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Original Article

Evaluation of Drug Resistant Genotypes to Fansidar and Chloroquine by Studying Mutation in *Pfdhfr* and *Pfmdr1* Genes in *Plasmodium falciparum* Isolates from Laghman Province, Afghanistan

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Received 09 Mar 2021 Accepted 25 Jun 2021	Abstract Background: Malaria is one of the major health problems in endemic countries like Afghanistan. Evidence has been reported about reducing the effects of chloroquine against <i>Plasmodium falciparum</i> in many endemic countries. The aim of this study was to investigate the resistance mutations in <i>pfmdr1</i> and <i>pfdhfr</i> genes of <i>P. falciparum</i> samples detected in blood samples of malaria patients in Laghman Province, Afghanistan. Methods: Samples were taken on DNA retention cards and 3 glass slides (thin and thick spread) from Laghman Province, Afghanistan in 2018. The <i>pfmdr1</i> and <i>pfdhfr</i> mutations in 30 <i>P. falciparum</i> positive samples were examined using PCR-RFLP techniques. The PCR product was then sequenced to determine the mutation at the N86Y and D1246Y mutations of the <i>pfmdr1</i> and N51, C59, I164, S108 and A16 points of <i>pfdhfr</i> genes. Results: In the <i>pfmdr1</i> gene, all samples were wild-type and no mutation was detected at point 86 and D1246Y. In the <i>pfdhfr</i> gene sequences using CLC main workbench software no mutations were detected at codons 16, 51. However, some mutation was observed at codons 59, 108 and 164. These mutations were L164I, S108N and C59R. Conclusion: Our findings provide evidence of the possible emergence of fansidar-resistant specimens in Laghman. The data of this study provide the basis for future prospective studies in other endemic areas of Afghanistan. The absence of significant mutations in <i>P. falciparum</i> samples of Laghman Province may indicate that this parasite may have switched to chloroquine re-sensitization in this area.
Keywords: Afghanistan; <i>Plasmodium falciparum</i> ; Chloroquine; Fansidar; Drug resistant	
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Introduction

According to the WHO, malaria transmission occurs in five regions of the world. It is estimated that 3.4 billion people in 92 countries are at risk for malaria and 1.1 billion are at high risk (1). Afghanistan has the third highest number of malaria cases in the world. It accounts for 11% of cases in the WHO Eastern Mediterranean region (1). In Afghanistan, 95% of malaria cases are attributed to *Plasmodium falciparum* and 5% to *P. vivax*. According to the WHO report in 2017, 91% of *P. falciparum* and 89% of *P. vivax* cases were in the six provinces of Nangahar, Laghman, Kunar, Nuristan, Khost and Paktika, of Afghanistan (2).

Chloroquine is one of the best-known anti-malarial drugs in the world and no alternative has been produced so far as it is economical, effective and of low toxicity. The first confirmed report of *falciparum* resistance to chloroquine was reported in South American countries (3). Harinasuta et al. then reported the first case of *P. falciparum* resistance to chloroquine in Southeast Asia (Thailand) (4). In 1979, the first cases of *falciparum* resistance to chloroquine was reported from the eastern regions of Tanzania and Kenya (5, 6). Nearly 30 years after the first report of chloroquine-resistant *falciparum* in 1989, the first cases of chloroquine-resistant *falciparum* were reported in Afghanistan (7).

The *P. falciparum* multidrug resistance gene 1 (*Pfmdr1*) is involved in the development of resistance to chloroquine, amodiaquine, and amino-alcohols (mefloquine, lumefantrine; *Pfmdr1*) (8, 9). "The mode of action of chloroquine and other quinoline-like drugs involves an interference with the plasmodial heme metabolism in the digestive vacuole. Some studies have suggested that point mutations in *Pfmdr1* are associated with resistance to quinoline-like drugs" (8, 9).

Various studies have been performed on the *P. falciparum* multidrug resistance protein 1

(*pfmdr1*) and *P. falciparum* dihydrofolate reductase (*pfdbhfr*) genes by different researchers (10). Their results showed that mutations in the *pfmdr1* gene (especially Tyr86 and Asp1024) were essential for the development of resistance in *P. falciparum* strains (8-17). Some studies on the prevalence of *pfmdr1* gene mutations were investigated in Chabahar, Iran (11, 12). Furthermore, three studies of polymorphisms of this gene were conducted in Pakistan (13-15). Additionally, two studies were directed on resistance *pfmdr1* gene mutations in the Afghan refugee population living in Pakistani camps and on prevalence of antifolate resistance mutations in people living in Afghanistan (16,17).

