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Review Article

The Drug Resistance of *Plasmodium falciparum* and *P. vivax* in Iran: A Review Article

Aliehsan Heidari [1], *Hossein Keshavarz [2,3]

1. *Department of Medical Parasitology, School of Medicine, Alborz University of Medical Sciences, Karaj, Iran*
2. *Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*
3. *Research Center for Endemic Parasites of Iran, Tehran University of Medical Sciences, Tehran, Iran*

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***Correspondence**
Email:
hkeshavarz@tums.ac.ir

Abstract

Background: One of the main obstacles to malaria control in the world has been the emergence of resistance in *Plasmodium falciparum* to chloroquine and other antimalarial drugs. This study aimed to review studies in Iran on resistance in *P. falciparum* and *P. vivax* to drugs, and to reveal the mechanisms and molecular markers of resistance of these two species.

Methods: The databases of PubMed, Scopus, Google Scholar, Magiran, and reputable Iranian journals were searched to find published studies on the resistance in *P. falciparum* and *P. vivax* to antimalarial drugs in Iran.

Results: There is a significant relationship between resistance to chloroquine in *P. falciparum* and the emergence of K76T mutation in the *P. falciparum* chloroquine-resistance transporter gene in Iran. Resistance to sulfadoxine-pyrimethamine (SP) in *P. falciparum* is also significantly associated with the development of mutations in the dihydrofolate reductase and dihydropteroate synthase genes. Resistance to chloroquine in *P. vivax* has not been reported in Iran and it is used as a first-line treatment for *P. vivax* malaria.

Conclusion: *P. falciparum* has become resistant to chloroquine in different regions of Iran and is not currently used to treat malaria. Besides, cases have emerged of *P. falciparum* resistance to SP in different parts of southern Iran, and SP is not administered alone for treating *P. falciparum*.

Introduction

Drug resistance is a serious concern for the control and elimination of malaria in the world (1). Malaria has existed since ancient times, was first recorded by Hippocrates four centuries BCE, and Avicenna described it as an intermittent fever in the Canon of Medicine circa 1000 CE (2). However, as one of the most life-threatening infectious diseases in the world, malaria is still prevalent (3). Malaria infected about 219 million people and killed 409 thousand cases throughout the world in 2019 (4).

Five species of *Plasmodium falciparum*, *P. vivax, P. malaria, P. ovale, and P. knowlesi* cause malaria in human (5). Two of these species, *P. vivax* and *P. falciparum,* are common in Iran with *P. vivax* accounting for more than 90% of malaria cases. Iran's southern provinces including Sistan-Baluchestan, Hormozgan, and southern parts of Kerman are affected by indigenous cases of malaria (Fig. 1) with most cases found in Sistan and Baluchistan Province (6).

