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Original Article

Induction of Artesunate Resistance in *Plasmodium falciparum* 3D7 Strain Using Intermittent Exposure Method and Comparing *P.fk13* Sequence between Susceptible and Resistant Strains

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Abstract

Background: Resistance to artemisinin has threatened major achievements in malaria control, more investigations is needed about resistant strains and related genes. We aimed to induce resistance to artesunate in the *Plasmodium falciparum* 3D7 strain using intermittent exposure method and comparing *P.fk13* gene sequence between susceptible and resistance strains.

Methods: *P. falciparum* 3D7 strain was cultured according to Trager & Jensen method with some modifications. Serial concentrations between 10^{-2} mol/l, to 10^{-7}mol/l were prepared, then *P. falciparum* 3D7 was exposed to each of the dilution to determine IC50 and lethal dose. Sensitivity reduction process was started from the concentration of 10^{-7}mol/l and ended at 10^{-2}mol/l. Exposed parasites were collected after at least 27 days after cultivation in each drug concentration. DNA extraction, PCR and sequencing process were performed to investigate any possible mutations in the *P.fk13* gene sequence.

Results: Effectiveness of 10^{-2}mol/l concentration of artemisinin was found as a lethal dose. IC50 value was equal to $5^{\times}10^{-4}$ mol/l. The resistant strain was provided in the lab, sequenced and registered in the gene bank as *P.f Art* $^{-2}$, (accession number MH796123. 1). Alignment of this registered sample showed no mutation in *P.f kelch13* gene in comparison with standard strain submitted in the GenBank.

Conclusion: Resistance to artesunate in malaria parasite may occur but with no mutation in the *P.f kelch13* gene. Therefore, whole genome sequencing should be applied to determine mutations in resistant strains.

Introduction

Malaria is a significant health issue in many countries, particularly in tropical and subtropical areas (1). The disease is prevalent in 90 countries, and 40% of the world's population live in areas where there is a risk of transmission (2, 3). In 2017, 216 million people were exposed to malaria, resulting in 445,000 deaths (4). Over the past 50 years, *Plasmodium falciparum* has become resistant to several antimalarial drugs, including chloroquine, sulfadoxine-pyrimethamine, quinine, piperquine, and mefloquine. The recent emergence of resistance to some derivatives of artemisinin and the failure of combination therapy with artemisinin has threatened all major achievements in malaria control (5).

Delays in clearing parasites from the infected individuals' blood is the first alarm of spreading of malaria resistance in malaria areas that ultimately will lead to a complete failure of treatment. Following frequent historical chain of events that have occurred in the field of drug resistance to malaria, many efforts have been taken to consider such phenomenon. Experimental induction of resistance in malaria parasites against one or more antimalarial drugs and considering results of the process can guide malaria policy makers to make appropriate decisions when they encounter with the real problem. Producing a drug resistance line of *P. falciparum* against artesunate can provide a new field of research for challenging the mentioned problem and can demonstrate likely mutations in the relevant genes (6-8).

Therefore, the aim of this study was to reduce the sensitivity of *P. falciparum* 3D7 strain to artesunate using intermittent exposure method and comparing the sequence of *P.fk13* gene between the sensitive and probable resistant strains. The findings of this study may help to better understand drug resistance in malaria parasites and inform the development of new treatments to combat the disease.

Materials and Methods

Parasite cultivation

This study was conducted at the National Malaria Laboratory, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. The procedure was as follow: continues culture of *P. falciparum* 3D7 was performed according to the Trager and Jenson procedure with some modifications (9). The parasite was cultured in CCM medium with 10% O^+ human RBCs hematocrit and Albumax (0.5%) as a growth factor. The plates incubated at 37 ^{0}C in a candle jar and fresh medium was added every 48 hours. Thin blood smears were prepared from the sedimentation of each plate and then percentage of parasites was counted against 10000 red blood cells. When the parasitemia reached to 10 percent, artesunate was added to the plates by the following procedure:

