REVISTA DO INSTITUTO DE MEDICINA TROPICAL DE SÃO PAULO

JOURNAL OF THE SÃO PAULO INSTITUTE OF TROPICAL MEDICINE

ORIGINAL ARTICLE

http://doi.org/10.1590/S1678-9946202163059

Surveillance of molecular markers of antimalarial drug resistance in *Plasmodium falciparum* and *Plasmodium vivax* in Federally Administered Tribal Area (FATA), Pakistan

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Received: 30 April 2021

Accepted: 16 June 2021

ABSTRACT

This molecular epidemiological study was designed to determine the antimalarial drug resistance pattern, and the genetic diversity of malaria isolates collected from a war-altered Federally Administered Tribal Area (FATA), in Pakistan. Clinical isolates were collected from Bajaur, Mohmand, Khyber, Orakzai and Kurram agencies of FATA region between May 2017 and May 2018, and they underwent DNA extraction and amplification. The investigation of gene polymorphisms in drug resistance genes (*dhfr*, *dhps, crt,* and *mdr1*) of *Plasmodium falciparum* and *Plasmodium vivax* was carried out by pyrosequencing and Sanger sequencing, respectively. Out of 679 PCR-confirmed malaria samples, 523 (77%) were *P. vivax*, 121 (18%) *P. falciparum,* and 35 (5%) had mixed-species infections. All *P. falciparum* isolates had *pfdhfr* double mutants (C59R+S108N), while *pfdhfr/pfdhps* triple mutants (C59R+S108N+A437G) were detected in 11.5% of the samples. About 97.4% of *P. falciparum* isolates contained *pfcrt* K76T mutation, while *pfmdr1* N86Y and Y184F mutations were present in 18.2% and 10.2% of the samples. *P. vivax pvdhfr* S58R mutation was present in 24.9% of isolates and the S117N mutation in 36.2%, while no mutation in the *pvdhps* gene was found. *Pvmdr1* F1076L mutation was found in nearly all samples, as it was observed in 98.9% of isolates. No significant anti-folate and chloroquine resistance was observed in *P. vivax;* however, mutations associated with antifolate-resistance were found, and the chloroquine-resistant gene has been observed in 100% of *P. falciparum* isolates. Chloroquine and sulphadoxine-pyrimethamine resistance were found to be high in *P. falciparum* and low in *P. vivax.* Chloroquine could still be used for *P. vivax* infection but need to be tested *in vivo,* whereas a replacement of the artemisinin combination therapy for *P. falciparum* appears to be justified.

KEYWORDS: Malaria. FATA. *Plasmodium falciparum*. *Plasmodium vivax*. Antimalarial drug resistance. Pakistan.

INTRODUCTION

Malaria is still a public health concern despite tremendous efforts to control and eradicate malaria. About 229 million malaria cases were reported by the World Health Organization (WHO) in 2020, with 0.41 million deaths globally[1]. About 205 million people in Pakistan live in the malaria-endemic areas, and 0.3 million confirmed cases are reported annually. According to the Malarial Control Program, *Plasmodium vivax* corresponds to 84% of all malarial infections, the proportion of *Plasmodium falciparum* is 15%, and mixed-species infections are around 1%

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(*P. falciparum* and *P. vivax*). A Federally Administered Tribal Area (FATA) of Pakistan has the second-highest malaria incidence (23%) in the country, after the Khyber Pakhtunkhwa (KPK) province (31%)[2]. Political instability and war against terrorism during the last two decades have destroyed the health care system and its infrastructure in FATA. Other factors like militancy, Talibanization, Afghan refugees, migration of internally displaced people, and poor health system setup have ruined all efforts made by malaria control programs in this remote, neglected and malaria-endemic region[3].