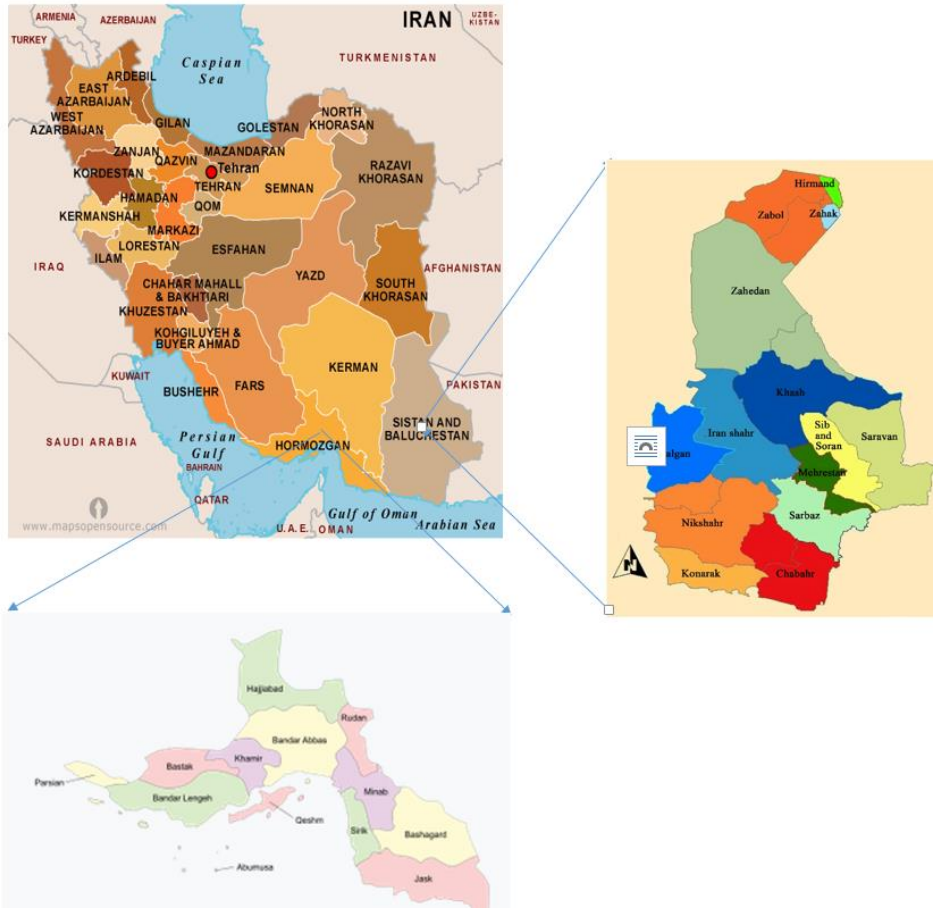

Fig. 1: Map of Iran

Extensive measures taken in Iran have brought malaria on the verge of elimination. Accordingly, no cases of indigenous transmission were reported in 2018, 2019, and 2020 (4).

Iran's neighboring Pakistan and Afghanistan with the highest rates of malaria cases in the Eastern Mediterranean Region after Sudan, and the resulting border contacts, migration and travel of citizens of these countries to Iran have made imported malaria particularly important (4, 7, 8). Some studies on the genetic diversity of *P. falciparum* and *P. vivax* genes in Iran indicate excessive diversity of their genes, which is not commensurate with the malaria situation in Iran and its low transmission rate, confirming the exchange of *Plasmodium* strains between neighboring countries and Iran (9-14)

One of the reasons for the persistence of malaria in the world is the decrease in the sensitivity of *Plasmodium* to anti-malarial drugs and the emergence of drug resistance in this protozoan, especially in *P. falciparum* (15). Although the highest malaria mortality is due to *P. falciparum*, severe and even more fatal cases can occur due to *P. vivax* (5, 16). This article aimed to review studies on the drug resistance in *P. falciparum* and *P. vivax* in Iran and to reveal the mechanisms and molecular markers of resistance in these *Plasmodium* species.

Methods

The databases of PubMed, Scopus, Google Scholar, Magiran and reputable Iranian journals were searched to find published studies on the resistance in *P. falciparum* and *P. vivax* to anti-malarial drugs and studies on molecular markers associated with anti-malarial drug resistance. All articles related to resistance to antimalarial drugs were selected and analyzed. Duplicate and insignificant articles were not reviewed.

Definition of drug resistance in malaria parasites

Drug resistance in *Plasmodium* is defined as its ability to survive or reproduce although the drug reaches the infected parasite or red blood cell sufficiently and that the drug is administered and absorbed in doses equal to or even higher than those tolerable for humans (17). Accordingly, the treatment failure which may be due to insufficient dose of the drug or insufficient drug delivery to the parasite is not considered *Plasmodium* resistance to antimalarial drug (18, 19). Besides, drug resistance must be distinguished from recrudescence.

WHO has defined the antimalarial treatment failure in *P. falciparum* in human as follows (20):

- Early Treatment Failure (ETF): The onset of clinical symptoms and/or the presence of parasitemia during the first three days of follow-up.
- Late Clinical Failure (LCF): The recurrence of symptoms in the presence of parasitemia within 4 to 28 d after starting the treatment.
- Late Parasitological Failure (LPF): Parasitemia from day 7 to 28 with axillary temperature less than 37.5 °C.