Dihydrofolate reductase (*dhfr*) is known target of fansidar (pyrimethamine and sulfadoxine), respectively (18-19). These drugs specifically inhibit the enzymes of the folate pathway to kill the parasites. Several mutations in the *P. falciparum* dihydrofolate reductase (*pfdbhfr*) gene (e.g., triple mutations at codons 51, 59 and 108 of *pfdbhfr*) has been reported associated with fansidar resistance. In addition, the *dhfr* mutation have been reported in different countries (20-22).

So far no study has been carried out on the prevalence of polymorphisms in *pfmdr1* and *pfdbhfr* genes in *P. falciparum* samples in Laghman Province, Afghanistan. The aim of this study was to investigate the resistance mutations in *pfmdr1* and *pfdbhfr* genes of *P. falciparum* samples detected in blood samples of malaria patients in that province.

Materials and Methods

Study area

Laghman is one of the eastern provinces of Afghanistan with its headquarters in Mehtarlam. The province covers an area of 843.3 square kilometers. It located in a mountainous area, the city is 772 m above sea level. The

province has five cities of Mehtarlam (center), Ali Sheng, Ali Negar, King Shah and Qargahi. The population of the province is estimated 435000 individuals (23) (Fig.1).



Fig. 1: Map of Laghman Province, Afghanistan

Sampling

Since the most *P. falciparum* cases in Laghman Province occur from July to December, this time interval was chosen for sampling in 2018. Totally, 324 blood samples were collected from people attending the Malaria and Leishmania Center in Laghman Province in Mehtarlam. All sampling was done with the full consent of the individuals and adhering to all the principles of medical ethics. Samples were taken on DNA retention cards and 3 glass slides (thin and thick spread).

Ethics approval

Ethical approval application with appropriate experimental protocols was taken from Medical Ethic Committee of Tarbiat Modares University, Tehran, Iran

(IR.TMU.REC.1395.401). Informed agreement were taken from all patients.

Molecular analysis

DNA extraction

DNA extraction was performed on DNA banking cards (DBC) (24).

Real-time polymerase chain reaction coupled with high-resolution melting analysis (qRT-PCR-HRM)

The high resolution melting analysis was performed on 18S SSrRNA gene for detection of *Plasmodium* species (25). After qRT-PCR-HRM test, 30 *P. falciparum* positive samples were selected for mutation study.

Mutation in *pfmdr1* Gene

Totally, 30 samples were screened for detecting the mutations of points 86 and 1244 of *pfmdr1* Gene. Nested PCR-RFLP and sequencing method were used to evaluate mutation at point 86 (26).

Pfdhfr gene mutations

The nested PCR and sequencing methods were used to investigate mutations of *pfdhfr* gene. Thus, on 30 positive *P. falciparum* samples, *pfdhfr* gene mutations (codons 16, 51, 59, 108, 164) were evaluated by a nested PCR method. In the first round, Forward primer1 and Reverse primer1 primers were amplified for the 635bp fragment and then in the second round, Forward primer2 and Reverse primer2 primers were used to amplify the 616 bp fragment. The primers used are shown in Table 1.

Table 1: List of primers used to determine *Pfdhfr* gene mutations

Variable	Sequence (5'->3')	Length	Tm	GC%	MW
Forward primer1	TCCTTTTATGATGGAACAAG	21	52.01	33.33	6435
Reverse primer1	AGTATATACATCGCTAACAGA	21	52.01	38.10	6422
Forward primer2	TTTATGATGGAACAAGTCTGC	21	53.97	27.27	6460
Reverse primer2	ACTCATTTTCATTTATTTCTGG	21	50.94	33.33	6656

PCR program for 635 bp fragment was done, including initial denaturation at 95 °C for 5 min, then 35 denaturation cycles at 95 °C for 30 s, annealing at 52 °C for 30 s, and elongation at 72 °C for 30 s. Final elongation was also performed for 10 minutes at 72°C. In the second round to amplify the 616-bp fragment, initial denaturation steps at 95 °C for 5 min, then 35 denaturation cycles at 95 °C for 30s, annealing at 51 °C for 30 s, and elongation at 72 °C for 72 s. 30 seconds. Final elongation was also performed for 10 min at 72 °C. After electrophoresis and band observation, PCR product of positive samples was sequenced.