Chloroquine resistance (CQR) has first appeared in Thailand in 1957, then spread throughout South and Southeast Asia, eventually appearing in Sub-Saharan Africa and South America by the 1970s[4]. CQR in *P. falciparum* was initially documented in Pakistan in the early 1980s, and according to a 1997 survey, CQR in the Pakistani population ranged from 18% to 62%[5]. According to a study conducted in Punjab province, 34% of CQR was found in five districts[6]. In 2007, the national treatment guidelines for *P. falciparum* were released, contraindicating the use of CQ and encouraging the use of artemisinin with sulfadoxine-pyrimethamine (SP). the artemisinin-based combination therapy (ACT)[1]. SP had been used as a monotherapy to treat malaria infections, and it had been also used as a partner drug to ACT before 2018. However, according to the WHO guidelines, artemether-lumefantrine plus primaquine is now the first-line treatment for uncomplicated confirmed *P. falciparum* malaria, while artesunate is indicated for the treatment of severe malaria in Pakistan[1].

Single Nucleotide Polymorphisms (SNPs) in *P. falciparum pfcrt* and *pfmdr1* genes are well documented molecular markers related to the *in vitro* CQR. Polymorphism at codon K76T of the *pfcrt*[7] gene, and N86Y, Y184F, S1034C, N1042D and D1246Y in the *pfmdr1* gene confer CQR[8]. SP partner drug of ACT targets the *P. falciparum* folate metabolism pathway in which two enzymes act: dihydrofolate reductase (PDHFR) and dihydropteroate synthase (DHPS)[9], that have been used as molecular related to the *in vitro* drug susceptibility[10]. In 2018, WHO recommended the replacement of the ACT partner drug from SP by lumefantrine due to the development of *P. falciparum* resistance to SP in Pakistan[11]. Polymorphism in the *pfdhdr* gene at C50R, N51I, C59R, S108N, I164L codons as well as at S436A, A437G, K540E, A581G,A613T/S codons in *pfdhps* genes of *P. falciparum* have been associated with reduced SP susceptibility. Molecular markers of drug resistance against SP have been reported in a few studies on blood samples representing different Pakistan provinces except for the Federally Administered Tribal Area (FATA) in Pakistan[12,13]. In *in vitro/in vivo* investigations have revealed the artemisinin resistance along the Thailand-Cambodia border, in Vietnam, Myanmar and other Southeast Asian countries[14] but it is still an effective antimalarial drug in Pakistan[12,15].

In Southeast Asia, malaria due to *P. vivax* is prevalent, and 80% of the malaria burden come from India, Indonesiaand Pakistan[1]. The CQR mutation has been found in 100% of samples with *P. falciparum;* however, chloroquine plus primaquine is the first-line treatment for malaria due to *P. vivax* in Pakistan. SNPs in the *pvmdr1* gene have been associated with CQR in *P. vivax,* and two mutations at position Y976F and F1076L have been linked to chloroquine amodiaquine and 4-aminoquinolones resistance in *P. vivax*[16]. Resistance *in P. vivax* against SP has been associated with mutations in dihydrofolate reductase (*pvdhfr*) and dihydropteroate synthetase (*pvdhps*) genes. *Pvdhfr* mutations I13L, P33L, F57L/I, S58R, T61M, S117N/T, I173L/F, and *pvdhps* mutations S382A/C, A383G, K512M/T/E, and A553G/C genes have been linked to reduced susceptibility to pyrimethamine and sulfadoxine, respectively[17].

In Pakistan, previous molecular antimalarial surveillance studies focused on different regions of Pakistan and FATA were neglected due to military operations and the war against terrorism. In this study, molecular markers associated with antimalarial drug resistance were investigated in *P. falciparum* and *P. vivax* clinical isolates collected from five FATA agencies/districts. Samples collected in 2017-2018 provided information regarding the prevalence of antimalarial resistant genotypes in the two predominant *Plasmodium* species: *P. falciparum* (*pfdhfr, pfdhps, pfcrt,* and *pfmdr1*) and *P. vivax* (*pvdhfr, pvdhps* and *pvmdr1*). The study findings will contribute to a better understanding on the patterns of molecular drug resistance markers in *P. falciparum/vivax* in this region.