Meanwhile, the anti-malarial treatment failure in *Plasmodium* was previously defined at three levels of R1, R2, and R3 (21).

P. falciparum resistance to antimalarial drugs

Quinine, extracted from the leaves of the cinchona tree, was used as an important drug to treat malaria from the 17th century until the 1920s (22). During World War II, synthetic chloroquine was developed due to insufficient access to quinine. The German scientist Hans Andersag, who worked for the German pharmaceutical company Bayer AG, synthesized chloroquine as an antimalarial drug in 1934 (Fig. 2).

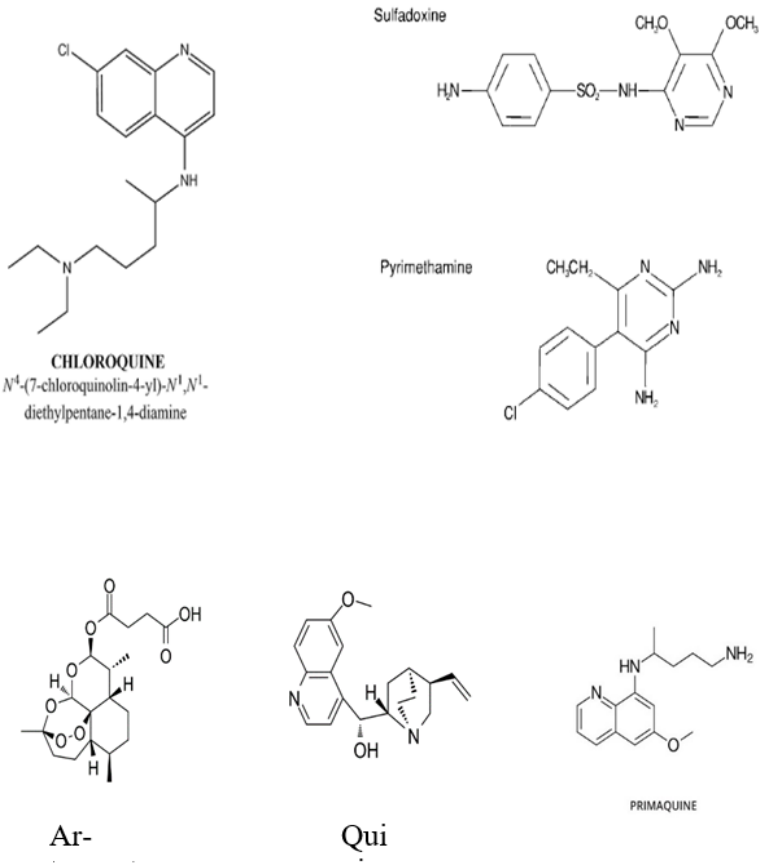

Fig. 2: Chemical structure of some anti-malarial drugs (Ar =Artesunate, Qui=Quinine)

It was used as 7-chloro-4-quinoline in the 1940s to treat malaria (23-25). By adding the hydroxyl group to chloroquine, the hydroxychloroquine compound was also used as an alternative for chloroquine. Since 1946, both chloroquine and hydroxychloroquine have been used as effective inexpensive drugs with fewer side effects as a first-line treatment for malaria worldwide (23-25) However, the effectiveness of chloroquine decreased with the emergence and spread of chloroquine-resistant *P. falciparum* isolates, and in 1959, almost a decade after its use, the first cases of *P. falciparum* resistance to chloroquine were reported in Colombia and Southeast Asia. Currently, resistance in *P. falciparum* has been reported almost from all over the world except in small remote areas (17, 24, 26). *Plasmodium* feeds on hemoglobin such that young trophozoites consume up to 75% of the hemoglobin in red blood cells (27). In *P. falciparum*, hemoglobin is transferred to the digestive vacuole at a pH of approximately 5.2. In the digestive vacuole, *P. falciparum* breaks down the hemoglobin to obtain amino acids (28). *P. falciparum* prevents the accumulation of free toxic heme in the digestive vacuole and, by polymerizing heme, converts it to a crystalline substance called hemozoin or pigment (23). Chloroquine prevents the conversion of heme to hemozoin in the digestive vacuole (Fig. 3).

