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Genetic diversity of *Plasmodium falciparum* and *Plasmodium vivax* field isolates from the Nowshera district of Pakistan

Chandni Hayat[1], Atif Kamil[2], Asifullah Khan[1], Aniqa Sayed[3], Kehkashan Akbar[4] and Sahib Gul Afridi[1*]

Abstract

Background The genetic diversity of malaria parasites contributes to their ability to adapt to environmental changes, develop drug resistance and circumvent the host immune system. This study aimed to analyse the genetic diversity of the *Pfmsp1* and *Pfmsp2* genes in *Plasmodium falciparum* and the *Pvmsp-3α* gene in *Plasmodium vivax* isolates from District Nowshera in Pakistan.

Methods Blood samples from 124 consenting patients with uncomplicated malaria presenting to different hospitals from the Nowshera district were collected between March and August 2019, representing 28 *P. falciparum* and 96 *P. vivax* isolates. The genomic DNA extracted from the isolates were subjected to nested PCR and allele-specific analysis. *Pvmsp-3α* amplified fragments were further treated with restriction fragment length polymorphism (RFLP)-based *Hha1* restriction enzyme.

Results Of the analyzed *P. falciparum,* 21 distinct alleles were detected, including 14 alleles for *Pfmsp-1* and 7 alleles for *Pfmsp-2*. The sub-allelic families MAD20 (50%) of *Pfmsp-1*and FC27 (75%) of *Pfmsp-2* were predominant. The multiplicity of infection (MOI) was calculated as 1.4 and 1.2 for *Pfmsp-1* and *Pfmsp-2,* respectively, with an overall mean MOI of 1.34. In *P. vivax*, 4 allelic variants, *Pvmsp*-3α types *A, B, C* and *D*, were detected, while RFLP digestion of amplicons, detected 9 sub-allelic variants (*A1-A4*, *B1*, *B2*, *C1*, *C2* and *D1*) at the *Pvmsp-3α* locus.

Conclusion This first ever report of molecular characterization of *P. falciparum* and *P. vivax* genotypes from District Nowshera, Pakistan reveals moderate to high allelic diversity in parasite population from District Nowshera, Pakistan.

Keywords Malaria, *Pfmsp-1*, *Pfmsp-2*, *Pvmsp-3α*, Genetic diversity, Nowshera, Pakistan

Background

Despite huge investments and efforts to control and eradicate malaria, it is still a major concern for human health and wealth in resource-limited countries of the world. Malaria is one of the most important protozoan infections around the world, causing 249 million cases and 608,000 deaths in 2022 [1]. Pakistan is one of the malaria-endemic countries in the world, where 2.65 million cases of malaria were reported by the World Health Organization (WHO) in 2022 [1]. Malaria is moderately endemic in Pakistan with unstable transmission [2, 3]. Approximately 60% of its populace lives in malaria-endemic areas, with 177 million

*Correspondence:
Sahib Gul Afridi
drafridi@awkum.edu.pk
[1] Department of Biochemistry Abdul Wali, Khan University Mardan, Mardan, Pakistan
[2] Department of Biotechnology Abdul Wali, Khan University Mardan, Mardan, Pakistan
[3] School of Public Health, University of Alabama at Birmingham, Birmingham, USA
[4] Department of Biochemistry, Abbottabad International Medical College, Abbottabad, Pakistan

people at risk of contracting malaria [3]. The prevalence of malaria differs in provinces and even in different districts of Pakistan with different climates. Khyber Pakhtunkhwa is one of the most endemic provinces in Pakistan, where 159,912 of malaria cases were reported in 2022 [4].

Among the five human malaria species, *Plasmodium falciparum* and *Plasmodium vivax* are the most prevalent in the world; the former remains the most dominant species in Africa and is responsible for most severe clinical cases and is associated with 95% of malarial deaths, while the latter is the most widely distributed parasite in tropical regions, especially in Asia and America [5, 6]. *Plasmodium falciparum* remains the major focus by researchers, as the most virulent, genetically complex and contributes to majority of malaria-associated deaths, whereas *P. vivax* has been comparatively less explored and used to be classified as benign [7]. However, recent studies have revised this classification of *P. vivax* malaria by reporting severe vivax malaria in most malaria-endemic regions, causing about 300 million episodes of clinical malaria annually [7–10]. *Plasmodium vivax* has a wider geographical distribution and is increasingly being recognized as less responsive to malaria control measures than *P. falciparum* [11]. A number of distinctive features of *P. vivax* biology are believed to assist in the evasion of control measures, such as infection relapse [12, 13], the early appearance of transmission stages [12, 14] and prompt acquisition of clinical immunity [14, 15]. *Plasmodium vivax*-based malaria transmission is, therefore, likely to be more stable over time and during control efforts than *P. falciparum* malaria transmission [15].