Comparison of sequences determined with reference sequences in different regions was performed using CLC main workbench software.

Results

Out of 347 samples collected from Laghman Province by real-time PCR-HRM 71.75%, 22.76% and 5.47%, were infected with *P. vivax*,

P. falciparum and mixed infection with both types of species respectively.

Pfmdr1 gene mutation

In the N86Y mutation of the *pfmdr1* gene, analyzed by sequencing, all 30 samples were wild-type and no mutation was detected at point 86 (Fig. 2).

In the D1246Y mutation study, the RFLP assay did not detect any mutation at the 1212 point and all samples were found to be wild. No enzymatic digestion was found in any of the samples (Fig. 3).

In the N86Y and D1246Y mutations of the *pfmdr1* gene, analyzed by sequencing, all samples were wild-type and no mutation was detected at point 86 and D1246Y.

The PCR product was then sequenced to determine the mutation at N51, C59, I164, S108 and A16 points. In the *pfdhfr* gene sequences, using CLC main work bench software did not detect any mutations at codons 16, 51. However, some mutation was observed at codons 59, 108 and 164. These mutations are L164I, S108N and C59R.

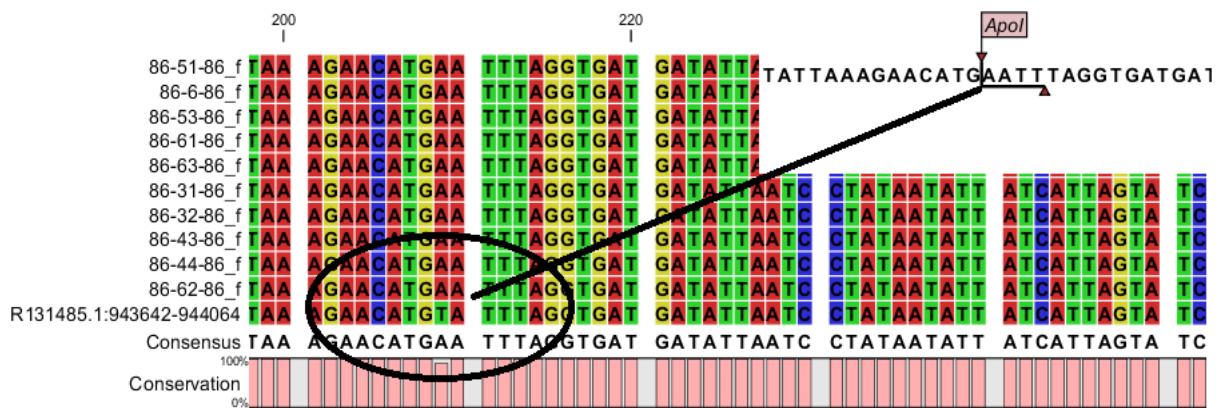


Fig. 2: Comparison of the sequences of the present samples with the mutation reference sequence of the 86 point mutation in the *pfmdr1* gene using CLC main work bench software. As can be seen in all the samples studied (rows 1-10), the enzyme is able to cut the sequence of amplified sequences (the ApoI enzyme is able to cut the wild type). However, the reference sample (row 11 or R131485.1... ..) is mutant

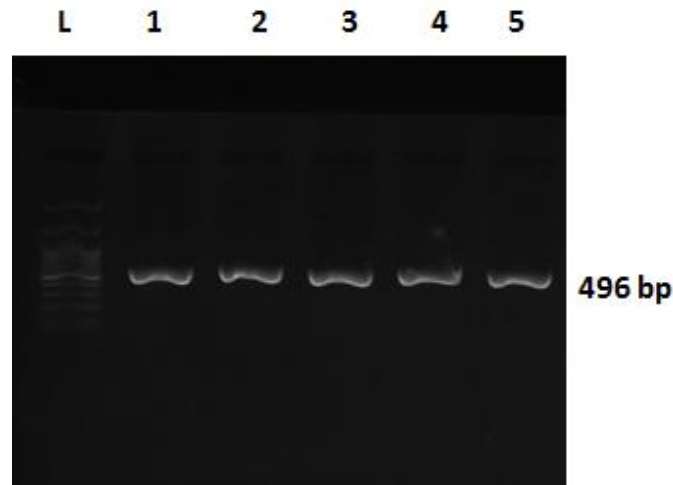


Fig. 3: A band 496bp after enzymatic digestion with EcoRV for 1246 point mutation in the *pfmdr1* gene (wild-type samples do not cut by this enzyme). L: Ladder; 1-5 samples