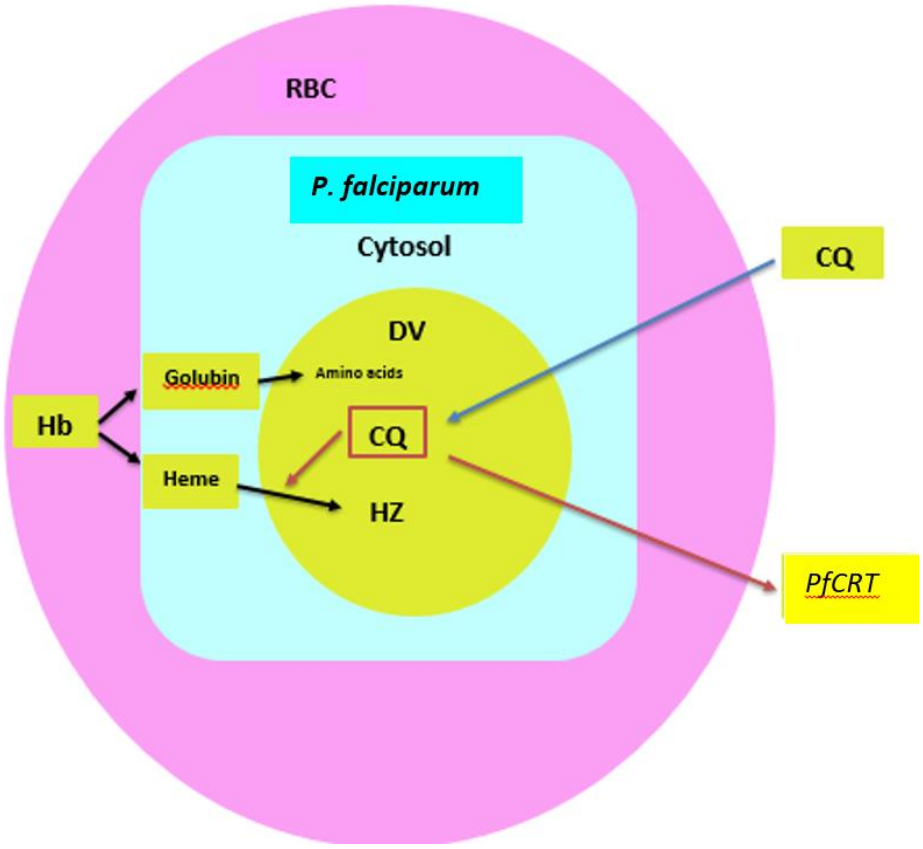

Fig. 3: Conversion of heme to hemozoin in the digestive vacuole in *P. falciparum* and the role of *Pfcrt* mutation in inhibiting chloroquine action

In other words, proteolysis of hemoglobin releases heme, which is soluble and is a form that can inhibit the activity of various *Plasmodium* enzymes (23, 24). K76T mutations in the *P. falciparum* chloroquine resistance transporter (*Pfcrt*) have been associated with *P. falciparum* resistance to chloroquine in various global studies. This mutation converts K (lysine) to T (Threonine). The *Pfcrt* gene encodes an integral protein membrane that is concentrated in the digestive vacuole (29). This mutation generally prevents the entry of chloroquine, a weak alkaline drug 4-aminoquinoline, into the acidic organelle of the digestive vacuole of *P. falciparum* or causes it to leave the digestive vacuole quickly, preventing chloroquine from binding to heme and preventing its detoxification. Finally, chloroquine will not be able to prevent the conversion heme to either hemozoin (27, 30).

Another factor that plays a role in resistance to antimalarial drugs that target hemoglobin is the P-glycoprotein homologue of *P. falciparum* multidrug resistance (Pfmdr) 1, a protein encoded by the *Pfmdr1* transporter gene. *Pfmdr1*, like *Pfcrt*, is located on the membrane of the digestive vacuole (27). Adequate drug delivery is essential for maximum drug activity. Mutations in *Pfmdr1* probably prevent the transfer of the antimalarial drug from the cytosol into the digestive vacuole where it reduces the concentration of drugs that target hemoglobin, such as chloroquine and amodiaquine (31). Mutations in codon 86 (N86Y) of the *Pfmdr1* gene are commonly involved in chloroquine resistance, and there are few studies on the polymorphism of other codons (32).