Determination of artesunate IC50 on P. falciparum 3D7

Artesunate (MW = 384.42, cat No. A3731, sigma Aldrich,) was used for preparing different concentrations for exposing the parasite. The substance was dissolved in 60% ethanol in PBS to obtain 10^{-2} mol/L concentration as a lethal dose, serial concentrations of artesunate as 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} and 10^{-10} mol/L, were made from stock solution (10^{-2} mol/L) in CCMto investigate IC50 of artesunate against the parasite. The drug sensitivity test was conducted in 96-well microtitre plate. Each plate was designed as follow: negative control including 10 µl fresh-washed O+ RBC plus 190 µl of CCM. Positive control was charged with 10 µl infected RBC with 6 % parasitemia yield in continuous culture and 190 µl of CCM. Groups of 3 to 9 (groups Pi test): 10µl of infected RBC (6% parasitemia) plus 90 µl CCM and 100 µl drug dilution

(from 10^{-4} mol/l concentration to 10^{-10} mol/l for each test respectively). Negative and positive control were respectively designed to check fresh red blood cells and parasite growth enhanced during the incubation. The plates were kept in a 37 °C incubator for 24 hours. Thin smear was made as explained above. The tests were repeated three times and mean of parasite percentage was calculated. Rate of parasitemia and inhibitory effect of each drug concentration was calculated as following and IC50 was achieved using SPSS 20 software.

$$\text{Inhibitory rate} = 100 - \left(\frac{\text{mean of parasitemia in each test goup}}{\text{mean of parasitemia in control group}} \times 100\right)$$

Induction of drug resistance in P. falciparum 3D7:

The procedure of establishing drug resistance in *P. falciparum* against artesunate was accomplished with some modifications (10). Briefly, parasites in the culture media were exposed to the above-mentioned concentrations of artesunate. Induction was started from a concentration lower than IC50. In each tests, 100 µl of artesunate was added to 2.5 ml parasitized culture with 10% hematocrit, when the parasitemia rate reached 6-7%. Every 24 hours the medium removed and fresh CCM-artesunate were added. The rate of parasitemia was calculated by preparing thin blood smears and staining them. When the parasitemia rate was reduced by 50% compared to the control group, a free of drug break with normal medium was administered to the drug-exposed parasites to allow the parasite's growth to renew. This procedure was continued for at least 27 days, during which the drug concentration was increased up to 10^{-2}mol/l. At the end of each phase of resistance development some of the resisted parasites were preserved in liquid nitrogen for further investigation, also about 200 µl of each sample was kept in -20 °C for molecular analysis. Overall, this procedure aimed to induce and study the resistance of *P. falciparum* to artesunate to understand better drug resistance in malaria parasites.

DNA extraction and Nested PCR test:

Those samples that became resistant against artesunate in the culture media were selected for DNA extraction processes. Moreover, a case control was applied for molecular analysis. It was a blood sample achieved from a patient who had a travel to African countries infected with *P. falciparum*. No recovery observed after artesunate administrated, so this sample was considerate as a resistant strain to compare with created resistant strain in the lab. The extraction process was performed according to the manufacture instructions (SinaClon, Cat No: EX6001, Lot No: 9660027).

Nested PCR method was employed to detect Pfk13 gene. For this purpose, two pairs primer were prepared (Table 1) by CinnaGen company based on previous study (11). Gene amplification was designed in two steps; a pair of primer including K13F1 and K13R1 were used for the first round. PCR reactions were performed in 25 µl volumes containing 10 µl master mix, 1 µl of each primer (10 pmol), 10 µl distill water and 3 µl of DNA template (50 ng) with the following protocol: initial denaturation 94ºC for 5' followed by 40 cycles including denaturation 94 ºC for 30 sec, annealing 60 ºC for 90″, extension 72°C for 90″ and last step containing 10' in 72°C for final extension. PCR products were electrophoresed on the agarose gel and a band corresponding to *P. falciparum* 3D7 *P.fk13* gene was detected.

The PCR product obtained from the first stage was used as a template for the second round of amplification using the same materials. The results were detected and observed through horizontal electrophoresis on a 1% agarose gel in a UV Transilluminator room. A 849 bp band corresponding to *P.f kelch13* was observed in the second step of the nested PCR and photographed using a Gel Documentation System. All PCR product samples were sequenced by the Biotechnology Re-

search Center of Germany (MWG) through the SinaClon Company and submitted to the GenBank.

