Research in Molecular Medicine

Evaluation of *Dihydrofolate Reductase (Dhfr)* Gene, Related to *Plasmodium Falciparum Pyrimethamine* Resistance in Imported Malaria Cases in Iran

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ABSTRACT

Background: Antimalarial drug resistance is one of the important challenges for governments in the fight against malaria. Molecular surveillance of antimalarial drug resistance supports early detection of how the recommended treatments work. This allows immediate action to reduce any threat and prevent it from spreading. Therefore, the aim of this study was to evaluate the frequency of *dihydrofolate reductase* (*dhfr*) mutants in *Plasmodium falciparum* resistance to pyrimethamine in Iranian malaria patients.

Materials and Methods: In 2020, 27 patients (21 males and 5 females) with imported *P. falciparum* cases were studied. The nested-PCR technique first confirmed the species in all samples and then amplification was done by the semi-nested-PCR method in order to detect single nucleotide polymorphisms (SNPs) in *dhfr* gene related to pyrimethamine resistance.

Results: All samples in the *18S rRNA* gene had species-specific bands for *P. falciparum* strains. In the sequence analysis of *pfdhfr* gene amplification after comparison with the standard strain (wild type), 21 patients had a double mutation (C59R+S108N) and six patients had a triple mutation (N51I+C59R+S108N) of pyrimethamine resistance.

Conclusion: The results of this study showed that the susceptibility of *P. falciparum* to pyrimethamine in the treatment of malaria is significantly reducing. These findings can raise concerns about pyrimethamine resistance in *P. falciparum*. Due to the high emergence of double and triple mutants related to pyrimethamine resistance, the malaria surveillance and treatment systems in Iran, the use of pyrimethamine should be considered.

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Introduction

Malaria is a health problem for 40% of the world's population (about 2.4 billion people) [1]. According to the latest malaria report from the World Health Organization (WHO), there were 241 million cases, of whom 627,000 died [2]. Malaria is still one of the most important infectious diseases in Iran. The total number of registered cases of indigenous malaria from 2015 to 2017 was less than 200, 90, and 89, respectively. According to the latest report of the WHO, the number of indigenous malaria in Iran has reached zero, but due to its geographical proximity to Afghanistan and Pakistan, 878 imported cases have been reported [2, 3].

Resistance to antimalarial drugs in *Plasmodium falciparum* is one of the challenges in the fight against malaria. Monitoring the effectiveness of antimalarial drugs supports the early detection of changes in the performance of recommended treatments. This monitoring provides rapid action to reduce any effect of resistance and prevent its spread. Therapeutic Effectiveness Studies (TESs) provide a measure of a patient's clinical and parasitic outcomes. TESs are the primary source of data for National Malaria Programs to make decisions about treatment policies [4].

In Iran, pyrimethamine is one of the first-line drugs for the treatment of *P. falciparum* [2]. Molecular monitoring of drug resistance has been ongoing in Iran's elimination program since 2002, and related data have led to a change in malaria treatment policy in Iran since 2005 [5]. Resistance to anti-malarial drugs is a major obstacle to controlling the disease. The emergence of *P. falciparum* resistance to anti-malarial drugs has led to an increase in treatment failure rates in some parts of the world (Southeast Asia and sub-Saharan Africa) [6, 7]. Monitoring molecular markers of resistance play an important role in evaluating effective treatment methods and inhibiting the spread of malaria drug resistance [8]. In the *Plasmodium falciparum* dihydrofolate reductase (*pfdhfr*) gene, mutations in codons N51I, C59R, S108N, and I164L are associated with pyrimethamine resistance [9].

Continuous monitoring to prevent resistance to antimalarial drugs, especially pyrimethamine as the first line of treatment can significantly reduce the incidence of *P. falciparum* in these areas [10, 11]. The high rate of imported malaria cases in Iran shows a serious threat to the elimination program [12]. Therefore, in these cases, preventive, continuous, and effective monitoring in the field of drug resistance is a necessity for elimination programs [13].