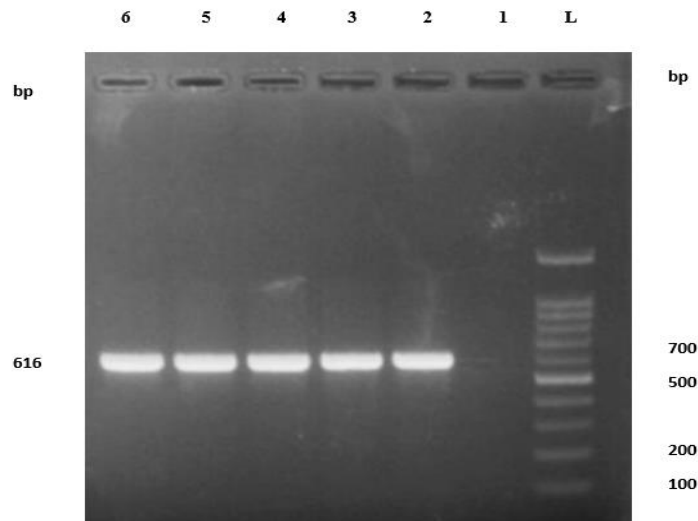


Fig. 4: The 616-bp *pfdhfr* gene banding of *P. falciparum* in Nested PCR. Line L: marker, 1: negative control, 2: positive control, 3-6 samples studied

Pfdhfr gene mutations

To investigate mutations in this gene, the gene was amplified by Nested PCR in two steps. In the first stage, the band 635 and in the second and final stage the band 616 was obtained (Fig. 4). The PCR product was then sequenced to determine the mutation at N51, C59, I164, S108 and A16 points. In the *pfdhfr* gene sequences, using CLC main workbench

software did not detect any mutations at codons 16, 51. Nevertheless, some mutation was observed at codons 59, 108 and 164. These mutations are L164I, S108N and C59R (Fig. 5).

At codon 59, TGT cysteine is changed to CGT arginine, at codon 108, AGC serine to AAC aspartate, at codon 164, TTA leucine to ATA isoleucine.