Malaria control is confounded by different factors, including drug and insecticide resistance, and lack of vector control measures [1]. Moreso, the unavailability of an effective multivalent vaccine against *Plasmodium* species is contributory to the high malaria burden [16]. Investigation of *Plasmodium* parasite structure and genetic diversity is essential for designing promising vaccines against malaria and for understanding the evolution of parasite virulence and the role of polymorphisms in malaria transmission [17]. During the blood stage of the malaria life cycle, merozoite surface proteins are expressed and are the main potential targets for the assessment to develop an effective vaccine [18]. For *P. falciparum*, *Pfmsp-1* and *Pfmsp-2,* and *P. vivax, Pvmsp-3α* and *Pvmsp-3β* are among the promising target antigens for candidatevaccine development [19]. The *Pf*MSP-1 (190 kDa protein) has a pivotal role in erythrocyte invasion by merozoites, and its block 2 is considered the most polymorphic region [20]. *Pf*MSP-2 is a glycoprotein consisting of 5 blocks, in which block 3 is a polymorphic region [21]. The *Pfmsp*-1 gene comprises 3 main allelic variants, K1, MAD20, and RO33, and the *Pfmsp*-2 gene comprises FC27 and 3D7 allelic variants [22].

Similarly, merozoite surface proteins expressed on the *P. vivax* parasite surface are used to study *P. vivax* genotypes, among which *Pvmsp-3α* and *Pvmsp-3β* are highly immunogenic and hence, are important vaccine candidate antigens. The *Pvmsp*-3α is composed of three blocks of alanine-rich domains with heptad repeats [23]. Thus, the *Pvmsp-3α* and *Pvmsp-3β* genes are highly polymorphic due to the addition and removal of alanine-rich regions [24]. The genetic diversity of *Plasmodium* parasites and multiplicity of infection (MOI) have been reported to vary with the severity and transmission intensity of malaria in different regions [25]. Worldwide genetic diversity and genotyping of *P. falciparum* and *P. vivax* field isolates have been extensively studied using their MSPs [26–35]. Although there is very limited information available regarding the genetic diversity of *P. falciparum* and *P. vivax* field isolates from Pakistan [26, 32, 36–38] no such study delineating the genetic diversity of *P. falciparum* and *P. vivax* from district Nowshera of Khyber Pakhtunkhwa Province in Pakistan has been reported thus far. Therefore, the current study seeks to generate baseline information on *P. falciparum* and *P. vivax* genetic diversity among Pakistani isolates from District Nowshera using their corresponding polymorphic markers, *Pfmsp-1, Pfmsp-2* and *Pvmsp-3α* and the multiplicity of infection (MOI).

Methods

Ethics statement and consent for participation

The present study was approved by the Ethical Committee of the Department of Biochemistry Abdul Wali Khan University Mardan *(AWKUM/Biochem/Dept/Commit/18),* and all the participants or their parents/guardians were asked to provide written informed consent before recruiting them for this study.

Study area

The current study was conducted in District Nowshera of Khyber Pakhtunkhwa (KP) Province in Pakistan, located between 33° 04′ 00″ to 34° 10′ 00″ N latitude and 71° 44′ 50″ to 72° 15′ 05″ E longitude. District Nowshera is situated in the centre of KP and is bordered by district Swabi from the north and district Charsadda from the northwest, while to its east district Attock of Punjab Province, in the south district Kohat and in the west district Peshawar are located (Fig. 1). Its total area is 1748 $km^2$with a total population of 1,518,549 persons in 2017. In Nowshera, summers are hot and humid, and winters are cool and rainy. Its annual rainfall is 131.34 mm, and its temperature typically ranges from 43°F to 111°*F* [39]. Its location is along the River Kabul, and its rainfall and

Fig. 1 Map showing the study area of District Nowshera Pakistan

weather conditions are apparently favourable for malaria transmission.

