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SELECTIVE SWEEP AND PHYLOGENETIC MODELS FOR THE EMERGENCE AND SPREAD OF SULFADOXINE-RESISTANCE MUTATION IN PLASMODIUM FALCIPARUM MALARIA WITH IN THE TRIBAL AREAS OF KHYBER PAKHTUNKHWA PAKISTAN

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ABSTRACT

Background: Malaria remains a significant global health challenge, particularly affecting populations in tropical and subtropical regions. In Pakistan, malaria is highly prevalent and pose a substantial burden on public health especially in the Tribal Areas of Khyber Pakhtunkhwa province, which shares borders with Afghanistan. However, due to political instability and challenges in implementing malaria control initiatives, resistance to sulfadoxine has emerged in this region, subsequently spreading to other parts of the country. Here, we present for the first time evidence of emergence and spread of signal nucleosides polymorphisms (SNPs) of dihydropteroate synthetase (DHPS) gene in plasmodium falciparum (P.f) in tribal areas of Khyber Pakhtunkhwa (KP) province, Pakistan.

Methodology: After the microscopic and rapid diagnostic test, DNA of P. falciparum was extracted from infected patients blood samples and a 711-base pair segment of the DHPS gene having polymorphic codons, using nested PCR. The PCR products were then subjected to Sanger sequencing and sequences were further verified using Bioedit software to confirm peak patterns, and a phylogenetic tree was constructed using MEGA12 software. Selective sweep models were employed to examine the soft and hard selective sweep model for sulfadoxine resistance gene in nine localities with in each tribal area of Khyber Pakhtunkhwa. Pakistan.

Results: Sixteen mutations were identified in the DHPS gene of plasmodium falciparum. Notably, E556K and K582K mutations were observed in all the samples, while R610K were occurred in

82.35%, M538R in 76.47%, and R608K in 64.71% of the samples respectively. Conversely, H536L and H630L mutations exhibited the lowest occurrence at 5.88%. Phylogenetic analysis revealed 16 unique haplotypes, with FATA regions of Dera Ismail Khan and Kohat each displaying a single haplotype. Bajaur Agency exhibited a higher degree of similarity with a specific haplotype.
Conclusion: This study sheds light on the dynamics of sulfadoxine resistance emergence and spread in P. falciparum DHPS gene within tribal areas of Khyber Pakhtunkwa, highlighting the importance of continued molecular surveillance in guiding malaria control strategies. The findings underscore the urgent need for targeted interventions to mitigate the spread of sulfadoxine-resistant strains and preserve the efficacy of antimalarial treatments in these high-risk areas.

Keywords: Malaria, plasmodium falciparum, DHPS gene, haplotypes, sulfadoxine-resistance, selective sweep model, emergence and spread.

INTRODUCTION

Malaria continues to pose a significant global health challenge, with an estimated 229 million cases and 0.409 million deaths (WHO World Malaria Report 2020). The disease is caused by Plasmodium falciparum (P. falciparum) and Plasmodium vivax (P. vivax), with P. vivax being the most widespread species, while P. falciparum is associated with higher fatality rate. (Menkin and Winders, 2024). P. vivax is responsible for 84% of malaria infections, while P. falciparum accounts for 15%, with mixed-species infections constituting about 1% in Pakistan (Directorate of Malaria Control, 2019).

In Pakistan, malaria is particularly prevalent in the tribal areas of Khyber Pakhtunkhwa province, where resistance to antimalarial drugs like sulfadoxine has emerged as a major concern (Directorate of Malaria Control, 2019). The rise of drug resistance, notably within P. falciparum, poses significant challenges to malaria management and control programs. Understanding the molecular mechanisms underlying sulfadoxine resistance in P. falciparum populations within tribal regions is therefore of significant importance for guiding effective treatment strategies and preventing the spread of resistant strains (Karim et al., 2016; Rini et al., 2021).