MATERIALS AND METHODS

Study site and patient enrollment

This study was carried out in the FATA agency, and in May 2018, this region was merged with the KPK province. FATA has unique geographical importance as it shares its border with Afghanistan to the West, KPK to the Northeast, and Balochistan to the South. It is located at latitude 34° 28' 24.59" North and Longitude 71° 17' 16.20" East, stretching for a maximum length of about 450 kilometers. Blood samples from malaria suspect cases were collected by the basic health units and different private health units from five agencies/districts (Bajaur, Mohmand, Khyber, Orakzai, and Kurram Agency) of FATA (Figure 1), KPK between May 2017 to May 2018. After explaining the study's purpose, informed verbal consent was taken from all patients or their

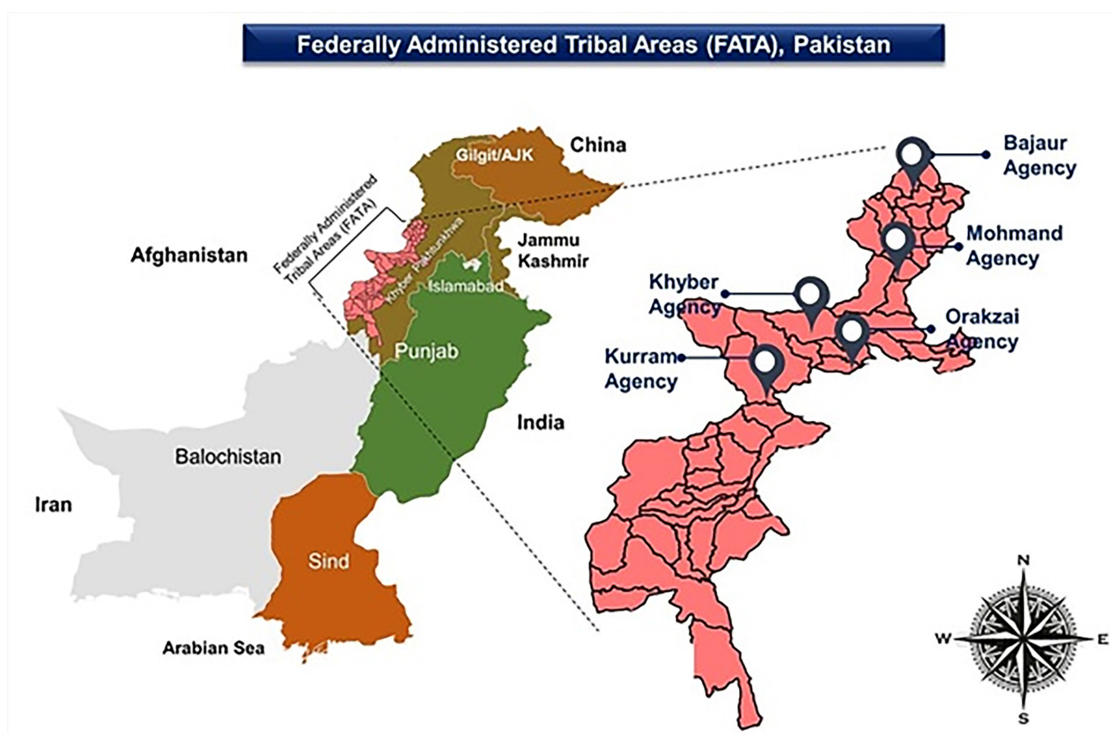

Figure 1 - Geographical map of Pakistan indicating the provinces and the FATA region.

legal guardians in the case of patients under the age of 18. The verbal consent was taken because it is a war-affected area, and the study population was mainly illiterate and the study would be of minimal risk to the study participants. All the questions raised by the participants were answered, and they were given time to decide if they wanted to participate. This consenting process was applied in the presence of an impartial witness. Ethical approval for this study was obtained from the ethical committee of the Department of Biochemistry & Biotechnology, University of Gujrat, Gujrat, Punjab, Pakistan.