Sample collection

Blood samples (2 mL) were collected from 124 malaria patients after being confirmed positive for *P. vivax* (96 samples) and *P. falciparum* (28 samples) by microscopy using thick and thin blood smears, stained with Giemsa. Study samples were collected from patients (aged 6–65 years), visiting three major hospitals of District Nowshera, i.e., DHQ Hospital, Nowshera Medical Complex (NMC) and Combined Military Hospital (CMH), between March and August 2019. Among 124 blood isolates, 114, including 90 *P. vivax* and 24 *P. falciparum* isolates, were further confirmed to be positive by RDT (CareStart™: Malaria HRP2/pLDH(Pf/PAN) Combo). All the samples were transferred to the Molecular Biology Laboratory in the Department of Biochemistry AWKUM for parasite genomic DNA isolation and genotyping analysis.

DNA isolation

Collected blood samples of *P. vivax* and *P. falciparum* infections were subjected to DNA extraction using theThermo Scientific Gene JET Genomic DNA Purification Kit (Cat No. K0721) followed by checking the quality and roughly estimating the quantity of extracted DNA through gel electrophoreses and visualization through an ethidium bromide-based UV gel documentation system.

PCR amplification of *Pfmsp-1 and Pfmsp-2* genes

A nested PCR protocol with species-specific primers targeting the 18S ribosomal RNA (rRNA) of *P. falciparum* and *P. vivax* was carried out as previously described [22]. PCR-confirmed isolates were further genotyped for the *Pfmsp-1* (block-2) and *Pfmsp-2* (block-3) polymorphic regions of *P. falciparum* was performed following

a previously reported protocol by Snounou et al. [22] (Table 1). For the negative control, deionized water was used, while for the positive control, DNA from commercially available parasite reference strain 3D7 (ATCC, cat# PRA-405D) was used. The final volume of the initial PCR mixture for both *Pfmsp-1* and *Pfmsp-2* was adjusted to 16µL by adding 7.5µL master mix (Solis BioDyne), 0.5µL each forward and reverse primer, 5.5µL double distilled water and 2µL genomic DNA. Using the product of the initial amplification as a template for the secondary reaction, 5 separate reactions were performed, in which specific primers (Table 1) were used to identify allelic variants of *Pfmsp-1* (K1, MAD20 and RO33) and *Pfmsp-2* (FC27 and 3D7). In the secondary reaction, 20µL mixture was prepared for each sample containing 6µL master mix, 10µL ddH_2O, 0.5µL each forward and reverse nested primers and 3µL PCR product of initial PCR as template. Nested products were analysed using2% agarose gel stained with ethidium bromide under UV light. The suballelic variants of the *P. falciparum* infections were determined from their banding pattern on agarose gels using a 100 bp DNA ladder.

Multiplicity of infection and heterozygosity (HE) of P. falciparum genotypes

The mean multiplicity of infection (MOI) was estimated by dividing the total number of distinct *Pfmsp-1* or *Pfmsp-2* genotypes detected by the number of positive samples for the same marker [40]. Samples with more than one allelic family were considered polyclonal infections, and those with a single allelic family were considered monoclonal infections. The expected heterozygosity (H_E) was calculated using the formula.\

$$H_E = \left[n/(n-1)\right]\left[1 - \sum P_i^2\right]$$

where n is the sample size and Pi represents the allele frequency of the -ith allele.

PCR–RFLP analysis of the *Pvmsp-3α* gene

The *Pvmsp-3α* gene of *P. vivax* was amplified by nested PCR followed by digestion of the PCR amplicons with RFLP restriction enzymes according to previously described protocols by Bruce et al. [41] and Yang et al. [18]. Briefly, the *Pvmsp-3α* gene was amplified using nested PCR conditions and primers (Table 1) in a total reaction volume of 20µL comprising 1 µL genomic DNA, 7µL ddH_2O, 10µL master mix and 1µL each of the forward and reverse oligonucleotide primers. In the nested reaction, 2 µL product of the primary reaction was used along with 8 µL master mix, 6 µL ddH_2O and 1µL each forward and reverse primers making a total volume of 18 µL reaction mixture. All PCR amplicons were confirmed by electrophoresis using a 2% agarose gel and visualized under UV light. The size of the PCR products (expected to be 0.5 to 2.5 kb) was estimated using a1 kb DNA ladder.