Iran has witnessed great successes in controlling malaria in many parts of the country since 1958 when the malaria eradication pro-

gram began, and chloroquine has been used since the 1950s as a first-line treatment for malaria caused by *P. falciparum* and *P. vivax* (33). However, this program did not lead to the eradication of malaria in Iran for various reasons, including parasite resistance to antimalarial drugs and vector resistance to insecticides (33).

An in vivo study conducted in Hormozgan Province in southern Iran in 1968 reported susceptibility of *P. falciparum* to chloroquine (34). Another study on 45 patients with malaria in the same province also showed that *P. falciparum* was still susceptible to chloroquine and no case of resistance was reported (35).

In Iranshahr, a city in Sistan and Baluchistan Province, *P. falciparum* resistance to chloroquine in 1983 was reported at 5.7% at R1 level. However, its resistance to chloroquine gradually increased to 52.1% in Iranshahr at three levels of R1, R2, and R3 (2).

P. falciparum resistance to chloroquine was also explored by in vivo studies in other areas, including Hormozgan Province and Kahnooj in Kerman Province. During 1997-2000, the resistance reached 68% at the R1 level and 84% at the R2 level in the mentioned areas (2, 36). Since most of the reported cases of chloroquine resistance were detected in people who were either Afghan nationals or had a history of traveling outside Iran, chloroquine-resistant strains might have been imported from other countries (17).

Chloroquine efficacy was investigated in compliance with the 28-day WHO standard protocol from 2002 to 2004 in 5 locations in Sistan and Baluchistan, Hormozgan, and Kerman provinces in southern Iran. On day 28 of the follow-up, 78.5% of cases were found to be resistant to chloroquine, with 17.4% having ETF, 34.7% LCF, and 26.4% LPF (37). Based on this study and previous reports, the use of chloroquine was discontinued for the treatment of malaria caused by *P. falciparum* in Iran.

P. falciparum resistance to chloroquine and genetic markers in Iran

Some studies have addressed the resistance of *P. falciparum* to chloroquine and its relationship with *Pfcrt* and *Pfmdr* genetic markers in Iran. A study reported 49 isolates with K76T mutation out of 50 isolates of *P. falciparum* in Sistan and Baluchistan Province (38). In another in vivo study conducted in the same province, 23 out of 25 patients with *P. falciparum* malaria treated with chloroquine showed resistance to chloroquine during the 28-day follow-up period. A correlation was reported between 76T mutant allele and resistance to chloroquine (39).

Furthermore, K76T mutation was observed in 60 isolates (93.75% of the participants) out of 64 patients with *P. falciparum* malaria in Sistan and Baluchistan Province using nested PCR. Only four *P. falciparum* isolates carried the wild K76 codon. Response to chloroquine was evaluated in 28 of these patients in vivo for 28 days. The response of *P. falciparum* isolates to treatment was as follows: ETF = 17.9%, LTF = 60.7%, and sensitive to chloroquine = 21.4%. In other words, 22 out of 28 patients (78.6%) showed resistance to chloroquine in vivo. All of these patients carried the 76T mutant allele (40).

In another study in Sistan and Baluchistan Province on 206 *P. falciparum* isolates, the 76T mutant allele in the *Pfcrt* gene was observed in 202 isolates (98%) while the 86 Y allele in the *Pfmdr1* gene was observed in 78 (37.8%) isolates (41). Finally, a study on 26 *P. falciparum* isolates that revealed treatment failure to chloroquine during the 28-day follow-up period showed that the N86Y mutation in the *Pfmdr1* gene was carried in six isolates (23.1%). No mutations were observed in the other four codons of this gene (42).

P. falciparum resistance to sulfadoxine–pyrimethamine

Pyrimethamine, commercially known as Daraprim, was developed in the early 1950s by

Gertrude Elion and George Hitchings. The two researchers won the 1988 Nobel Prize in Medicine for developing pyrimethamine (24).