Considering the importance of studies on drug resistance in various pathogenic species of Plasmodium in Iran and the report of a significant frequency of pyrimethamine resistance codons in recent years in Iran [14-19] and neighboring countries [20, 21], it is necessary to monitor the drug effectiveness by molecular markers of resistance and review the treatment policies, especially in the malaria elimination program. Therefore, the aim of this study was to evaluate the frequency *dhfr* mutants in *P. falciparum* resistance to pyrimethamine in Iranian malaria patients in 2020.

Materials and Methods

Ethical considerations

All patients filled the informed consent form to participate in the study. Demographic characteristics, including age and sex, were recorded. The current study protocol was accepted by the Ethics Committee of Mazandaran University of Medical Sciences (Code: IR.MAZUMS.REC.1399.8476).

Sample collection

Peripheral blood samples were taken from 27 patients with imported malaria (21 males and 5 females) who had suspected symptoms of malaria and were referred to the health center in 2020. Blood samples were taken from patients whose peripheral blood smear and rapid diagnosis kit were positive for *P. falciparum*.

DNA extraction

DNA extraction was performed using a Beta Bayern kit. In order to completely and correctly separate the blood from the paper tissue, the preparation step before DNA extraction was performed as follows: first, a diameter of 1 cm of paper impregnated with blood was cut and then crushed in a microtube containing a lysis buffer kit and incubated at 60°C for 3 h. This action completely separated the blood from the paper tissue and dramatically increased the quality and quantity of the DNA extraction. Then, 200 µL of the obtained solution was used for DNA extraction according to the kit instructions.

Confirmation of species by molecular method

The screening was performed by nested PCR technique of samples using special primers for accurate detection of *P. falciparum* and the absence of *P. vivax*

Table 1. Sequence of designed primers for amplification of pyrimethamine resistance gene (dhfr)

Gene	Primer Sequence 5′-3′	Size (bp)	PCR Conditions
Primary amplification	TTATGATGGAACAAGTCTGC TCGCTAACAGAAATAATTTGATAC	637	95°C 5 min/[95°C 30s, 58°C 45s, 72 °C50s] × 35 cycles, 72°C 10 min
Secondary amplification	TTATGATGGAACAAGTCTGC AACAACGGAACCTCCTATA	509	95°C 5 min/[95°C 30s, 59°C 40s, 72 °C350s] × 30 cycles, 72°C 7 min

[22]. In the first stage of PCR, rPLU5 and rPLU6 primers, and in the second stage, rFLA1 and rFLA2 primers were used to detect *P. falciparum*. Moreover, VIV1 and rVIV2 primers were used to detect *P. vivax*. Initial reaction was done at 94°C for 3 min in one cycle, 94°C for 60 s, 56°C for 2min, 72°C for 2min in 30 cycles, and finally, at 72°C for 7min in one cycle.

The first PCR was performed in a 25 µL reaction mixture consisting containing 3 µL of genomic DNA, 12.5 µL of 1X PCR mix (Taq PCR MasterMix, Amplicon, Denmark), and 1 µL (10 pmol/ µL) of primers. The thermal cycler program included the first cycle of denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 2 min and extension at 72°C for 2 min, and then a final step at 72°C for 10 min. The first PCR yields were diluted (1: 10) and were considered as DNA templates for secondary PCR. The second round of PCR reaction was conducted in a volume of 25 µL containing 1 µL of diluted primary PCR yields, 12.5 µL of 1X PCR mix, and 65 µL (6.5 pmol/ µL) of primers (VIV1 and r VIV2 for *P. vivax* and rFAL1 and rFAL2 for *P. falciparum*). The thermal cycler program for the second PCR was similar to the first round. The second PCR product was exposed to electrophoresis on a 1.5% agarose gel in a TBE buffer (tris boric acid-EDTA) at 100 V for 45 min and imagined by gel.