Laboratory methodology

A total of 762 malaria-positive blood samples, diagnosed by microscopy, were collected regardless of age and gender. About 3 mL of peripheral blood were drawn in EDTA tubes, and 50 µL of whole blood were spotted on Whatman filter paper, dried, and stored at room temperature, and EDTA blood samples were stored at -80 °C. Malarial DNA was extracted from the dried blood spots using the QIAmp 96 DNA kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

Plasmodium DNA amplification by nested PCR

Nested PCR was used to genotype the 18S rRNA *Plasmodium* gene with genus-specific and species-specific primers (*P. falciparum*, *P. vivax, P. malariae* and *P. ovale*) as previously described[18]. In addition, the PCR master mix, primer sequences, and thermal cycler conditions followed previously used protocols[19]. Nested PCR amplification products were electrophoresed on 2% agarose gels, along with a molecular ladder (Thermo Fisher Scientific, Waltham, MA, USA).

PCR amplification for pyrosequencing (*pfdhfr*, *pfdhps*, *pfcrt*, and *pfmdr1*)

Screening for polymorphism linked to anti-folate resistance, present at codons 50, 51, 59, 108 164 of the *pfdhfr* gene and 436, 437, 540,581, 613 of the *pfdhps* gene in *P. falciparum* was performed by pyrosequencing using primers from a previously reported protocol[20]. The CQR molecular marker *pfcrt* codon K76T containing the gene fragment and N86Y and Y184F of the *pfmdr1* gene were amplified using the Khattak *et al.*[11] protocol. PCR reagents and thermal cycler conditions were adopted from a previously described protocol with slight modifications. Briefly, in primary PCR reaction, 25 µL reaction volume was used having 2 µL of template DNA, 2x PCR buffer, 0.2 mM dNTPs, 1.0 mM magnesium chloride, 0.4 µM primer (forward and reverse), and 0.04 U/µL Taq DNA polymerase (Promega, Madison, WI, USA). Then, about 2 µL of the first PCR product were used as the template DNA for the nested PCR, 1× PCR buffer, 0.3 mM dNTPs,

1.0 mM magnesium chloride, 0.4 µM both primers, and 0.04 U/µL Taq DNA polymerase, in a total volume of 50 µL. Primers and thermal cycler conditions followed previous protocols[11,12]. PCR was performed using a BioRad thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA), and the amplification products were visualized on 2% ethidium bromide-stained agarose gels under UV lights. All samples were processed for pyrosequencing at the University of Maryland, School of Medicine facility in Baltimore, USA.

Pyrosequencing reaction

To investigate the mutations present at all codons of interest, pyrosequencing was carried on PyroMark® Q96 MD Pyrosequencer (Qiagen, Valencia, CA, USA) using a published protocol[20]. Depending on the intensity/quantity of the amplification product, 3 to 6 µL of the second PCR product or control samples (wild and mutant samples for each codon) were used in each pyrosequencing reaction. PyroMark® software of Pyrosequencer Q96 MD version 1.2 Qiagen (Biotage AB, Uppsala, Sweden) was used to achieve an allele quantification mode (AQ) for all SNPs. However, *pfcrt* (codons 72–76) and *pfmdr1* (codons N86Y and Y184F) were analyzed using the sequence analysis mode (SQA). After adjusting each allele cut-off value against the control DNA using a standard curve, allele frequencies greater than 10% were considered.