Sulfadoxine acts by inhibiting the enzyme dihydropteroate synthase (DHPS) and sequencing of the DHPS gene has revealed amino acid variations that could play a role in the mechanism of resistance to this drug (Triglia et al., 1997). Different regions of this gene have exhibited diverse mutations, such as SGEAA in East Africa and AGKAA in West and Central Africa (McCollum et al., 2008; Pearce et al., 2009). The dissemination of these alleles reflects various origins and continuous selection pressures (Alam et al., 2011; Vinayak et al., 2010). Molecular surveillance, particularly through selective sweep models, provides insights into the frequency, distribution, and genetic basis of resistance mutations (Chaudhry, 2015; Vanheer et al., 2023). Additionally, phylogenetic models have suggested that resistance conferring mutations may arise from a single or multiple origin, and potentially can spread through the parasitic populations via human or animal movement (Chaudhry, 2015).

All over Pakistan, including tribal areas of Khyber Pakhtunkhwa artemisinin-based combination therapy (ACT) with artesunate plus sulfadoxine–pyrimethamine (AS + SP) has been the first-line treatment for uncomplicated falciparum malaria since 2007 (Yaqoob, A., et al., 2018). Understanding the molecular mechanisms underlying sulfadoxine resistance in P. falciparum populations is crucial for guiding effective treatment strategies and mitigating the spread of resistant strains (Rini et al., 2021). The rise and dissemination of drug resistance, notably within P. falciparum have presented considerable obstacles to the management of malaria. Resistance typically develops through specific mutations in particular genes, gradually heightening levels of resistance (Heinberg et al., 2015).

Molecular markers associated with reduced susceptibility to sulfadoxine-pyrimethamine (SP) have been identified in various regions, but studies focusing on the tribal areas of Pakistan are very limited (Yaqoob et al., 2018; Khatoon et al., 2009). Understanding the dynamics of drug resistance mutations is therefore crucial for developing effective malaria control strategies, particularly in regions facing socio-political challenges. Currently there is no publish data available from Pakistan on the DHPS

resistance alleles of P. falciparum with respect to consequences of positive selection pressure on the emergence and spread. The information of this work will helpful for understanding of the sulfadoxine resistance mutations that may differ between tribal areas with implications for the development of high throughput diagnostic methods for resistance surveillance programs.

The objective of this study is to address this gap in knowledge regarding the emergence and spread of sulfadoxine resistance mutations DHPS resistance gene of P. falciparum in tribal areas of Khyber Pakhtunkhwa Pakistan. We also seek to explore the emergence and spread of resistance haplotypes through selective sweep model and phylogenetic analysis. Single and or multiple time emergence of resistance mutations could suggest that resistance is "inevitable" for P. falciparum implications of target treatment or different drug combination strategies including new drug or the modification of current drugs, the spread of sulfadoxine resistance mutations may depend on its reproductive isolation of the human carrying resistance alleles.

MATERIALS AND METHODS

Ethical approval and Study Area

Ethical approval for the study was obtained from the ethical committee of Kohat University of Science and Technology, Pakistan (KUST/EC/1379). All participating patients provided signed informed consent. The selected regions for sample collection included strategic border areas such as Orakzai agency border (Shanaweri Zargiri), Ali Masjid-Landi Kotal-Khyber agency the border of Pakistan-Afghanistan, Kurram Agency, North Waziristan agency (Wanna), AFR Kohat (Ara Khil-Jamu Sultan Khil), AFR Kohat (Dar Adm Khil), AFR Peshawar, AFR DI Khan, Bajaur agency, Mohmand agency, AFR Kohat (Ara Khil-Shin Dhand), AFR Kohat (Bosti Khil) as depicted in Figure 1. Specimens positive for P. falciparum were collected from selected nine tribal areas of Khyber Pakhtunkhwa (KP), Pakistan, spanning from January 2022 to June 2023. The study site's coordinates were measured at latitude of 34.9526 N and a longitude of 72.3311 E. The region sustains a population of 35.53 million within an area of 101,741 km², with an annual rainfall of 384 mm primarily during March-May and August-November. Average temperature ranges from 20°C to 40°C. In the epidemiological context, KP Province has exhibited a substantial malaria burden, constituting 31% of cases per the NMCP 2019, corroborated by a 2018 annual parasite index (API) of 6.3 (Directorate of Malaria Control, 2019). All the data collectors were provided with comprehensive training on the specific research study design before proceeding for the collection of samples and geographical data from the tribal areas, to equip them with the necessary knowledge and skills for effective processing. In addition to the required training, essential supplies were distributed among the data collectors to facilitate the fieldwork, including swabs, blood syringes, ethylenediaminetetraacetic acid (EDTA) tubes, and data collection forms to ensure readiness and efficiency during the data collection process.