Sulfadoxine was developed in the early 1960s (24), but it was not used for a long time because the malaria parasite was resistant to it. Sulfadoxine-pyrimethamine (SP) was approved in 1981 for the treatment of malaria and was sold under the brand name Fansidar. Both drugs specifically target the folate biosynthesis pathway. Pyrimethamine inhibits the dihydrofolate reductase (dhfr) and sulfadoxine inhibits the dihydropteroate synthase (dhps) (24). SP is used as a single dose.

Following *P. falciparum* resistance to chloroquine in Iran, SP was used to treat *P. falciparum* malaria. However, *P. falciparum* resistance to SP was reported very quickly due to mutations in *dhfr* and *dhps* genes (Figs. 4 and 5). Since 2007, the Center for Infectious Diseases Management modified the treatment regimen and SP-artesunate replaced the previous treatment. An in vivo and in vitro study on the response of *P. falciparum* to SP showed that out of 26 chloroquine-resistant isolates that used amodiakin+standard dose of SP, three cases were in R1 and three cases were resistant to SP at the R2 level. Another study administered a standard dose of SP for the treatment of 43 chloroquine-resistant *P. falciparum* isolates in three provinces of Sistan and Baluchistan, Hormozgan, and Kerman, and reported 11.6% of the isolates were resistant to SP (43).

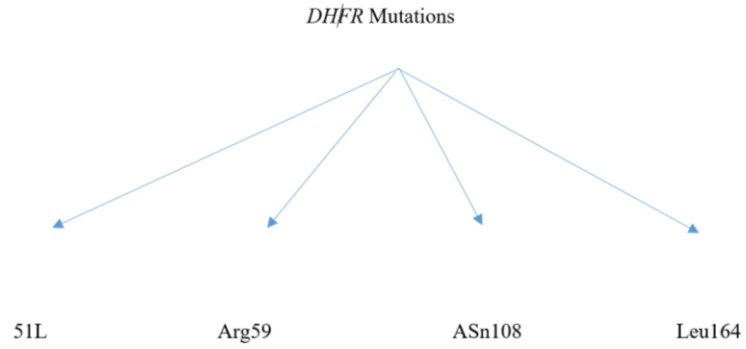

Fig. 4: Mutation in codons 51, 59, 108 and 164 in Iranian *P. falciparum* isolates that confer resistance to pyrimethamine

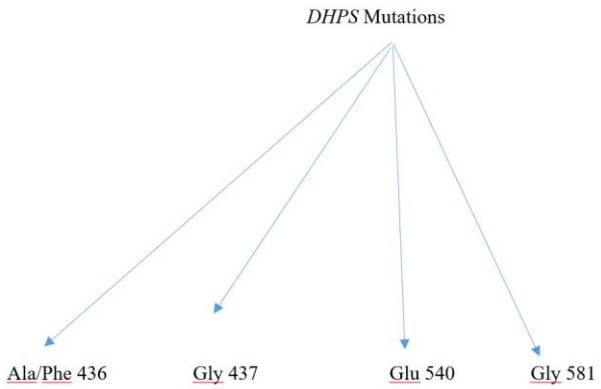

Fig. 5: Mutation in codons 436, 437, 540 and 581 in Iranian *P. falciparum* isolates that confer resistance to sulfadoxine

Mutations in P. falciparum dhfr and dhps genes

In a study of 35 *P. falciparum* malaria patients in Hormozgan Province receiving SP for the treatment of malaria, two patients failed treatment, one of the two had a Glu 540 mutation in the *dhps* and Asn-108 and Arg 59 mutations in the *dhfr* gene (44).

A study in Chabahar of Sistan and Baluchistan Province, on 206 *P. falciparum* isolates revealed that all isolates in the *dhfr* gene carried 108N mutations and 98.5% of the isolates had both 108N and 59R mutations. In total, 20.4% of the isolates had 59R/108N simultaneous mutations in the *dhfr* gene and 437G in the *dhps* gene (41).