For RFLP analysis, PCR products (6µL) of *Pvmsp-3α* gene were digested with 2 units (1µL) of *Hha* I restriction enzyme in 16µL reaction volume including 7µL PCR water and 2µL buffer supplied with the enzyme, at 37 °C for 4 h. Alleles were classified based on undigested PCR

Table 1 The primer sequences, base pairs and PCR conditions for *P. falciparum* and *P. vivax* genes in District Nowshera, Pakistan

Primers	Sequences	Base pairs	PCR conditions	References
K1 family (N2)	F: 5′-AAATGAAGAAGAAATTACTACAAAAGGTGC-3′	30	95 °C 4 min/[95 °C 30 s, 55 °C 45 s, 72 °C 3 min]×30 cycles 72 °C 4 min	[22]
	R: 5′-GCTTGCATCAGCTGGAGGGCTTGCACCAGA-3′	30		
MAD20 family (N2)	F: 5 ′-AAATGAAGGAACAAGTGGAACAGCTGTTAC-3′	30	95 °C 4 min/[95 °C 30 s, 55 °C 45 s, 72 °C 3 min]×30 cycles 72 °C 4 min	[22]
	R: 5 ′-ATCTGAAGGATTTGTACGTCTTGAATTACC-3′	30		
RO33 family (N2)	F: 5 ′-TAAAGGATGGAGCAAATACTCAAGTTGTTG-3	30	95 °C 4 min/[95 °C 30 s, 55 °C 45 s, 72 °C 3 min]×30 cycles 72 °C 4 min	[22]
	R: 5 ′-CATCTGAAGGATTTGCAGCACCTGGAGATC-3	30		
Pfmsp-2 (N1)	F: 5 ′-ATGAAGGTAATTAAAACATTGTCTATTATA-3′	30	95 °C 5 min/[94 °C 1:45 s, 54 °C 30 s, 72 °C 3 min]×35 cycles 72 °C 5 min	[22]
	R: 5′-CTTTGTTACCATCGGTACATTCTT-3′	24		
FC27 family (N2)	F: 5′-AATACTAAGAGTGTAGGTGCARATGCTCCA-3′	30	95 °C 4 min/[95 °C 30 s, 55 °C 45 s, 72 °C 3 min]×30 cycles 72 °C 4 min	[22]
	R: 5 ′-TTTTATTTGGTGCATTGCCAGAACTTGAAC-3′	30		
3D7 family (N2)	F: 5 ′-AGAAGTATGGCAGAAAGTAAKCCTYCTACT-3′	30	95 °C 4 min/[95 °C 30 s, 55 °C 45 s, 72°C3 min]×30 cycles 72 °C 4 min	[22]
	R: 5 ′-GATTGTAATTCGGGGGATTCAGTTTGTTCG-3′	30		
Pvmsp-3a (N1)	F: 5 ′-CAGCAGACACCATTTAAGG-3′	19	95 °C 3 min/[94 °C 40 s, 54 °C 30 s, 68 °C 30 s]×35 cycles 68 °C 5 min	[41]
	R: 5′-CCGTTTGTTGATTAGTTGC-3′	19		
Pvmsp-3a (N2)	F: 5′-GACCAGTGTGATACCATTAACC-3′	22	95 °C 5 min/[94 °C 30 s, 55 °C 30 s, 68 °C 30 s]×30 cycles 68 °C 5 min	[41]
	R: 5′-ATACTGGTTCTTCGTCTTCAGG-3′	22		
	R: 5′-GCTGCTTCTTTTGCAAAGG-3′	19		

product size and RFLP banding patterns after running on 2% agarose gel and visualizing the fragments under UV light. The digested and undigested amplicon sizes ranging from 120 bases to 2 kb, were estimated using 100 bp and 1 kb DNA ladders (New England Biolab), respectively. The mean MOI and H_E index for *Pvmsp*-3α genotypes were calculated.