Blood sampling, Microscopy and RDT

A comprehensive survey was performed across various Basic Health Units (BHUs) and laboratories in the endemic tribal areas of KP to collect blood specimens from suspected individuals. The sample collection process was carefully randomized to ensure representation across diverse tribal areas with in KP. Five milliliters of intravenous blood sample were aseptically drawn into EDTA tube following the established medical protocols from each patient undergoing malaria infection examination at the testing facility. Collected samples were transported to the Laboratory of the Department of Microbiology, Kohat University of Science and Technology, Pakistan, followed by a comprehensive microscopic scrutiny involving both thin and thick Giemsa-stained blood films, along with Rapid Diagnostic Testing (RDT) (Wahab et al 2020).

Genomic DNA Extraction and PCR Amplification

Genomic DNA was extracted from all the samples by using the QIAamp DNA Mini Kit (Qiagen) following the manufacturer recommended protocols, and the extracted DNA was stored at -20°C for further use. A 711-base pair (bp) segment of DHPS encompassing polymorphic codons was

independently amplified through nested PCR using a 96-well plate format. The PCR primer sequences and reaction conditions are provided in table 1. The PCR mixture of 25 µL comprised primers at a final concentration of 0.25 µM, 2 mM MgCl2, 250 µM of each deoxynucleoside triphosphate, and 1x Bioline Taq polymerase. For the outer reaction mixtures, 1 µL of template DNA was introduced. Subsequently, 1 µL of three-fold diluted DHPS outer PCR product was introduced into a 25 µL inner amplification reaction mixture (Table 1) (Pearce et al., 2003).

Gel Electrophoresis

The PCR amplified products were run in a 1.5% agarose gel for 35 minutes at 500 MA current and a voltage of 90 volts. The gel was visualized under a UV transilluminator, and the ladder 100bp (Promega) marker was compared to the PCR amplified products.

DNQ Quantification and DNA Purification

The PCR amplified products were quantified using Nano drop, and the samples were subjected to DNA purification through a DNA purification Kit. The PCR products were purified by using the kit Wizard™ SV Gel and PCR Clean-Up System (Promega), following the manufacturer's procedure (Cubides, J.R., et al.,2018).

Sequencing and Phylogenetic Analysis

The PCR confirmed products a 711-base pair (bp) segment of DHPS encompassing polymorphic codons were independently subjected to Sanger sequencing by genetic analyzer ABI3700. The sampled sequences were further run by Bioedit for confirmation of the peaks, followed by the construction of phylogenetic tree through MEGA12. A network comprising 54 P. falciparum genes was constructed, analyzing the genetic distance with in the 18S rRNA gene of DHPS haplotypes. The construction was carried out using the Neighbor-Joining method within Network 4.6.1 software developed by Fluxus Technology Ltd. To enhance clarity, unnecessary median vectors and links were removed through star contractions. The methodology employed in this study entails the categorization of haplotype classes based on branch counts in networks, computation of adjusted degree of distribution, and the introduction of haplotype network branch diversity metric for the purpose of complexity comparison. Haplotype network branch diversity (HBd) is a statistic that calculates the complexity of haplotype networks by adding Haplotype diversity (Hd) and Branch diversity (Bd), both of which are frequencies. Thus, HBd is the likelihood that two randomly chosen individuals from a population will have separate haplotypes with different number of branches in a haplotype network (Gracia et al., 2021).