To investigate the association between molecular markers of *P. falciparum* resistance to SP and in vivo *P. falciparum* resistance, 53 *P. falciparum* isolates were examined, in which 11.3% of the isolates (6 patients) were resistant to SP (45), indicating a higher level of resistance than the rate (5.77%) reported by Eskandarian (44). Mutant codons of Asn108 in 100% and Arg59 in 81.1% of isolates were observed in the *dhfr* gene. Besides, 85% of the isolates in the *dhps* gene carried the 436Ala/Phe mutant codon. All SP-resistant cases had at least three mutations in both genes (45). This study showed that the effect of SP in Iran was decreasing. The prevalence of Gly437 mutant codon was 32%, a significant increase when compared with 17% in the previous study (46). Mutations in codon 436 in *dhps* gene and mutant codon Leu-164 in *dhfr* gene were also reported in this study in Iran (45).

Another study conducted in Hormozgan Province on 16 *P. falciparum* isolates revealed that all isolates had double mutations in codons 108 and 59 (allele 59R /108N) in *dhfr* gene (47). These mutations indicate possible *P. falciparum* resistance to pyrimethamine.

A study conducted on 107 isolates after the introduction of SP and artesunate in Iran as the first-line therapy for *P. falciparum* malaria showed that the mutant codons 108N, 59R, and 51I were 100%, 95.9%, and 4.1% in *dhfr* gene of the isolates, respectively. The 437G mutant codon was observed in *dhps* gene in 26.9% of isolates, a reduction compared with 55.5% before the introduction of the SP and artesunate combination (48). Cumulative mutations 59R, 108N in *dhfr*, and 437G in *dhps* were observed in 42.4% of isolates; meanwhile, the same cumulative mutations decreased to 38% (48).

Artemisinin and its derivatives

Artemisinin was first isolated from *Artemisia annua* in 1971 by Tu Youyou. This plant has been widely used in traditional Chinese medicine to treat malaria. Tu Youyou received the Nobel Prize in Medicine in 2015 for discovering a new treatment for malaria (24). Artemisinin is effective against multidrug-resistant *P. falciparum* strains and has become the first-line malaria treatment in the world (27). Artesunate is a semi-synthetic derivative of artemisinin, used in Iran together with SP to treat falciparum malaria (49).

Artemisinin-based combination therapies (ACTs) are the current strategy to combat falciparum malaria. ACTs have significantly reduced the burden of malaria and its mortality in the world (50). Despite the efficiency of ACTs, the first case of partial artemisinin-resistant *P. falciparum* was reported in Western Cambodia in 2008 (51). Artemisinin-resistant *P. falciparum* was again reported in Western Cambodia, western Thailand, southern Myanmar, and southern Vietnam (18, 52). In another study in 2018, 30 cases of artemisinin-resistant *P. falciparum* were reported in Southeast Asia (53).

There is no consensus over the artemisinin mechanism of action. One theory explains that the drug molecules are activated by heme to produce free radicals, which destroy the proteins needed for the parasite survival (24). Artemisinin acts as an effective inhibitor of phosphatidylinositol-3-kinase (54). Artemisinin-resistant mutations affect one of the B

sheets of the Kelch domain (52). Upregulation-resistant K13 mutant parasites have shown oxidative stress (50).

An in vivo study in Iran examined the susceptibility of *P. falciparum* to SP/artesunate in 38 symptomatic malaria patients and reported full treatment of all 38 patients, and no reduction in susceptibility to this drug during the 28-day follow-up period (55).

P. vivax resistance to antimalarial drugs

Annually, *P. vivax* causes about 14.3 million malaria cases with clinical signs worldwide (56). Chloroquine has been used to treat *P. vivax* malaria for over 60 years. *P. vivax* resistance to chloroquine was first reported in Australian soldiers returning from Papua New Guinea (16, 57). There have been reports of *P. vivax* resistance to chloroquine in many endemic areas (16).