Table 1: Primers for nested PCR of K13 gene in *P. falciparum*

Name	*Seg- (5-3)*	*MW*	*OD (1000 µl)*	*nmol*	*Water/tube (µl)*	*TM*	*GC%*	*Mer*	*Product size*
K13F1	GGGAATCTGGTGGTAACAGC	6222.07	2.5	13.26	132.59	59.35	55	20	2097bp
K13R1	CGGAGTGACCAAATCTGGGA	6191.06	4	21.32	213.21	59.35	55	20	
K13F2	GCCTTGTTGAAAGAAGCAGA	6190.07	3	15.99	159.93	55.25	45	20	849bp
K13R2	GCCAAGCTGCCATTCATTTG	6067.94	3.5	19.03	190.34	57.30	50	20	

Results

Parasite cultivation, lethal dose and IC50

The successful cultivation of the *P. falciparum 3D7* strain was shown in Fig. 1, which displayed healthy ring and schizont stages. The number of parasites increased over time (Fig. 2A). At the beginning of the study, a concentration of 10^{-2} mol/L was found to be a lethal dose, as shown in Fig. 2B, which resulted in the death of all parasites. However, a resistant strain to the same concentration was later produced.

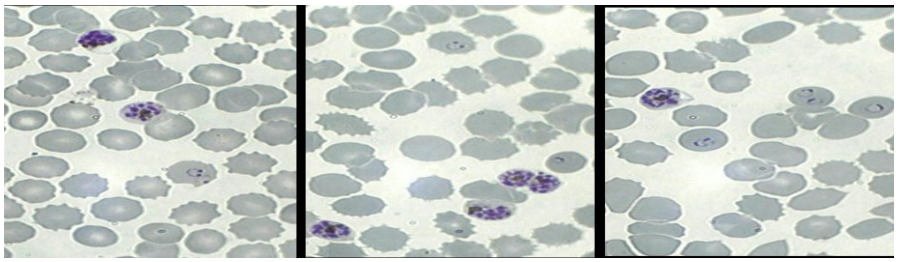

Fig. 1: Ring and schizont stages of P. falciparum 3D7 in continuous culture (thin blood smears)

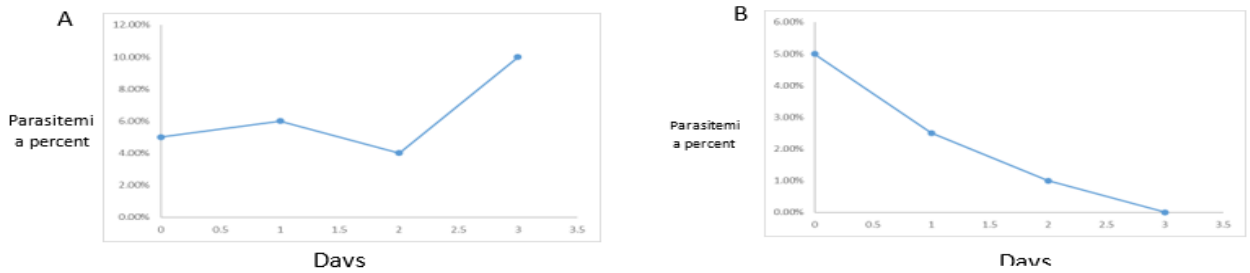

Fig. 2: Determination of artesunate lethal dose. The control group (A) (non-receiving drug) and test group (B) (plus 10^{-2} mol/l artesunate, lethal dose) of P. falciparum 3D7 continues culture

Table 2 displays the effects of different concentrations of artesunate on *P. falciparum 3D7* parasite to determine the IC50. The growth inhibitory percentage was calculated, and the IC50 value was estimated to be between 10^{-4} mol/l and 10^{-5} mol/l, with growth inhibition percentages of approximately 56% and 45%, respectively. To obtain a more precise result for IC50, these outcomes were analyzed using SPSS software. Fig. 3 shows the results of this analysis, indicating that the IC50 value was equal to $5^{\times}10^{-4}$ mol/l.