RESULTS

Allele frequencies of sulfadoxine resistance-associated SNPs in P. falciparum DHPS

A 711-base pair (bp) segment of P. falciparum DHPS locus was successfully amplified from 54 individual isolates from nine localities sourced from tribal areas were sequenced, comprising six samples from each area with in the tribal areas of Khyber Pakhtunkhwa province. These samples were sequenced to study mutational frequencies in the P. falciparum DHPS gene. Among the identified mutations, E556K and K582K were found in 100% of the samples, followed by R610K (82.35%), M538R (76.47%), and R608K (64.71%). Conversely, the mutations H536L and H630L had the lowest frequencies rate at 5.88% (Figure 2).

Overall combinations of mutational frequencies of sulfadoxine resistance-associated SNPs in P. falciparum DHPS

We identified a total of 16 variants in the DNA sequence making 17 combinations of mutations and haplotypes were generated accordingly in the studied areas. The most common combinations involved mutations G1666A and G1746A. Mutation A1607T was found in only one combination. Additionally, mutations G1666A and G1746A were present in all 54 isolates (100%). Among the 54

collected samples, the highest frequency occurred in the combination C-1 (G1310C / A1605C / G1666A / G1743A / G1746A / G1823A / G1829A / A1875C) which was observed in 16.67% of the isolates. This combination was most prevalent in isolates from FR DI Khan6, FR Kohat5, FR Kohat6, Kurram Agency5, Kurram Agency6, Orakzai Agency1, Orakzai Agency2, Orakzai Agency3, and Orakzai Agency4. The combination C-2 (T1613G / G1666A / G1746A / G1823A / G1829A) had a frequency of 14.81% in isolates from Khyber Agency2, FR Peshawar1, FR Peshawar2, Khyber Agency1, FR DI Khan3, FR DI Khan4, Orakzai Agency6, and Orakzai Agency5. Other combinations, such as C-3 (G1579T / A1605C / T1613G / G1642T / G1666A / G1673A / A1726T / G1743A / G1746A / G1823A / G1829A / A1875C), C-4 (G1310C / T1575G / G1666A / G1673A / G1746A / G1823A / G1829A / A1875C) and occurred at a frequency of 9.26% in isolates from Mohmand Agency1, Mohmand Agency2, Mohmand Agency3, Khyber Agency3, Bajaur Agency5, FR DI Khan1, FR DI Khan2, Kurram Agency1, Kurram Agency2, and Kurram Agency3. The lowest frequency (1.85%) was observed in isolates with combinations C-12 (T1613G / G1666A / G1746A), C-13(G1579T / A1605C / T1613G / G1642T / G1666A / G1673A / G1743A / G1746A / G1823A / G1829A / A1875C), C-14 (G1310C / A1605C / G1666A / G1673A / G1743A / G1746A / G1823A / G1829A / A1875C), C-15(G1310C / G1579T / A1605C / T1613G / G1666A / G1673A / G1746A / G1823A / G1829A / A1875C), C-16 (G1310C / T1613G / G1666A / G1746A / G1829A), and C-17 (T1575G / T1613G / G1666A / G1746A / G1829A), with single isolates from Khyber Agency5, Bajaur Agency2, and Kurram Agency1. It should be noted that the DNA variants A1605C (P535P), G1743A (A581A), G1746A (K582K), and A1875T (T625T) are silent in nature and may have not any observable effect on the organism's phenotype (synonymous), and thus were not accounted in the analysis when referring to the amino acid changes in the resulting protein (Table 2). On the basis of this, only 12 mutations were observed at the protein level.