Results

Allelic frequency distribution of *Pfmsp-1, Pfmsp-2* and multiplicity of infection

For genotyping of *P. falciparum* parasites in district Nowshera, a total of 24 isolates were successfully amplified for *Pfmsp-1* alleles (K1, RO33, MAD20) and *Pfmsp-2* alleles (FC27 and 3D7). The *Pfmsp-1* and *Pfmsp-2* allelic families were classified on the basis of PCR-amplified fragments size. Both *Pfmsp-1* and *Pfmsp-2* with respect to their corresponding genotypes were highly diverse (Table 2). A total of 21 alleles were detected, including 14 from *Pfmsp-1* and 7 alleles from *Pfmsp-2,* respectively. At the *Pfmsp*-1 locus, 4 K1 alleles (180–350 bp), 4 RO33 alleles (150–300 bp) and 6 MAD20 alleles (100–300 bp) were detected, while at the *Pfmsp-2* locus, 6 FC27 alleles (150–700 bp) and 1 3D7 allele (300 bp) were observed. Among the *Pfmsp-1* allelic families, MAD20 was predominant, comprising nearly half of the isolates, while FC27 was the most dominant (75%) of *Pfmsp*-2. The mean MOIs for *Pfmsp-1* and *Pfmsp*-2 were calculated as 1.4 and 1.2, respectively, while the overall mean MOI was 1.34 for both genes. The expected H_E index was higher for *Pfmsp*-1 genotypes (0.68) compared to *Pfmsp-2* genotypes (0.26) (Table 2).

Genetic diversity of *Pvmsp*-3α

A total of 90 *P. vivax* field isolates were successfully analysed using the PCR–RFLP technique to investigate the genetic variation in their highly polymorphic marker *Pvmsp*-3α gene from the study area. Briefly, after nested PCR amplification of *Pvmsp-3α*, 4 major allelic variants were detected, labelled Type A (2.5 kb) in 36% of isolates, Type B (1.7 kb) in 32% of isolates, Type C (1.5 kb) in 30% of isolates and Type D (~0.65 kb) in 2% of isolates(Table 3). Among these variants, Type A was predominant, followed by types B and C at the *Pvmsp*-3α locus. Amplicons of the *Pvmsp-3α* gene acquired from the nested PCR were further digested with the restriction enzyme *Hha1* to obtain better resolution at the diversity level. Following amplicon digestion with *Hha1*, 9 allelic variants designated as A1-A4, B1, B2, C1, C2 and D1 were detected, where A4 [17% (16/90)] and B2 [15% (14/90)] were dominant among *Pvmsp-3α* sub-allelic variants (Table 3). Mixed infections with more than 1 sub-allelic variant of the *Pvmsp-3α* gene were also detected with 11% frequency in the study isolates (Fig. 2).

Table 2 Multiplicity of infection and expected heterozygosity (H_E) of the *Pfmsp-1* and *Pfmsp-2* genes in *P. falciparum* isolates

Genes	Alleles	No of alleles observed (%)	Alleles size	Mean MOI	H_E
Pfmsp-1(n = 14)	K1	4 (28.5)	180–350 bp	1.40	0.68
	MAD20	4 (28.5)	100–300 bp		
	RO33	6 (42)	150–300 bp		
Pfmsp-2(n = 7)	FC27	6 (85.7)	150–700 bp	1.20	0.26
Pfmsp-1 + Pfmsp-2	3D7	1 (14.3) 21	300 bp 100–750 bp	1.34	

Discussion

The genetic diversity of malarial parasites plays a major role in malaria transmission intensity, drug resistance, and the effectiveness of control measures. Hence, understanding the genetic population structure of these parasites in malaria-endemic regions is essential [28].

Globally, several vaccine candidate genes in *P. falciparum* and *P. vivax* have been extensively studied to understand parasite genetic diversity, population dynamics, drug resistance mechanism, and to inform malaria elimination strategies, with particular focus on polymorphic antigenic markers like merozoite surface proteins (MSPs) as promising vaccine targets [34, 42–45]. PCR-based genotyping has been successfully applied for genetic diversity analysis of *P. falciparum* and *P. vivax* parasites based on their corresponding antigenic markers, such as *Pfmsp-1, Pfmsp-2, Pfglurp* [34], *Pvmsp-3α, Pvmsp-3β* [29], *Pvcsp* and *Pvmsp-1* [46].