Sequencing of *P. vivax* genes (*pvdhps*, *pvdhfr*, and *pvmdr1*)

A subset of 75 *P. vivax* samples from five FATA agencies/districts (study sites) were amplified targeting *pvdhps* (767 bp), *pvdhfr* (632 bp), and *pvmdr1* (547 bp) genes harboring mutations associated with CQR and SP resistance. Using previously described PCR master mix reagents protocols and cycling conditions, the first and second amplifications were carried out to amplify *pvdhps, pvdhfr,* and *pvmdr1* genes of *P. vivax* using blood samples and control samples (positive and negative)[21]. The first and second round PCRs master mix concentrations and thermal cycler conditions were the same for all three genes. Purified DNA samples were sent to the Sanger Sequencing Service at Macrogen Korea. All electropherograms were first visualized, then assembled, and finally analyzed by the Sequencher 4.1.10 (Gene Codes Corporation, Ann Arbor, MI, USA). Wild type genes *P. vivax* ARI/Pakistan isolated (GenBank accession Nº X98123), Sal-1 strain (GenBank accession no AY618622), and a Brazilian clinical isolate (GenBank accession Nº AY186730) were used for *pvdhfr, pvdhps,* and *pvmdr1* genes as reference sequences.

RESULTS

Out of 762 malaria- positive blood samples by microscopy, 679 samples were positive for *Plasmodium* DNA by PCR. From this total of samples, 523 (77%) were positive for *P. vivax*, 121 (18%) were positive for *P. falciparum,* and 35 (5%) were found to harbor mixed infections. Among patients who tested positive for PCR, 468 (68%) were male, and 211 (32%) were female; the male to female ratio was 2.21. The patients' ages ranged from 6 months to 70 years, with a median of 22 years.

Polymorphisms in *pfdhfr*, *pfdhps*, *pfmdr1* and *pfcrt* genes of *Plasmodium falciparum*

Pyrosequencing was used to detect SNPs in the *pfdhfr, pfdhps, pfmdr1* and *pfcrt* genes in 156 malaria-positive, PCR-confirmed isolates [121 *P. falciparum* mono-infections and 35 infections with mixed species (*P. falciparum* plus *P. vivax*)]. All blood samples harbored C59R and S108N mutations; consequently, *pfdhfr* double mutants (C59R + S108N) were 156/156 (100%), indicating a complete predominance of this haplotype in the study population. No mutation was observed at position I164L of the *pfdhfr* gene. However, the double mutant C50R + N51I was found in 17/156 (10.9%) of the isolates, and no triple *pfdhfr* (N51I + C59R + S108N) mutant was observed. In the *pfdhps* gene, S436A/F and A437G mutations were found in 16/156 (10.3%) and 24/156 (15.1%) of the isolates. All *P. falciparum* blood samples were wild-type at codon K540E, A581G and A613T/S. The prevalence of a combination of triple *dhfr/dhps* (C59R+S108N+A437G) mutant was 18/156 (11.5%) (Table 1). *Pfmdr1* polymorphism data showed that the frequency of N86Y and Y184F was 29/156 (18.2%) and 16/156 (10.2%), respectively. The

Table 1 - Frequency of genotype at various sites and SNPs in *Plasmodium falciparum pfdhfr, pfdhps, pfmdr1* and *pfcrt genes.* Number of samples = 156.

Gene	Mutation/Haplotype	Samples No. (%)
pfdhfr	C59R	156 (100)
	S108N	156 (100)
	C50R+ N51I	17 (10.9)
	C59R + S108N	156 (100)
pfdhps	S436A/F	15 (10)
	A437G	24 (15)
pfdhfr + pfdhps	59R + 108N + 437G	18 (11.5)
pfmdr1	N86Y	29 (18.6)
	Y184F	16 (10.3)
pfcrt	K76T	152 (97.4)

Polymorphism analysis of the *pfcrt* gene fragment showed 152/156 (97.4%) K76T mutations.