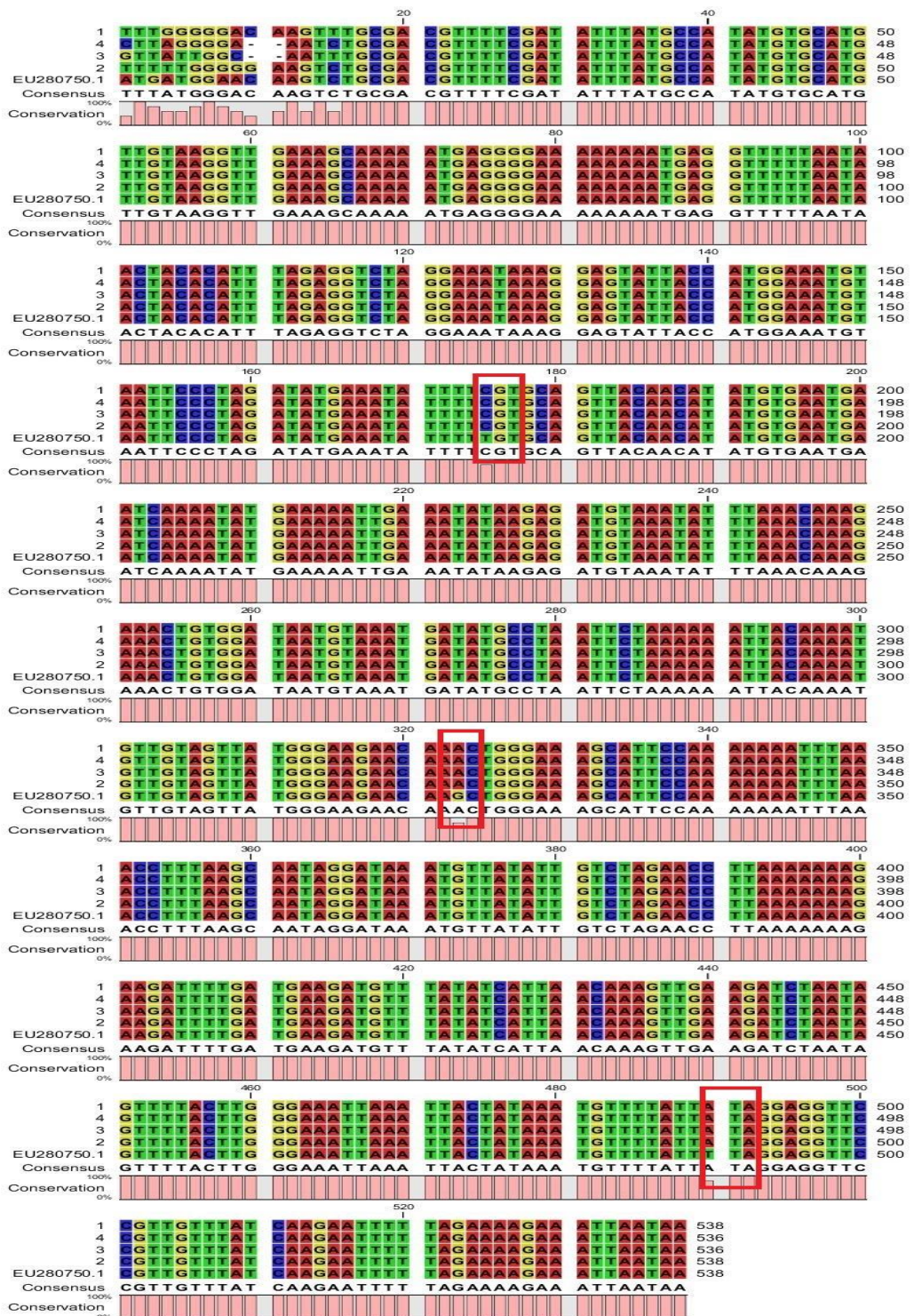


Fig. 5: Comparison of the sequences of the present study with the reference sample. The L164I, S108N and C59R mutations can be seen using CLC main workbench software. Table 2 shows the number of genotypes of *P.falciparum* in Laghman province with regard to *pfdmr1* and *Pfdhfr* gene mutations

Table 2: Number of genotypes of *P.falciparum* in Laghman province with regard to *pfmdr1* and *Pfdhfr* gene mutations

Gene	Mutation	Genotype No.		
		Mutant	Wild	Mix
<i>Pfmdr1</i>	N86Y	0	30	0
	D1246Y	0	30	0
	A16	0	30	0
<i>Pfdhfr</i>	N51	0	30	0
	C59	1	29	0
	S108	1	29	0
	I164	1	29	0

Discussion

P. falciparum is usually flexible in its genome. The parasite to survive can remove a gene or part of a gene that is not essential for growth. It is capable of altering its genes at different stages of the life cycle, as well as in adverse environmental conditions (27). The half-life of new drugs can be increased if combined with other drugs, so the focus of multi-drug resistant malaria management programs has been on combination therapy. The advantages and disadvantages of the proposed drug regimens should be carefully examined in each specific endemic area. Today, drug selection can shape future drug strategies. The ability to prevent epidemics and control malaria-related mortality depends on the choice of appropriate drug.

In the past decades, significant advances have been made in identifying the molecular basis of drug resistance in *P. falciparum*. The focus of studies on drug resistance was to identify mutations in the two *pfmdr1* and *pfcr1* genes, which are specifically related to chloroquine resistance. In addition, the polymorphisms of these two genes are also associated with susceptibility to halofantrine, quinine, and artemisinin (28). The *P. falciparum* multi-drug resistance protein 1 (*pfmdr1*) N86Y mutation has been linked to chloroquine and amodiaquine resistance (29). However, point mutations in certain genes of *P. falciparum* nec-

essarily exist to reveal the drug resistance phenotype (29).