This study aimed to decipher the genetic diversity of *P. falciparum* and *P. vivax* isolates collected from Nowshera district, Khyber Pakhtunkhwa, Pakistan. *P. falciparum* and *P. vivax* isolates from Nowshera showed moderate to high genetic diversity. In current study, a higher number of alleles was detected for *Pfmsp-1* compared to *Pfmsp*-2. This result is in agreement with previous reports from Burkina Faso [47, 48], Côte d'Ivoire and Gabon [49], and

Table 3 PCR-based allelic polymorphism of the *Pvmsp-3α* gene in *P. vivax* isolates

Allelic variants	Size in kb (Frequency)	Mean MOI	H_E
Type A	2.5 kb (36%)	1.11	0.685
Type B	1.7 kb (32%)		
Type C	1.5 kb (30%)		
Type D	0.65 kb (2%)		

H_E = Expected heterozygosity

MOI = Multiplicity of infection

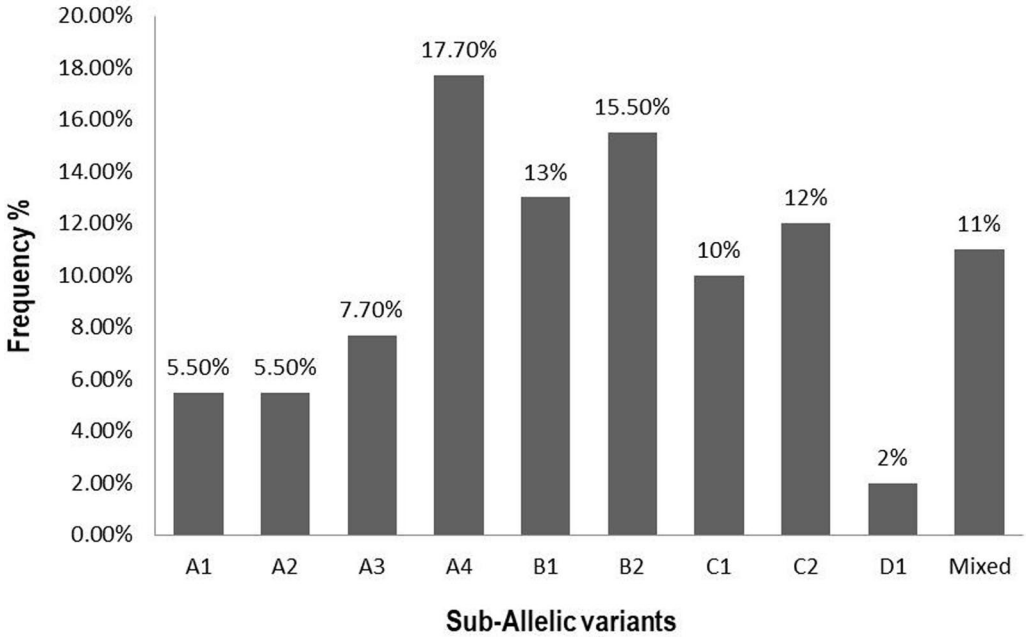

Fig. 2 Frequency distribution of *Pvmsp-3α* suballelic variants in the Nowshera district

Pakistan [38]. Conversely, some studies from Ethiopia [36], Myanmar [50] and Sudan [51] reported higher frequency of *Pfmsp*-2 than *Pfmsp-1 genotypes*.