Polymorphisms in *Plasmodium vivax pvdhfr*, *pvdhps* and *pvmdr1* genes

Amplification and sequencing of the *pvdhfr* gene revealed that about 27/75 (36.2%) of *P. vivax* isolates harbored the S117N mutation while the incidence of the S58R single mutant was about 19/75 (25%). A double *pvdhfr* (S58R+S117N) mutant was observed in 17/71 (23.9%) of *P. vivax* blood samples (Table 2). Concerning the pvdhfr wild type gene sequence (*P. vivax* ARI/Pakistan isolate GenBank accession no: X98123), the 18-bp double insertions (insertion I and II) and one 18 bp deletion were observed. Insertion I was located between the 91-92 codons, insertion II was between 102-104 codons, and deletions were between 92-97 codons after local alignment with the wild type *pvdhfr* gene sequence. The tandem repeat region of the *P. vivax pvdhfr* gene was aligned between amino acids 84 and 106 (Figure 2). Insertion II was the most common, accounting for 8/75 (10.6%), followed by deletions, which were observed in 5/75 (6.6%) isolates, and insertion I was observed in 3/75 (4%) isolates.

Table 2 - Frequency of genotype at various sites and SNPs in *Plasmodium vivax pvdhfr, pvdhps* and *pvmdr1* genes. Number of samples = 75.

Gene	Haplotype	No. samples and (percentage)
pvdhfr	IPFSTN I	27 (36)
	IPFR TN I	19 (25.3)
pvdhps	SAKAV (WT)	73 (97.3)
	SG KAV	2 (2.1)
pvdhfr + pvdhps	**IPFSTSISAKAV (WT)**	29 (38.7)
	IPFSTN ISAKAV	26 (35.5)
	IPFR TN ISAKAV	17 (23.8)
	IPFSTN ISG KAV	1 (1.7)
pvmdr1	YF (WT)	1 (1.1)
	YL	75 (98.9)

Pvdhfr = wild type haplotype (IPFSTSI) I13L, P33L, F57L/I, S58R, T61M, S117N/T and I173L/F; ***Pvdhps*** = wild type haplotype (SAKAV) S382, A383, K512, A553 and V585; ***pvmdr1*** = wild type haplotype (YF) Y976F and F1076L.

The majority of samples sequenced to investigate point mutations associated with the sulphadoxine resistant gene (pvdhps) were wild-type reference sequences (Brazilian clinical isolate accession no: AY186730). Only 2.1% of samples carrying the A383G mutation; however, none of the isolates presented with mutations at codon A553G in the *pvdhps* gene. The incidence of *pvdhfr* + *pvdhps* double mutants (S117N+A383G) was observed in 1.7% of specimens collected from the FATA region.

P. vivax CQR-associated mutations, Y976 and F1076, were screened after sequencing the *pvmdr1* gene region between codons 931 and 1095 (Reference Sal-1 Gen-Bank accession Nº AY618622). The amplified gene fragments indicated 98.9% of mutations at codons F1076L and only 1.1% of samples harboring the Y976F mutation.

DISCUSSION

Malaria infection, the country's second most commonly reported disease, affects 205 million Pakistani. Molecular analysis of SP resistance-associated marker gene (*pfdhfr*) in *P. falciparum* showed 100% prevalence of the double *pfdhfr* (C59R/S108N) mutant, indicating a complete predominance of this haplotype. Two previous studies from the FATA region reported 96% and 97.6% prevalence of the double *pfdhfr* mutant[12,22]. In addition, a high incidence of the *pfdhfr* double mutant has also been reported in different Pakistan cities[12,13].