Table 2: Inhibitory effectiveness of different dilutions of artesunate on parasite growth to determine IC50. Group 1 (negative control), group 2 (positive control), groups of 3 to 9 (groups Pi test) containing drug dilution (from 10^{-4} mol/l concentration to 10-10mol/l in each test respectively)

Groups	*Drug concentration*	*Average growth inhibitory percentage*
1	Negative control	-
2	Positive control	0
3	10^{-4} mol/lit	56
4	10^{-5} mol/lit	45.3
5	10^{-6} mol/lit	40.08
6	10^{-7} mol/lit	24.61
7	10^{-8} mol/lit	14.49
8	10^{-9} mol/lit	11.27
9	10^{-10} mol/lit	-17.45

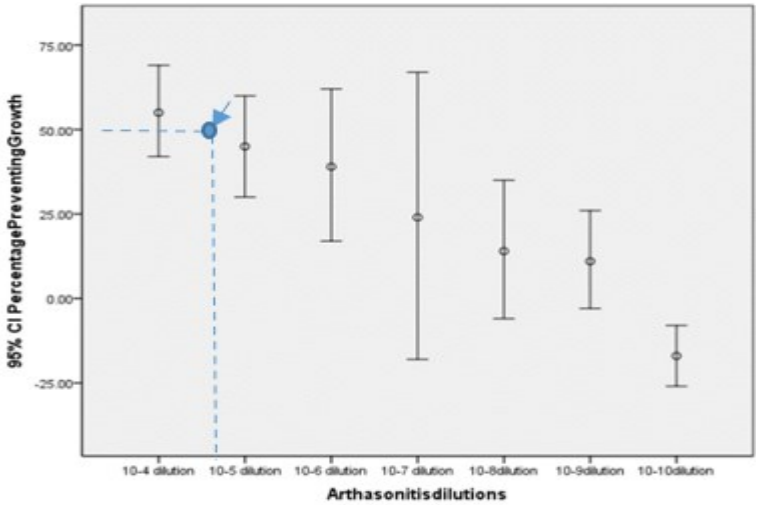

Fig. 3: IC50 determination using different concentrations of artesunate by SPSS software

The results of sensitivity reduction in P. falciparum 3D7 against artesunate using the intermittent exposure method

When sensitivity reduction test was performed for parasite, the patterns of decreasing and increasing parasitemia in the culture for each concentration (from 10^{-7} mol/l to 10^{-2} mol/l) was more or less the same. This pattern of induction of drug resistance has been shown in Fig.4 (parasitemia percentage was 6-7% at the beginning of this process). The line of parasite which was successful to growth with the presence of drug, saved and pre-

served in comparison with control group (Fig. 5). Some times in this process a line of parasite was going to the death completely, so it was coming out of the study automatically (Fig. 6).

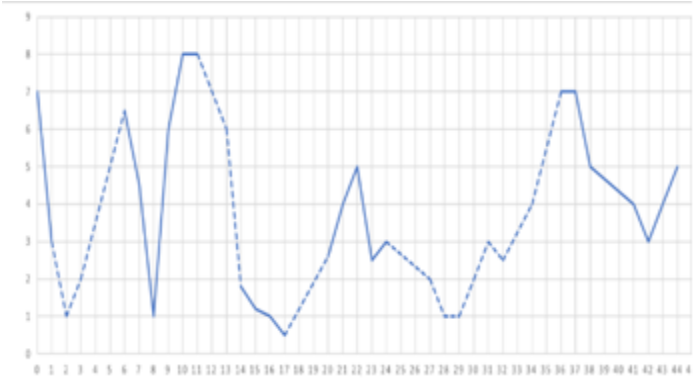

Fig. 4: Sensitivity reduction for different concentration of artesunate on P. falciparum 3D7, the test was started when parasitemia percentage was 6-7%

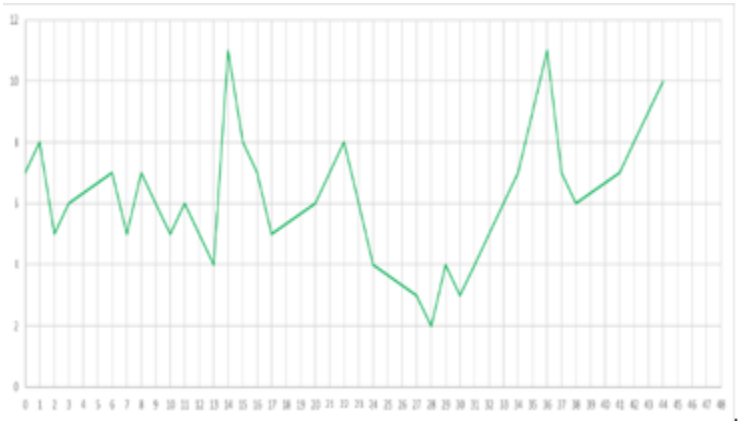

Fig. 5: Control group of *P. falciparum* 3D7, control group included only the culture medium and parasite, which was not affected by any drug