After definitive confirmation of species (*P. falciparum*) by molecular method, primers designed with the CLC Genomic Work Bench software, version 20 were amplified to evaluate the resistance codons of pyrimethamine in the *pfdhfr* gene. For molecular analysis by nested PCR, first, a 637 bp fragment was amplified in the *pfdhfr* gene and then a 509 bp fragment was amplified with second (internal) primers. The sequence of designed primers and temperature program are described in Table 1.

The second PCR products were sequenced by the Sanger sequencing method. The sequences were registered in GenBank. For investigation of mutations in *pfdhfr* gene, obtained sequences were compared with the standard sample available in GenBank with accession number PF3D7_0417200, and resistance codons were evaluated and analyzed using Mega or Chromas software.

Results

Out of 27 studied malaria patients, 21 patients were men and six cases were women. The mean age of patients was about 28 years with an age range of 16 to 43 years. The results of molecular screening showed that out of 27 samples taken from the patients, all samples belonged to *P. falciparum*. In all samples, a 205 bp band was observed in *18S rRNA* gene. No bands were observed in the range of 120 bp by amplification of rVIV1 and rVIV2 primers (Figure 1), indicating no mixed infections of *P. falciparum*/*P. vivax*

In the analysis of sequences obtained from *pfdhfr* gene amplification, all isolates had the same band in the weight range of 509 bp. After comparison of the sequences obtained with the standard strain (wild type), 21 patients had double mutations in 59 and 108 codons and six pa-

Table 2. Demographic characteristics of the patients resistance to pyrimethamine patterns (double and triple mutants)

Pattern of Mutation	Sex/No.		Age		Origin of the Patients, No.	
	Male	Female	Range	Mean±SD	Afghanistan	Pakistan
Triple mutant	3	3	16-32	21.50±5.81	1	5
Double mutant	19	2	17-43	29.42±5.81	3	18

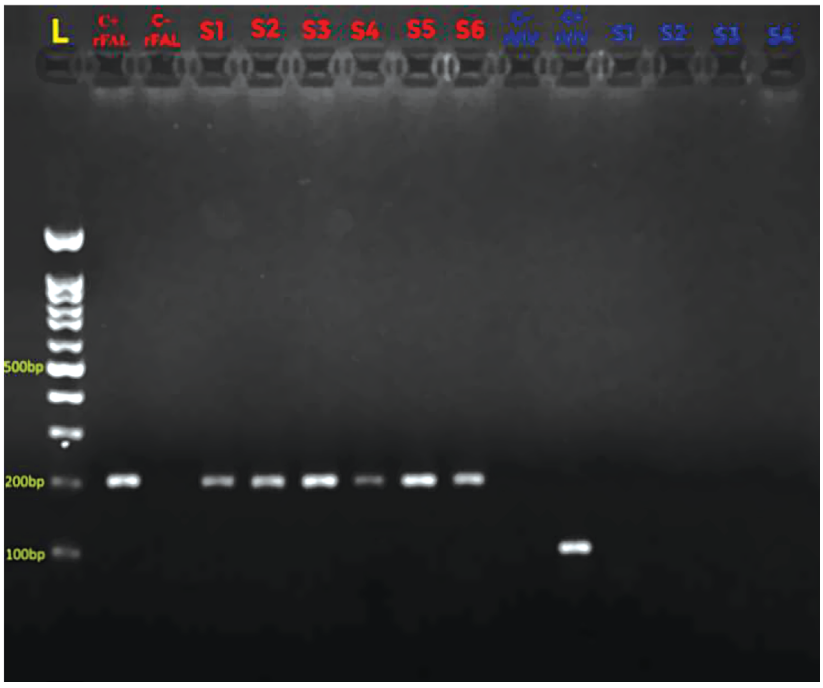

Figure 1. The fragment of the polymerase gene was amplified by Nested PCR with two rounds of amplification

Blue: amplified fragments with rFAL primers, Red: amplified fragments with rVIV primers, L: marker 100 bp, C+: positive control, C-: negative control, and S: sample.

tients had triple mutations (51, 59, and 108 codons) in *P. falciparum* resistance to pyrimethamine. A chromatogram of one of the sequences related to the triple mutant is shown in Figure 2. Demographic characteristics of patients related to both resistances to pyrimethamine patterns (double and triple mutants) are given in Table 2.