In the present study, the A437G mutation was observed in 15% of the isolates. Similar results were obtained when samples collected from the FATA Khyber Agency, reporting a 10.9% prevalence of the A437G mutation[12]. In this study, 12% of *P. falciparum* isolates harbored *pfdhfr* + *pfdhps* triple (C59R + S108N and A437G) mutation. A survey conducted in the FATA region has reported a 9.8% prevalence of triple mutants, and it was comparatively lower than in the Balochistan province (83.3%)[12]. A study published in 2013 reported a 51% prevalence of *pfdhfr*+

Amino Acid Position	84													98					103			106
Insertion I	S	Q	G	G	G	D	N	T	S	G	G	D	N	T	H	G	G	D	N	A	D	K
Insertion II	S	Q	G	G	G	D	N	T	S	G	G	D	N	T	S	G	G	D	N	A	D	K
Deletion	S	Q	G	G	G	D	N	T	S	G	G	D	N	*	*	*	*	*	*	A	D	K

Figure 2 - Alignment of *P. vivax pvdhfr* gene sequences, focusing the tandem repeat region between amino acids 84 and 106. The pink color indicates the tandem repeat, and the asterisk (*) in green colored boxes represent the tandem repeat deletion between positions 98 and 103.

pfdhps triple mutation in all four Pakistan provinces, but samples from FATA were not included[11]. The decrease in the incidence of the *pfdhfr* + *pfdhps* triple mutant haplotype was also observed in Iran, decreasingfrom 53% to 38% between 2008 and 2010 after exchanging CQ and SP monotherapy for AS+SP[23]. No polymorphism was observed in *pfdhps*, codons K540E, A581G, and A613S/T while a study reported 4% and 3% of polymorphisms at K540E and A581G codons in Pakistan[21]. The highly-resistant quintuple mutant [combination of the *pfdhfr* triple mutant (51I+59R+108N) and the *pfdhps* double mutant (437G+540E)] associated with SP treatment failure[24] was not reported in this study or in earlier reports from the FATA region in Pakistan[12].

Analysis of mutations in the *pfmdr1* gene showed that 18% of isolates had a mutation at codon N86Y, and 10% of isolates were mutated at codon Y184F; this findings contrasts with the ones from studies from Pakistan in which 25% of samples included mixed mutations (if both the wild-type and mutant alleles were detected)[11]. In Asia and Africa, the association of CQR with the N86Y codon mutation is not clear. There are still gaps to increase the understanding on the association of SNPs in *pfmdr1* gene with CQR[25]. Our results showed that *P. falciparum* CQ resistant K76T *pfcrt* mutation has completely predominated or is near the 100% frequency as this mutation was observed in 97% of isolates collected by five FATA agencies. In 2009, a study of samples gathered from the Bannu area, which is near the FATA zone, found that 100% of samples had the *pfcrt* K76T mutation[13]. Similarly, a study conducted in Pakistan in 2013 found the same results[8]. A survey of clinical isolates collected from all over Pakistan in 2018 found that 98.3% of the country had the CQR[15], and comparable results have been reported from adjacent countries[26].

In *P. vivax* samples, the S117N (IPFSTNI single mutant) *pvdhfr* haplotype was found in 36% of the samples in this study, and a survey from Pakistan in 2013 reported a 45% prevalence of this haplotype[21]. In contrast, another study from Bannu reported about 93% prevalence[13]. Quadruple 57L/58R/61M/117T, triple 57L/58R/117 and double *pvdhfr* (57L/117N) mutant haplotypes have been associated with *in vitro* drug resistance[16]. This study found *pvdhfr* double mutant S58R + S117N in 25% of clinical isolates reported from neighboring countries like India, Afghanistan and Iran[27-29].

In this study, two insertions (I and II) and one deletion of 18 base pairs have been observed. These nucleotides insertion/deletion events in the *pvdhfr* known repeated regions, as well as their roles, warrant more investigation. A study conducted in Southern Pakistan has also reported insertions/deletions in the *pvdhps* gene[30]. Many studies have used these repeated regions to study *pv*dhfr and *pv*dhps alleles based on their genetic diversity[31,32]. An apparent association of tandem repeat variants with mutant alleles and disease severity has been proposed[33]. According to the studies, these tandem repeat variants could be employed in genotyping analyses of *dhfr* and *dhps* alleles[31,34].