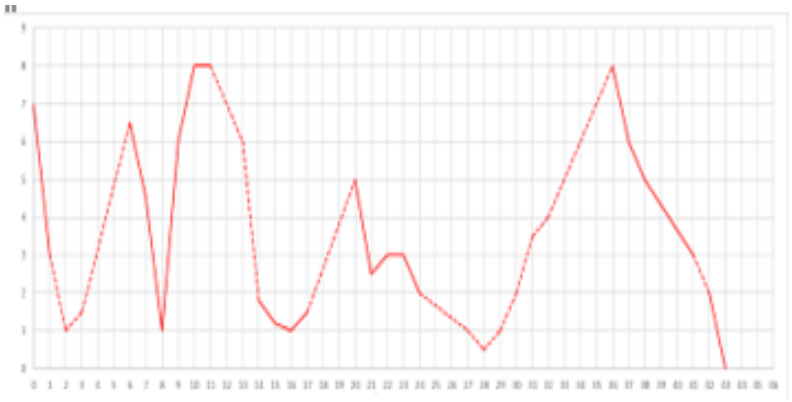

Fig. 6: Death curve of *P. falciparum* 3D7, following the exposure of artesunate

Molecular analysis

The results of gene proliferation, from *P. falciparum* 3D7 using the Nested PCR method showed a 2097bp band corresponding to *P.f kelch13* gene at the first round. Fig. 7 shows first stage amplification from seven samples including *P. falciparum* 3D7, which was sensitive to artesunate, four new strains, reduced sensitivity to artesunate with concentration of 10^{-5} mol/l, 10^{-4} mol/l, 10^{-3} mol/l, 10^{-2} mol/l, a sample from the patient who showed resistance reaction to artesunate administration and finally the Negative Control. In the second round of amplification an 849 bp band related to the middle part of *kelch13* gene was created and sequenced for molecular analysis (Fig. 8).

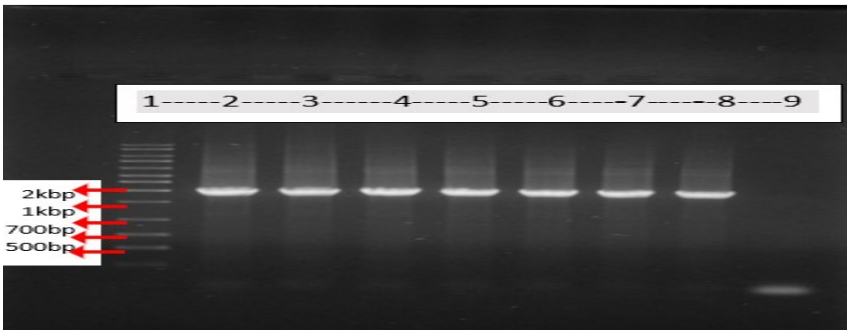

Fig. 7: Gel electrophoresis of PCR products corresponding to *P.fkelch13* gene. A 2097 bp band was yield at the first round. Samples were included: No. 1: DNA size Marker 1kb, No. 2: proliferation of *P.f kelch13* gene susceptible to artesunate drug. Nos. 3, 4, 5, 6: proliferations of *P.f kelch13* gene from four new strains reduced sensitivity to artesunate with concentration of 10^{-5}mol/l, 10^{-4} mol/l, 10^{-3} mol/l, 10^{-2}mol/l respectively. No.7: proliferation of *P.f kelch13* gene from a patient's blood sample with artesunate-resistant reaction. No. 8: Positive control. No. 9: Negative Control