Nine novel mutations were found in K27E, N34T, T36S, W48C, E67D, K74N, D135N, D139N, and Y159D loci. The results of alignment of the protein sequences with the reference strain in the GeneBank and the frequency of double and triple mutations are drawn in Figure 3. All registered sequences with access numbers ON411658 to ON411684 are available in GenBank.

Discussion

Iran has been introduced by the WHO as a country that is in the process of eliminating malaria [2]. Therefore, considering that NMP (National malaria program) in Iran is in the final stages of the elimination phase, drug resistance is a silent threat to the elimination program [23].

The evolution and development of studies on the combination of sulfadoxine and pyrimethamine have been considered because of the increase in resistance to sulfadoxine several years after the emergence of pyrimethamine resistance [24]. Also, mutations in the pfdhps gene play a minor role in the development of sulfadoxine-pyrimethamine resistance [25]. On the other hand, few studies have been performed on molecular evolution in the pfdhps gene [26, 27]; thus, assessing pyrimethamine-resistant lineages by *pfdhfr* is preferable to sulfadoxine by pfdhps [27].

Due to the reasons mentioned and most importantly, pyrimethamine is the first drug used to treat patients with *P. falciparum* in Iran. In order to monitor the effectiveness of pyrimethamine by molecular markers of resistance, in the present study, *pfdhfr* molecular markers were used to evaluate the genetic diversity of *P. falciparum* resistance to pyrimethamine in imported malaria patients.

The results of molecular screening of the samples showed that all the samples detected by *P. falciparum* microscopy were also positive by PCR. In the *pfdhfr* gene amplification, all samples had specific bands. These results indicate the strength of the surveillance system and diagnosis in the malaria elimination program in Iran. These results show that Iran's malaria elimination program has an acceptable performance in establishing a quality assurance system for malaria microscopy.

In this study, the frequency of codons with high resistance was reported. This study identified the highest