Hard and soft selective sweep model of SNPs in P. falciparum DHPS

The hard-selective sweep model implies that only a single resistant lineage survives and proliferates, effectively "sweeping" through the P. falciparum population, while the soft selective sweep model implies that resistance can emerge from multiple genetic backgrounds rather than a single lineage. Based on mutations in the current study, it was suggested that both hard and soft selective sweep were observed in the study population. The FR DI Khan showed the soft selective sweep the haplotypes emerged from FR DI. Khan, Khyber Agency and FR Peshawar and spread in all other tribal areas of Khyber Pakhtunkhwa Pakistan. While the Bajaur Agency showed the hard-selective sweep model and further no diversity was observed in the selective gene of DHPS figure 4.

Phylogenetic relationship of Plasmodium falciparum of sulfadoxine resistance-associated SNPs in P. falciparum DHPS

The phylogenetic tree shows the evolutionary relationships between different populations of Plasmodium falciparum, a parasite that causes malaria, from various areas in tribal areas of Khyber Pakhtunkhwa, Pakistan. The groups in the tree represent different regions with in tribal areas of KP, Pakistan. Overall, the 54 collected isolates are having 5 different groups of phylogenetic relationship. Based on mutations, the group 1 had five areas including FR DI Khan6, FR Kohat5, 6, Waziristan Agency5, 6, Kurram Agency4, 5, 6, Orakzai Agency1, 2, 3, 4. The group 2 had three areas isolates including Bajaur Agency1, 2, 4, 5, 6, Mohmand Agency1, 2, 3 and Khyber Agency3. The group 3 had three areas having the isolates closely similar to the group 2 based on mutations including FR DI Khan1, 2, Kurram Agency1, 2, 3 and Waziristan Agency1, 2, 3, 4. The group 4 had five areas isolates including FR Peshawar3, 4, 5, 6, Khyber Agency4, 5, 6, Mohmand Agency4, 5, 6, FR DI Khan5, Bajaur Agency3. The group 5 had 5 areas isolates including FR Kohat 1, 2, 3, 4, Orakzai Agency 5, 6, FR DI Khan 3, 4, FR Peshawar1, 2, Khyber Agency1, 2. This Phylogenetic relationship showed that the areas which were mostly near to each other and their borders were common with each other were found in same group. It can thus be inferred that the same isolates were circulating in the nearby tribal area. The haplotype G16664A, and G1746A were observed in all the tribal areas of Khyber

Pakhtunkhwa Pakistan. Due to the silent nature of G1746A, it will have no observable phenotypic effect, and thus the selective sweep model can only be claimed by the emergence and spread frequency of haplotype G1666A only (Figure 3).

Distribution network of sulfadoxine resistance-associated SNPs in P. falciparum isolates

A network of 54 P. falciparum gene DHPS haplotypes from 9 geographical tribal areas of Khyber Pakhtunkhwa region was constructed using the Neighbor-Joining method within Network 4.6.1 software by Fluxus Technology Ltd. Unnecessary median vectors and links were eliminated via star contractions (need reference). The size of each circle corresponds to the haplotypes, scaled to the frequency of sequences derived from various populations of the tribal areas of Khyber Pakhtunkhwa Pakistan. The genetic analysis revealed that Khyber Agency5 closely resembles the reference genome, with only four distinct haplotypes identified in isolate number 5. Across 54 isolates gathered from various regions of the tribal areas of KP, a total of 16 unique haplotypes were observed. For instance, FATA regions Dera Ismail Khan3 and Kohat1 each exhibited a single haplotype, as did Mohmand Agency4 and FR Peshawar. Bajaur Agency3 displayed a higher degree of similarity with one particular haplotype shared among other agencies, including Khyber Agency4, FR Peshawar3, FR DI Khan5. The largest population exhibiting identical isolates was found in FR DI Khan3, followed by FR Kohat6 and Bajaur Agency5. Notably, four haplotypes were identified between Bajaur1 and FR DI Khan6. Furthermore, with in the FR DI Khan area, four haplotypes were observed among isolates FR DI Khan6 and FR DI Khan1. Similarly, two haplotypes were noted among isolates from Bajaur Agency, specifically between Bajaur Agency5 and Bajaur Agency4. This information is illustrated in figure 4.