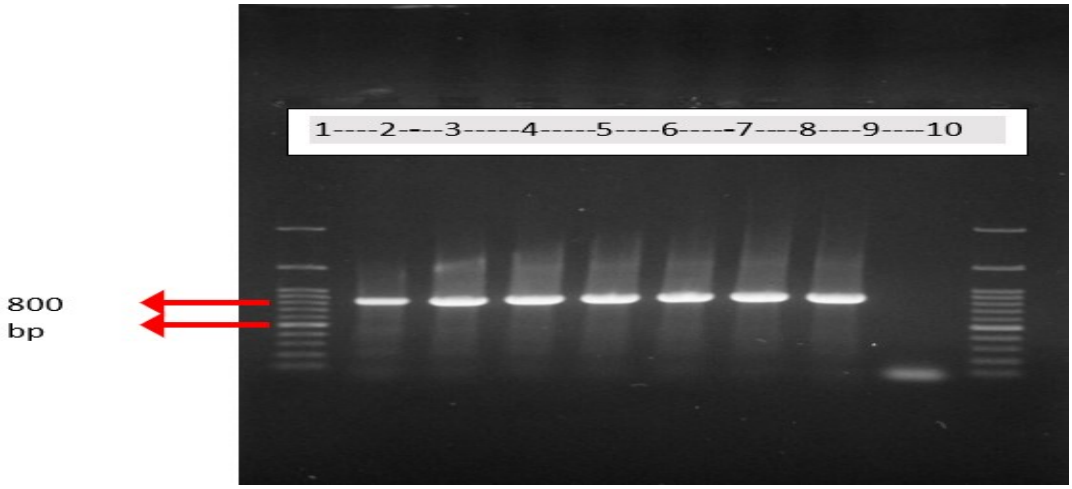

Fig. 8: amplification of PCR products from the second round: an 849 bp band related to *P.fkelch13* gene was yield on 1% agarose gel. Nos. 1 & 10: DNA marker size 100bp, No. 2: amplification of *P.fkelch13* gene from Plasmodium falciparum susceptible to artesunate. Nos. 3, 4, 5, 6: proliferations of *P.fkelch13* gene from four new strains reduced sensitivity to artesunate with concentration of 10^{-5} mol/l, 10^{-4} mol/l, 10^{-3} mol/l, 10^{-2}mol/l respectively. No.7: proliferation of *P.fkelch13* gene from a patient's blood sample with artesunate-resistant reaction. No. 8: Positive control. No. 9: Negative Control

P. fkelch13 gene sequencing

Gene sequencing was performed for all products of *P. fkelch13* gene on PCR product yield from second round, but according to the results just three of them registered in gene bank, accession numbers of MH746930 (the sensitive to drug and standard strain), MH796123 *(Pfkelch13* Ar^{-2}, which was created in this study and was resistance to artesunate 10^{-2} mol/l concentration) and MH796124 (*Pfkelch13*Ar^{-4}, a sample from the patient who was resistante to treatment with artesunate), it is important to note that the other lines which were created in resistance form to 10^{-5} mol/l, 10^{-4} mol/l, 10^{-3} mol/l were not registered due to their sequences similarity to *Pfkelch13* Ar^{-2}.

Nucleotide alignment was done for nucleotides sequence derived from *Pfkelch13* Ar^{-2}, *Pfkelch13* Ar^{-4} and sensitive strain in this study. Results showed no mutation in the relative strengthen lines and in one patient who showed resistance to artesunate administrating. Clustal multiple sequence alignment showed 100% Identity.

Discussion

To reduce the burden of malaria, correctly diagnosed and treated need to be employed (12). In this case reports about resistance to anti-malarial drugs specially artemisinin is one of the greatest fears for international attempt to control and eliminate malaria parasites. Producing experimental *P. falciparum* resistant strain against antimalarial drugs in the laboratory provides an invaluable opportunity to investigate the mechanism of parasite resistance to drugs. Moreover, these strains facilitate investigations about those substances that bears potential antimalarial activities. Previous reports suggested that mutations in the *Pfkelch13* gene could be resulted in *P. falciparum* resistance to artesunate. It is implied that this gene is the best candidate for the study of nucleotide mutations in the resistance of *P. falciparum* to artesunate (13-15), but in another report there was no mutation in the *kelch13* gene in African patients in spite of resistance to artemisinin (13).

There may be other genes related to drug resistance in *Plasmodium* species besides the Pfk13 gene. Mutations in genes such as *pfmdr1*, *pfctp* and *PfATPase6* genes can emerge in the presence of higher drug concentrations, leading to resistance in parasites (16-18). Further investigation is needed to identify the exact mechanism of resistance involving these genes. Parasites might show resistance through different methods, such as overexpressing specific genes involved in the process of inducing resistance and producing new proteins and substances. As mentioned, resistance can be induced through the cultivation of *P. falciparum* parasite and its long exposure to artemisinin or its derivatives.