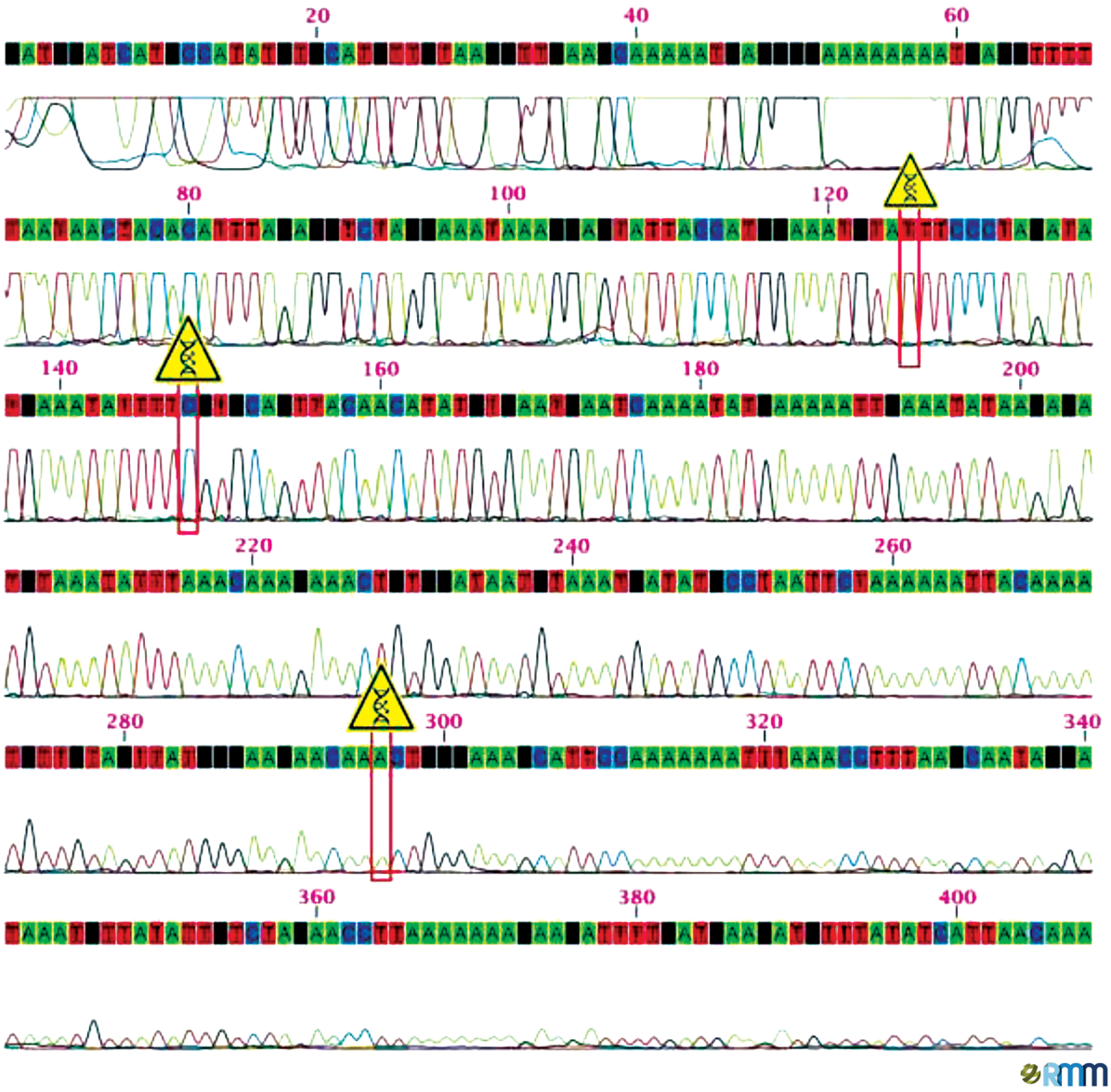

Figure 2. Chromatogram of one of the sequences related to triple mutant
The mutated nucleotides are marked in the figure.

number of triple mutant cases (22.2%) in Iran. This value indicates significant resistance of *P. falciparum* to pyrimethamine. Resistance codons to pyrimethamine in Iran were first studied in 2002 by Eskandarian et al. [14]. In this study, in one isolate, mutations related to resistance were reported at position 59 and in two isolates, mutations at position 108 were reported. In numerous studies from 2002 to 2020, many resistance codons were reported in Iran. The highest frequency was related to codon 108 [14-19].

In codon 108, changing the amino acid Ser to Asn was observed between 11 and 100% [14-19]. In the present study, this rate was 100%. Codon 59 had the highest frequency after codon 108 so the rate of change of amino acid Cys to Arg in the mentioned position was often 2.8 - 97% [14-19]. Only in the study conducted by Sharifi-sarasiabi et al., the frequency of this codon was reported to be 100%, in which the sample size was smaller than in the present study [19].

Regarding codon 51, most studies have not provided a significant report on the emergence of this haplotype. Only in the study conducted by Jafari et al. [15], changing amino acid Asn to Ile was reported to be 22% in codon 51 and other studies have not reported a significant amount (approximately 0.9 to 6%). In none of the studies in Iran and neighboring eastern countries, a report of codon 164 was presented.