A study on 270 symptomatic *P. vivax* malaria patients in Sistan and Baluchistan Province, Iran, showed that chloroquine was still effective against *P. viv*ax at the usual therapeutic dose of 25 mg/kg body weight. Besides, no case of resistance was reported in vivo during a 28-day follow-up (58). Previous studies have also confirmed the susceptibility of *P. vivax* to chloroquine (59-61). An in vivo study with a 28-day follow-up period in Sistan and Baluchistan Province, Iran, on 170 patients with *P. vivax* malaria showed that *P. vivax* was still susceptible to chloroquine and there were no reports of treatment failure (55). The first case of *P. vivax* resistance to chloroquine was reported in a 26-year-old pregnant woman in Pakistan: administration of standard doses of chloroquine did not result in parasite clearance (62).

Although molecular markers have been used to determine the resistance of *P. falciparum* to chloroquine, such studies have failed to show a correlation between the molecular markers Pvcrt and Pvmdr1 and the response of *P. vivax* to chloroquine (26, 63).

Several molecular studies investigated the status of mutations in the codons of *P. vivax dhfr* and *dhps* genes related to *P. vivax* resistance to SP in Iran. These studies have reported mutations in codons 57L, 58R, 117N, 117T, 93H, 33L, 61N in *dhfr* gene and mutations in codon 421 of *dhps* gene in southern Iran (47, 64-66). However, these studies have focused on the mutations and have not evaluated their association with in vivo resistance to SP in *P. vivax* isolates. SP are not currently recommended as the first-line treatment for *P. vivax* anywhere in the world. *P. vivax* appears to be resistant to this drug much faster than *P. falciparum* (63).

ACTs have a greater effect on *P. vivax* than on *P. falciparum* (67). However, they do not affect hypnozoites (63) and they have no role in preventing relapses (68). In countries where *P. vivax* is highly resistant to chloroquine, ACTs are used as the first-line treatment for *P. vivax* malaria (16).

Some forms of *P. vivax* hypnozoites in the liver are used to treat 8-Aminoquinoline compounds including primaquine. There are reports of malaria relapse in *P. vivax* endemic regions of the world (63). Although many cases of *P. vivax* are due to relapses, studies on the efficacy of primaquine in preventing relapses have led to controversies over the research design, implementation, and analysis (63). Some cases of *P. vivax* malaria relapse have also been reported in southern Iran (68, 69). There have been no reports of *P. vivax* resistance to primaquine in Iran.

Conclusion

One of the main obstacles to malaria control in the world has been the emergence of resistance to chloroquine and other antimalarial drugs in *P. falciparum*. Studies in Iran have reported a gradual decrease in susceptibility and then no response to chloroquine and SP used for the treatment of *P. falciparum*. *P. falciparum* has become resistant to chloroquine in different regions of Iran and it is not currently used

to treat *P. falciparum*. Besides, cases of *P. falciparum* resistant to SP have emerged in different parts of southern Iran and it is not applied alone for treating *P. falciparum*. In line with other studies in other parts of the world, there is a significant relationship between *P. falciparum* resistance to chloroquine and the emergence of K76T mutation in the *Pfcrt* gene in Iran. *P. falciparum* resistance to SP is also significantly associated with the development of mutations in the *Pfdhfr* and *Pfdhps* genes.

To date, there have been no reports of decreasing susceptibility in *P. falciparum* to ACTs, including artesunate, in Iran. Monotherapy is not recommended to prevent resistance to antimalarial drugs *in P. falciparum*.

An alternative approach is to use several compounds at the same time as used against other infectious diseases such as tuberculosis and AIDS. Molecules of several drugs used to fight malaria parasites with different functional mechanisms and different half-lives may reduce the likelihood of parasite survival. In this case, if the malaria parasite is less sensitive or semi-sensitive to a drug, it may be killed by the accompanying drug and resistance to the original drug may be delayed.

Chloroquine-resistant *P. vivax* has not been reported in Iran and this drug is the first-line treatment for *P. vivax* malaria. Reports of chloroquine-resistant *P. vivax* in Pakistan and other parts of Asia emphasize that ongoing studies should monitor susceptibility of *P. vivax* to chloroquine in Iran. There are several reports of relapse due to *P. vivax* hypnozoites in Iran, but so far no cases have been reported of their resistance to primaquine.

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Conflict of interest

The authors declare that there is no conflict of interest.

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