In the present study, a new strain of *P. falciparum* was created under the intermittent exposing the parasite to different concentrations of artesunate. The created strain cultured under the drug pressure from the least concentration to the maximum chosen dose for one month; then preserved in liquid nitrogen for further investigation. The line of *Pfkelch13* Ar^{-2} was the resistant line for the highest concentration of the drug, which is exposed to it (10^{-2} mol/l). Sequencing of the *Pfkelch13* gene derived from produced strain indicated that there is no mutation in comparison with the original ones. This idea was confirmed when compared with the sequence of an isolated sample from an artemisinin treatment failed patient. While some previous studies reported that nucleotide changes in this gene are responsible for phenotype resistance, others indicated that other interacting molecules may responsible for this mechanism, therefore, sensitivity reduction to the drug likely occurred in the line used in this study, but in the

Pfkelch13 piece has not happened any mutation.

In a study scientists tried to produce a resistant line to dehydroartemisinin (DHA). This product showed 25-fold reduction in susceptibility to DHA. They declared that resistance of *P. falciparum* to dehydroartemisinin firstly occurred in ring stage, and then propagate to schizont and trophozoite forms. Redundant proliferation of pfmdr1 gene locus of this new clone enhances production of antioxidant and expression of a chaperone in parasite (19).

Resistance to artemisinin can occur through various mechanisms. Exposure to increasing concentrations of artemisinin for five years led to the development of a highly resistant F32-ART cell line, which showed that artemisinin induces a stop in the extension of very young parasites (ring stage) leading to a silent phase. However, artemisinin can still kill parasites in other phases of the life cycle (6, 20). Mutations in the *kelch13* gene was firstly associated with resistance to artemisinin. This gene showed a crucial and fundamental function related to ART resistance and slow parasite clearance rates in patients (21).

In another study, two resistant clones (6A-R and 11C-R) against artemisinin were produced to detect and describe molecular factors that are responsible for resistance of *P. falciparum* to artemisinin. Both the above mentioned parasite lines showed decreases drug susceptibility and derived resistance phenotype(s) were predominant in the ring stage; although both of them carried the wild-type allele of the K13 gene (22). *P. falciparum* lines have the ability to improve resistance to artemisinin derivatives in vitro due to multiple mechanisms such as amplification and increased expression of Pfmdr1 (23). The method of the present study from this point of view that the parasite is intermittently exposed to drugs, was similar to the Rocamora et al (22), the parasite could be exposed to a concentration of 10^{-2}mol/l, while sequencing was not showed any mutation in *Pf kelch13*. Rocamora et al sequenced the whole parasite genome and found 5 mutations in others than *kelch13*. The mutation changes that were found by him were related to genes that have an expressive role, such as AP2-like transcription factors, PHD finger protein, and RNA helicase. Of course, both clones produced by him had wild *kelch13* genes confirmed by PCR and genotyping. As it mentioned above, molecular studies and alignment of the *Pfkelch13* Ar^{-2} produced in the present study showed a similarity of 100% with *Kelch13* gene sequence in the susceptible 3D7 strain at the nucleotide level and no mutation was observed at this level.

There is no local report for *P. falciparum* resistance with *Pfkelch13* mutations in the study zone in this research, but there are some investigations about resistance of *P. falciparum* to chloroquine and SP in different parts of southern Iran. Investigations about wild type, different alleles and Polymorphisms in the dihydrofolate reductase (*DHFR*) and dihydropteroate synthase (*DHPS*) genes showed association between molecular markers of SP resistance and in vivo drug resistance. However, chloroquine is not currently used to treat falciparum malaria in the country and SP is not administered alone for treating *P. falciparum* (24-26).

Conclusion

Creating experimental resistance strain of *P. falciparum* parasite against antimalarial drugs in the laboratory provides an invaluable opportunity for investigation the exact mechanism of parasite resistance to antimalarial drugs. Moreover, it offers an opportunity to investigate about new potential drugs with antimalarial activates.

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Competing interest

All authors of this manuscript declare no conflict of interest.

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