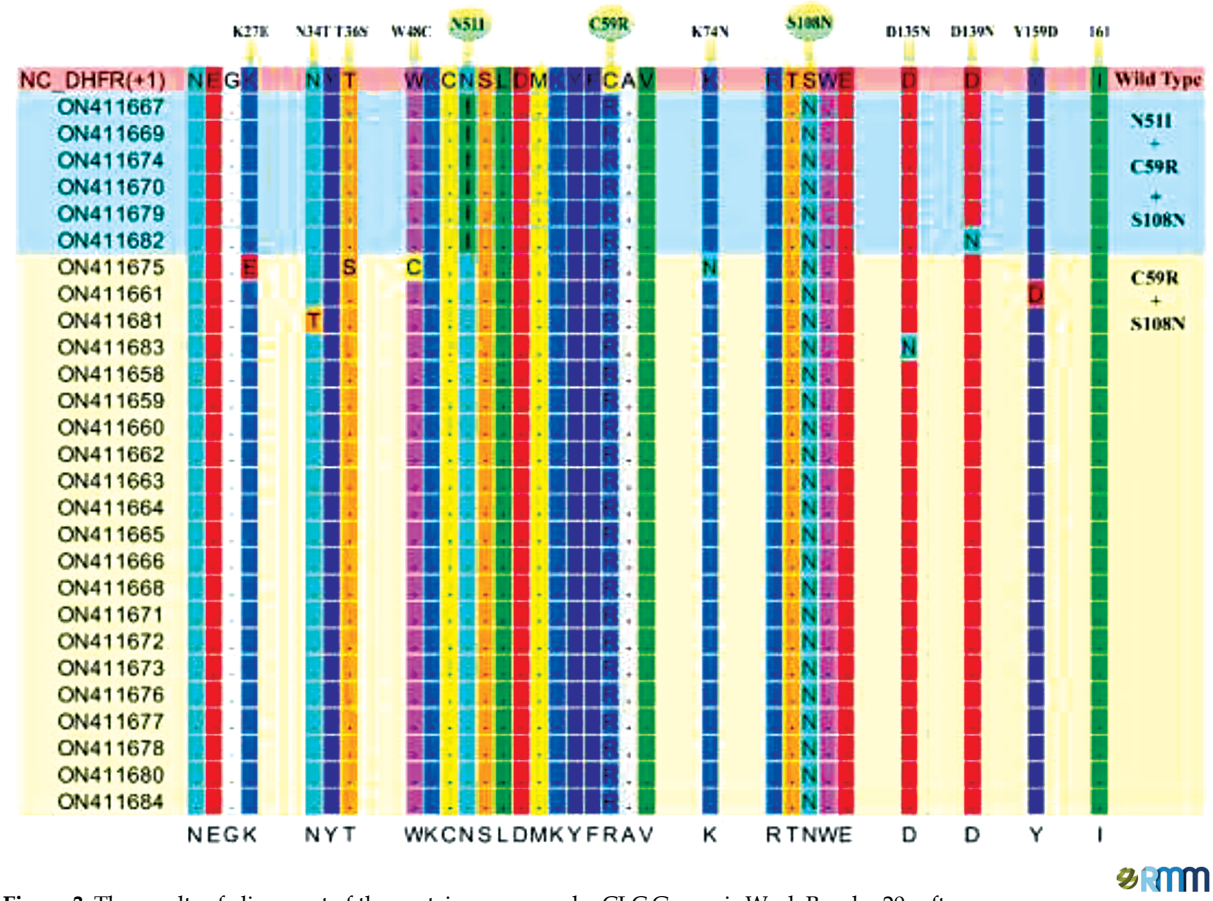

Figure 3. The results of alignment of the protein sequences by CLC Genomic Work Bench v20 software
Protein sequences obtained from this study were aligned with the reference wild-type strain (NC_DHFR).

In the present study, the frequency of codon 108 was 100%, which was slightly higher than in all previous studies. Moreover, regarding codon 59, similar to codon 108, an insignificant increase was reported compared to other studies conducted in Iran. In this investigation, the highest frequency of codon 51 was reported. In addition, nine novel mutations were found at various sites of *pfdhfr* gene.

Overall, the rate of *P. falciparum* resistance to pyrimethamine is similar in studies conducted in neighboring countries. In Pakistan, the frequency of codon 108 was between 87 and 100%, codon 59 was between 80 and 98% and finally, codon 51 was about 8% [21, 28, 29]. In a study conducted in Afghanistan, the frequency of codon 108 was 98% and codon 59 was 90%, but no resistance was reported in codon 51 [20]. The evaluation of imported malaria in Iran is important because the malaria-prone areas of Iran have a significant common border with Pakistan and Afghanistan, and in these countries, malaria cases are reported more than in Iran. The number of immigrants from these countries to Iran is high; thus, it can be concluded that molecular epidemiological factors for malaria in Iran are affected by malaria in these countries.

