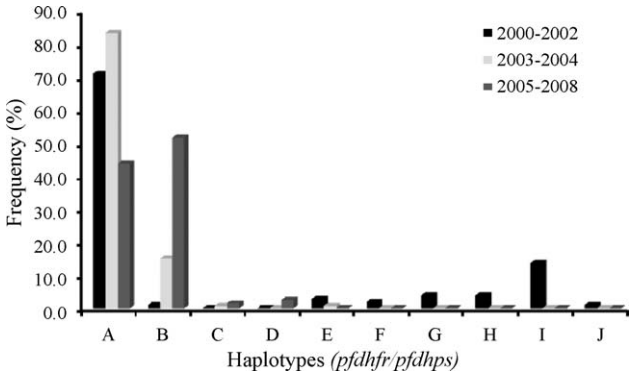

Fig. 1. Frequency distribution of the combination *pfdhfr/pfdhps* haplotypes obtained from isolates collected in Sistan and Baluchistan of Iran before (2000–2004) and after (2005–2008) the introduction of sulfadoxine–pyrimethamine as the first-line antimalarial treatment. The 10 haplotypes are labeled A to J. *dhfr/dhps* as follows: A = $N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}/S_{436}A_{437}K_{540}A_{581}A_{613}$; B = $N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}/S_{436}\mathbf{G}_{437}K_{540}A_{581}A_{613}$; C = $\mathbf{I}_{51}C_{59}\mathbf{N}_{108}I_{164}/S_{436}A_{437}K_{540}A_{581}A_{613}$; D = $\mathbf{I}_{51}C_{59}\mathbf{N}_{108}I_{164}/S_{436}\mathbf{G}_{437}K_{540}A_{581}A_{613}$; E = $N_{51}C_{59}\mathbf{N}_{108}I_{164}/S_{436}A_{437}K_{540}A_{581}A_{613}$; F = $N_{51}C/\mathbf{R}_{59}\mathbf{N}_{108}I_{164}/S_{436}A_{437}K_{540}A_{581}A_{613}$; G = $N_{51}\mathbf{R}_{59}S/\mathbf{N}_{108}I_{164}/S_{436}A_{437}K_{540}A_{581}A_{613}$; H = $N/\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}/S_{436}A_{437}K_{540}A_{581}A_{613}$; I = $N_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}/S_{436}A/\mathbf{G}_{437}K_{540}A_{581}A_{613}$; J = $N/\mathbf{I}_{51}\mathbf{R}_{59}\mathbf{N}_{108}I_{164}/S_{436}A/\mathbf{G}_{437}K_{540}A_{581}A_{613}$; mutated amino acids are shown in boldface type.

mutations ($I_{51}N_{108}$) were detected in only 4.3% of Iranian *P. falciparum* isolates, showing an increase from 0.7% to 4.3% after the introduction of SP as first-line drug. No mutation at codon 164 was detected in any of the examined isolates before or after the treatment policy changes in Iran. A similar result was obtained for Indian isolates.[25] Furthermore, a significant decrease in double mutations/wild-type of *pfdhfr/pfdhps* ($R_{59}N_{108}/A_{437}$) was observed in Chabahar districts from 2004 (83.5%) to 2008 (44%), showing a rapid increase in frequency of the single form of *pfdhps* at position 437*G* after the changes in treatment policy. In Iran, SP has not been used widely and has not been used as monotherapy in first-line treatment; it was used as a combination therapy with CQ for only two years and then replaced with 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 Iran. Therefore, the predominant *pfdhfr* haplotype in Iran appears to be $N_{51}R_{59}N_{108}$ rather than $I_{51}R_{59}N_{108}$. The low prevalence of triple mutations in the examined isolates in this study is similar to the findings from India,[26,27] Sri Lanka[28] and Papua New Guinea;[29] however, it is different from the isolates in Vietnam,[30,31] Malaysia,[32] Gabon,[33] and Brazil,[34] where the predominant haplotype has been found to be $I_{51}R_{59}N_{108}$. This may suggest that the *pfdhfr* allele has evolved independently due to drug pressure in geographically different regions of the world.

In contrast, most of the *P. falciparum* isolates (54.4%) revealed a single haplotype (437*G*) for *pfdhps* gene with no mutation at codons 436, 540, 581, and 613. In addition, three recently reported mutations at 587, 666, and 668 from Indian *P. falciparum* isolates were not detected by sequencing methods among the present examined isolates, which is in contrast to the work reported by Garg et al.[25] The results showed a rapid increase in frequency of the single pure form of *pfdhps* at position 437*G* and also in triple *pfdhfr/pfdhps* ($R_{59}N_{108}/G_{437}$) mutant haplotype after three years. In our previous work,[20] the prevalence of *pfdhps* 437*G*, and also the triple *pfdhfr/pfdhps* ($R_{59}N_{108}/G_{437}$) mutant haplotype, was higher in *P. falciparum* isolates collected from Sarbaz (33% and 35.3%, respectively), in the area of the border with Pakistan, than Chabahar isolates (17% and 15.8%, respectively) before the introduction of SP as first-line drug. In the present study, three years after the introduction of SP as first-line antimalarial drug, the frequency distribution of triple mutations has significantly

increased in the Chabahar districts. These data indicate that this mutant parasite might have spread through the Indian subcontinent to Pakistan and further to Iran. In fact, these results suggest the establishment of a coordinated network for antimalaria activities and the standardization of control measures between two countries, at least in border areas.

The work carried out by Kublin et al.[35] showed that quadruple mutations in *dhfr/dhps* loci are needed for the parasites to show clinical failure. Also, a previous study in Mozambique showed that two mutations at codon 59 in *dhfr* and codon 437 in *dhps* were actually enough to predict SP treatment failure.[36] However, although our results 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. In fact, the results of SP/artesunate efficacy in 75 patients (follow-up for 28 days) might support this suggestion. In addition, the present results were in accordance with our previous findings[20] in which double mutations at codon 59*R*/108*N* rather than 51*I*/108*N* were prevalent in our parasite isolates. Therefore, 51*I* mutation might be a good molecular marker for the triple mutant, indicating a failure of pyrimethamine in Iran and also the Indian subcontinent. However, the work carried out by Dokomajilar et al.[37] in Burkina Faso showed that *dhfr* 59*R* is more important than 51*I* as a marker of SP treatment failure. In addition, these two mutations with the mutation at position 437*G* in *dhps* indicate that the *P. falciparum* parasite populations have the potential to evolve into *dhfr/dhps* quintuple mutant polymorphism in the near future. Therefore, monitoring the status of *dhps* alleles as a predictor of the development of clinical resistance to sulfadoxine should be a high priority in this region.

In conclusion, the results of this study suggest that SP should remain the treatment of choice for uncomplicated malaria as a partner drug to ACT in Iran, but that regular surveillance for SP resistance should be continued in this country.

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