By promoting information about antimalarial drug resistance, newer and better molecular methods can be designed to detect drug resistance early. In addition, this information is used to limit the spread of resistance to other antimalarial drugs and to prevent the development of new drug-resistant foci (28). In the present study, of the 30 samples taken, no Y86N mutation was detected in the *pfmdr1* gene. In a study, among 200 samples of Iranian isolates collected from southeastern Iran during 2002–2005, a low rate of the mutant allele 86Y (44%) of the *pfmdr1* gene were detected (30). However, a study of *pfmdr1* gene mutations alone is not sufficient and simultaneous study of *pfcr1* and *pfmdr1* gene mutations usually yields better results. The Y86N mutation increases susceptibility to mefloquine and artemisinin and this should be considered in endemic areas of infection in drug therapy (31). In general, in these countries, these mutations, based on their prevalence and their association with drug resistance, can be used as differential markers for the detection of chloroquine resistant cases in sensitive cases. In *P. falciparum* isolates collected from Malaysia, Indonesia, Guinea, Nigeria and Sub-Saharan Africa, the Y86N mutation has been reported in chloroquine resistant samples. High prevalence of this mutation in Cambodian isolates has also been reported (32). In

Thailand, the Y86N and C1034S mutations of the *pfmdr1* gene have been reported at high significant levels (33).

The emergence and disappearance of *pfmdr1* mutant alleles are slower and shorter than *pfcr1* mutant alleles. Absence of significant mutations in the *pfmdr1* gene in samples taken from Laghman Province indicates that *P. falciparum* may have been susceptible to chloroquine after about thirty years. The resistance of *P. falciparum* to chloroquine gradually decreased over the time due to the eliminating the drug from the control program and have resulted in the recovery of chloroquine efficacy (34, 35). The same mechanism happened in Laghman province. According to our study, no mutation was observed in *pfmdr1* and *pfcr1* genes of *P. falciparum* but in *pfdhfr* gene related to fansidar the mutation observe. This means that the sensitivity of *P. falciparum* is recovered again.

The drug fansidar, has been used as an antimalarial chemotherapy in Southeast Asia and Africa. Mutations in the *P. falciparum* dihydrofolate reductase (*pfdhfr*) and *P. falciparum* dihydropteroate synthase (*pfdhps*) genes are concomitant with fansidar resistance (20). In the present work, only the *pfdhfr* gene was studied, and no mutation was detected at codons 16, 51 of the gene. However, some mutation was observed at codons 59, 108 and 164. These mutations are named L164I, S108N and C59R. In population studies, mutations at codon 59 of the *pfdhfr* gene could strongly predict resistance to fansidar resistance (21, 36). The prevalence of mutation in *pfdhfr* in Laisa and Paletwa towns in Myanmar was 100% and 83.3%, respectively, which was slightly lower than Banmauk town, indicating a strong selection pressure against fansidar in the western and northeastern Myanmar (22). Similarly, triple mutations at codons 108, 51, and 59 of *pfdhfr* is reported associated with fansidar treatment failure (20). Like in Banmauk (Myanmar), only one isolate had this quintuple mutation combination in, suggesting that *P. falciparum* from these asymptomatic carriers would be highly resistant to fansidar. The presence of the 164L

mutation has been detected in combination with the triple 51/59/108 mutant of *pfdhfr*, although at low frequency (1.4%) of *P. falciparum* related to resistance to fansidar in the Bata District (Equatorial Guinea) (37). The combined *pfdhfr* 51/59/108/164 mutation is common in South America and East Africa, related to high fansidar resistance (38).

Given the use of fansidar as an antimalarial drug in Afghanistan and the potency of this mutation, an effective control system is needed to prevent its spread throughout the country.

Conclusion

The findings of this study highlights evidence of the possible emergence of fansidar-resistant specimens, in Laghman, although further prospective studies such as studies on the *pfdhps* gene as well as their association with clinical conditions are needed. The absence of significant mutations in *P. falciparum* samples of Laghman Province may indicate that this parasite may have switched to chloroquine re-sensitization in this area. The data of this study provide the basis for future prospective studies in other endemic areas of Afghanistan. Certainly, more research is needed to obtain results that are more reliable by combining the results of molecular analysis of mutations and in vitro and clinical observations.

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Conflict of interest

The authors declare no conflict of interest, financial or otherwise.

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