P. vivax can be exposed to SP pressure (alone or in combination with artesunate) in an effect possibly involved in misdiagnosis, as mixed infections are frequently treated with SP or AS-SP.

Presumptive diagnosis is relatively high in Pakistan, especially in FATA where medical facilities are not adequate. However in some patients, *P. vivax* parasites are exposed to SP pressure at sub-therapeutic levels, which can favor the emergence of resistance to SP[4]. However, it seems that despite some use of SP or AS-SP, *P. vivax* isolates carrying the mutant *pvdhfr-pvdhp*s have not been selected to a large extent.

The majority (97.9%) of *P. vivax* isolates had the wild-type *pvdhps* haplotype (SAKAV), and only 2.1% of isolates had the mutant SGKAV haplotype; these findings are in line with a previous study[21]. The low prevalence of this *pvdhps* mutant haplotype SAKAV has been reported in Iran and Afghanistan[35,36]. The haplotype analysis revealed that IPFSTNI + SGKAV (*pvdhfr* + *pvdhps*) mutations are rare in the FATA region as these mutations are significantly associated with extensive drug resistance revealed by the molecular marker. Our study found four mutations in *pvdhps*, namely F365L, M367L, D459Aand V498A, and out of these four mutations, two mutations, F365L, M367L, have been reportedin India[33].

In the present study, only 1% of isolates had point mutation at codon Y976F, but 7% of mutations at this position Y976F have been reported in India[37]. Our findings are consistent with two other studies from neighboring countries that reported CQ susceptibility, but the mutant Y976F was not observed in these two studies[38,39]. The F1076L mutation in the *pvmdr1* gene of *P. vivax* isolates was highly predominant (99%) in all FATA study sites which showas a nearly complete predominance. Our findings are in line with previous reports from Pakistan, in which about 98% of mutations have been reported at position F1076L. Nearly all isolates were wild-type at the Y796F position, suggesting that CQ resistance in *P. vivax* has not yet emerged, but many isolates carried the F1076L mutant[13,21]. Our study found the F1076L mutation in all samples compared to the reference wild type sequence, but few studies reported that this mutation alone could be non associated with CQ resistance.

In order to express CQR in *P. vivax* isolates, both mutations must be present, however there is no evidence

that the Y976F mutation represents a molecular marker for CQ resistance[40].

CONCLUSION

This is the first comprehensive report on molecular genotyping and molecular patterns of drug resistance markers in *P. falciparum* and *P. vivax* isolates in FATA regions of Pakistan. Drug resistance findings suggest that although there is a high prevalence of *pfdhfr* double mutations in *P. falciparum* isolates across all FATA regions, these mutations are insufficient to transmit SP failure in clinical settings. In addition, a high prevalence of *P. falciparum* CQR markers has been found despite the discontinuation of drug pressure.

In *P. vivax* isolates, no primary chloroquine resistance-mediating mutation was found; however, a continuous monitoring of *pvmdr1* mutations is essential to identify the emergence of CQ resistant *P. vivax* in the FATA regions. Furthermore, the utility of parasite molecular markers to monitor drug resistance significantly impacts malaria control and elimination in the war-torn malaria endemic area, FATA. Therefore, to better understand malaria transmission, continuous molecular studies to determine genetic diversity are recommended.

ACKNOWLEDGMENTS

We thank the study participants for their involvement in the study.

AUTHORS' CONTRIBUTIONS

MFN, AAK, NZ and AY conceived and designed thestudy, analyzed and interpreted the data. UAA, SG, NMA and MFN were involved in the statistical analysis, writing of the first draft of the manuscript and interpretation of results; MFN, SA, WA, HZ and AY performed the experimental procedures, acquisition of data and collection of samples. AAK, UAA and NZ performed the final correction of the manuscript. All authors approved the final version of the manuscript.

CONFLICT OF INTEREST

None to declare.

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