The allelic families of *Pfmsp-1* (K1, RO33, MAD20) and *Pfmsp-2* (FC27, 3D7) were successfully amplified as previously reported from other districts of Pakistan [33, 36], Iran [52], Honduras [53], India [54], Burkina Faso [47] and southeast Gabon [55]. Conversely, results from this study differ from earlier findings reported from Khyber Agency in Pakistan [56], where only K1 and MAD20 alleles were reported for *Pfmsp-1*, and 3D7 for *Pfmsp-2,* showing low genetic diversity than the present study. The current study reports MAD20 as a highly prevalent variant of *Pfmsp-1* infections, showing close agreement with several studies reported from Bannu, Pakistan [33], Northwest Ethiopia [39], India [57], and Myanmar [31]. However, a remarkably incongruent result was reported from the Republic of Congo [58] and some southern regions of Khyber Pakhtunkhwa in Pakistan [36]. The allelic variation might be due to differences in the geographic and environmental conditions. The FC27 allele of *Pfmsp-2* was dominant in the present study, as previously observed in Northwest Ethiopia [39], Nigeria [59] and Gabon [55]. However, some studies conducted 13 years ago from the Bannu district of Pakistan [33] and from the Republic of Congo [58] have reported a higher prevalence of the 3D7 allele than FC27. The MOI is a potent tool to identify the number of distinct parasite clones and transmission intensity in different geographical regions. The mean MOI observed was 1.40 for *Pfmsp-1* and 1.20 for *Pfmsp*-2, with an overall mean MOI of 1.34, reflecting low to moderate malaria transmission intensity in the study area. These results are in close agreement with a number of previously reported studies [60, 61]. The MOI for *Pfmsp-1* and *Pfmsp*-2 infections in present study was lower than reported from Bioko Island, Equatorial Guinea (5.51) [28], Nigeria (2.6–2.8) [29] and Gabon (4.0) [62]. This huge difference in MOI confirms the high malaria transmission intensity in sub-Saharan Africa compared to Pakistan.

The *Pvmsp*-3α gene in *P. vivax shows* 4 distinct variants (A, B, C and D).Type A (2.5 kb) allele was the most prevalent, followed by type B (1.7 kb). This study agrees with previous studies that reported the type A allelic variant as the most frequent from Khyber Pakhtunkhwa, Sindh, Baluchistan and Punjab provinces [63] and from district Bannu [33], suggesting the uniform distribution of *Pvmsp-3α* allelic families in most of the provinces in Pakistan. However, in terms of the number of distinct allelic variants for *Pvmsp-3α*, the current study is different from those reported from Iran [46], Thailand [64] and Afghanistan [65], where only 3 allelic variants (A, B, C) for *Pvmsp*-3α were observed, which indicates the pattern of parasite diversity across the different geographical regions.

Restriction digestion of the *Pvmsp-3α* amplified product displayed the presence of 9 unique allelic families among the 91 resolved amplicons, with allelic variant

types A4 and B2 being the most frequent, while previous studies from Pakistan [33, 63] reported 12 allelic variant types for the *Pvmsp-3α* gene, with the A3 allele being the most frequent. PCR–RFLP data from *Pvmsp-3α* loci exhibited 11% mixed-strain infections, revealing a comparatively higher frequency than that reported from China (5.6%) and much lower than that found in Thailand (20.5%) and FATA Pakistan (30%) [18, 46]. The higher rate of mixed infections from FATA Pakistan is reflected by the fact that FATA shares a border with Afghanistan, owing to which human migration was at its peak at that time. Unlike present study, no mixed infections were reported from Iran [46] and Hongshuihe (China) [18].

The limitations of this study include small sample size of *P. falciparum* isolates and the use of only one RFLP marker for *Pvmsp-3α* genotyping, which may underestimate genetic diversity. Future studies should involve larger sample size and cover broader region, utilizing multiple restriction enzymes and sequencing to provide a more comprehensive assessment of genetic diversity.

Conclusion

Overall, moderate to high genetic diversity was observed in *P. falciparum* and *P. vivax* field isolates from the Nowshera district of Khyber Pakhtunkhwa, Pakistan, with a low value of MOI for *P. falciparum* and *P. vivax* infections, inferring low to moderate malaria transmission intensity in the region.

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Author contributions
Conceptualization: S. G. A. Data curation: C. H., A. K., A. K. Formal analysis: C. H., K. A., S. G. A. Investigation: C. H., S. G. A., A. K. Methodology: C. H., S. G. A., A. K.. Resources: A. K., A. S. Supervision: S. G. A., A. K. Writing – original draft: C. H., K. A., A. S. Writing – review & editing: A. K., S. G. A.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The study protocol was approved by the Ethical Review Committee of Abdul Wali Khan University Mardan under the letter of AWKUM/ERC/578. Consent was obtained from all participants before conducting the study.

Consent for publication
All authors have given their consent for publication of this manuscript.

Competing interests
The authors declare no competing interests.

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