Area wise frequency distribution of mutations in DHPS gene of P. falciparum

The distribution of mutations across different regions or populations was found to be highest in Bajaur Agency, followed by Mohmand Agency, Khyber Agency, FR Kohat, Waziristan, FR DI Khan, and Kurram Agency. Conversely, the occurrence of mutations was observed to be lowest in FR Peshawar. This indicates that genetic variation, as measured by mutations in the dihydropteroate synthetase haplotypes, varies across different geographic regions or populations, with some regions showing higher levels of genetic diversity compared to others figure 5.

DISCUSSION

In the tribal area of Khyber Pakhtunkhwa, Pakistan, we applied molecular genetics techniques to investigate the emergence and spread of resistance following strong positive selection pressure at the dihydropteroate synthetase locus. We selected tribal areas of Khyber Pakhtunkhwa (KP) province, Pakistan because here, we present for the first time evidence of emergence and spread of signal nucleosides polymorphisms (SNPs) of dihydropteroate synthetase (DHPS) gene in plasmodium falciparum (P.f) in also due to the negligence and inappropriate treatment of patients, frequently utilizing low-quality generic sulfadoxine medications. The emergence and spread of drug resistance in Plasmodium parasites cause significant challenges to malaria control efforts worldwide. Understanding the genetic basis of resistance mutations and their distribution across different geographical regions is crucial for devising effective strategies to combat malaria. In this study, we investigated the emergence and spread of sulfadoxine-resistance mutations in P. falciparum populations from different nine tribal areas in Khyber Pakhtunkhwa by analyzing SNPs in P. falciparum DHPS. Here, we identified 15 novel genetic variations and one reported. Additionally, we explored the selective sweep model associated with these mutations to gain insights into the evolutionary dynamics of drug resistance. Plasmodium falciparum, the deadliest malaria parasite, has developed resistance to several antimalarial drugs, posing a significant challenge to global malaria control efforts. Mutations in key genes, such as DHPS play a crucial role in conferring resistance to sulfadoxine, a frontline antimalarial drug (Thu et al., 2017)). In this study, we identified multiple

mutations in the DHPS gene of P. falciparum, including E556K, R610K, M538R, and R608K, shedding light on their potential impact on drug resistance and malaria transmission dynamics.

We have identified novel mutations in P. falciparum indicating the importance of molecular such ignorance and remote areas where less healthcare and diagnostic facilities available affecting the management of P. falciparum infections. This may cause expand the drug resistance pattern of P. falciparum in such areas. Conducting molecular surveillance in tribal areas presents unique challenges, including logistical constraints, limited access to healthcare facilities, and socio-cultural barriers. However, advances in molecular biology techniques, coupled with collaborative efforts between local health authorities, research institutions, and international partners, offer opportunities to overcome these challenges and establish robust surveillance systems.

We identified 16 mutations in gene sequence of P. falciparum DHPS leading to only 12 mutations in the respective gene product, as four out of the 16 were silent in nature. The genetic mutation G1666A was observed across all tribal areas of KP, suggesting a soft selective sweep model based on the emergence and frequency of the haplotype. This underscores the persistence and dissemination of sulfadoxine resistance alleles in tribal areas of KP, Pakistan, highlighting the urgent need for effective malaria control measures in the endemic areas.

Interestingly, the frequency of resistance mutations varied between geographical regions, which may be attributed to differences in drug usage patterns and selective pressures. Variability in drug dosing regimens and treatment practices can influence the prevalence of resistance mutations with in local parasite populations. Areas with higher drug usage or suboptimal treatment practices are more likely to exhibit elevated frequencies of resistance alleles due to continuous selection pressure.