Regarding the genetic pattern of pyrimethamine resistance and the emergence of resistant codons, the results of studies conducted in these countries are relatively consistent with the findings of the current study. Regarding novel codons found in this investigation, although they were not related to resistance, significant changes in the number of amino acids, especially in a drug resistance gene, require further investigation *in vitro* and *in vivo*, and also emerging resistance codons in other malaria-prone areas should be considered by other researchers.

In this investigation, the triple mutant frequency was at a high level, which indicates a definite resistance to pyrimethamine. The emergence of haplotype 51, which acts as a complement to definite resistance to pyrimethamine, puzzles this region.

In this study, the frequency of haplotype 51 in our sample size was 22.2%, with the normal distribution of imported malaria cases throughout Iran. This abundance

means not only an increase in a resistance codon but also the emergence of significant triple mutant cases that indicate definite resistance and a synergistic level of *P. falciparum* resistance to pyrimethamine [30].

In most studies, due to more malaria cases in recent years, larger sample sizes have been studied in one or two areas, but in the present study, the sample size was smaller in Iran due to the decreasing trend of malaria in recent years, but it has a comprehensive distribution (taken from all malaria-prone areas of the country) and to some extent can lead to a better understanding of the resistance to pyrimethamine in Iran.

Because malaria treatment protocols are usually performed in the countries of the world, such as mass drug administration (MDA) [31] and artemisinin-based combination therapies (ACTs) [32], the emergence of treatment failure has not been observed significantly, because the partner drugs used in treatment lines, such as artemisinin contribute to the competency and efficacy of the treatment lines and finally help to the timely clearance parasite.

However, in many endemic areas, due to the spread of drug resistance, another drug sulfadoxine-pyrimethamine is used simultaneously, but in some cases, malaria prophylaxis is recommended during pregnancy [33, 34]. Therefore, timely monitoring and control of this drug combination are essential.

Conclusion

The susceptibility of *P. falciparum* to pyrimethamine in the treatment of malaria is significantly decreasing and has raised concerns about drug resistance to *P. falciparum*. Pyrimethamine is often used in the first line of treatment in neighboring countries. Due to the high emergence of dual resistance codons, in the treatment of malaria, prescribing pyrimethamine should be more considered. However, the lack of a comprehensive study on codon 51 is becoming more apparent. Because in most studies, the occurrence of the double mutant has been confirmed, there is also a need for comprehensive and regional investigations on pyrimethamine and the frequency of codon 51 as a complement to the puzzle of definitive resistance to pyrimethamine in the region. Accordingly, it is finally suggested that conducting a comprehensive regional study on codon 51 by creating a better understanding and correct prognosis of the definitive resistance to pyrimethamine can prevent the widespread emergence of resistance to this drug and the overwhelming problems in the malaria control system in the region.

Ethical Considerations

Compliance with ethical guidelines

All patients filled the informed consent form to participate in the study. The study protocol was accepted by the Ethics Committee of Mazandaran University of Medical Sciences, Sari, Iran (Code of ethics: IR.MAZUMS.REC.1399.8476).

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Authors contribution's

Conceptualization and Supervision: Seyyed Ali Shariatzadeh, Ahmad Raeisi, Shirzad Gholami and Reza Valadan; Methodology: Reza Valadan, Abbas Shahbazi, Seyed Abdollah Hosseini, Adel Spotin; Investigation, Writing–original draft, and Writing–review & editing: All authors; Data collection: Seyyed Ali Shariatzadeh, Fattaneh Montazeri, and Davood Anvari; Funding acquisition and Resources: Shirzad Gholami and Seyyed Ali Shariatzadeh.

Conflict of interest

The authors declared no conflict of interest.

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