Moreover, our study investigated the selective sweep model associated with sulfadoxine resistance mutations in P. falciparum populations. Selective sweeps occur when a beneficial mutation rapidly increases in frequency with in a population, leading to a reduction in genetic diversity surrounding the locus under selection. Previous studies have demonstrated both "hard" and "soft" selective sweeps in P. falciparum populations, with the former characterized by a single resistance haplotype and the latter by the presence of multiple haplotypes (McCollum et al., 2007, 2008). In our study, we observed evidence of both soft and hard selective sweeps in P. falciparum populations from different tribal areas of KP, Pakistan. The presence of a single resistance haplotype in certain populations suggests a hard-selective sweep, indicative of strong selection pressure favoring a specific mutation. Conversely, the presence of multiple haplotypes in other populations indicates a soft selective sweep, suggesting a more gradual emergence and spread of resistance alleles. The presence of both hard and soft selective sweeps highlights the complex evolutionary dynamics underlying the emergence of drug resistance in malaria parasites. While pre-existing mutations may contribute to the initial emergence of resistance alleles, ongoing selection pressures and genetic drift can lead to the accumulation of additional mutations and the diversification of resistance haplotypes within parasite populations (Chaudhry et al., 2016).

CONCLUSIONS

Our study has highlighted the prevalence and distribution of DHPS mutations in P. falciparum isolates from tribal areas of Khyber Pakhtunkhwa, Pakistan. We observed a high frequency of mutations, indicating the presence of sulfadoxine resistance alleles.

Phylogenetic analysis revealed distinct haplotypes and suggested the circulation of similar isolates within geographically proximate regions. The two mutations (G1746A) and (G1666A) were observed as a soft selective sweep in each area of tribal areas of Khyber Pakhtunkhwa Pakistan. In a nutshell the mutations identified in the DHPS gene are known to be associated with sulfadoxine resistance in P. falciparum . Among these, mutations at positions E556K, and R610K have been extensively studied and linked to reduced drug susceptibility, leading to treatment failures in malaria-endemic regions. Additionally, mutations at positions M538R and R608K have also been implicated in

modulating sulfadoxine resistance, albeit with varying degrees of prevalence and clinical significance across different geographical settings.

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Table 1: Primer list and PCR condition for DHPS

Gene and primer	**Primer sequence**	**PCR conditions**
DHPS Outer, N1 770 bp, N2	5' GATTCTTTTTCAGATGGAGG 3' 5' TTCCTCATGTAATTCATCTGA 3'	94°C × 1 min, 51°C × 2 min, 72°C × 1 min, 40×; 72°C × 10 min

Inner, R2 711 bp, R	5' AACCTAAACGTGCTGTTCAA 3' 5' AATTGTGTGATTTGTCCACAA 3'	94°C × 1 min, 51°C × 2 min, 72°C × 1 min, 40×; 72°C × 10 min

Primers were used as previously (Pearce et al., 2003**).**

Table 2: Overall combinations of mutations in DHPS

Combination No.	Mutations combined	No. of occurrences	Frequency (%)	Isolates
C-1	G1310C / A1605C / G1666A / G1743A / G1746A / G1823A / G1829A / A1875C	9	16.67	FRDIKhan6, FRKohat5, FRKohat6, KurramAgen5, KurramAgen6, OrakzaiAgen1, OrakzaiAgen2, OrakzaiAgen3, OrakzaiAgen4
C-2	T1613G / G1666A / G1746A / G1823A / G1829A	8	14.81	KhybAgen2, FRPesh1, FRPesh2, KhybAgen1, FRDIKhan3, FRDIKhan4, OrakzaiAgen6, OrakzaiAgen5
C-3	G1579T / A1605C / T1613G / G1642T / G1666A / G1673A / A1726T / G1743A / G1746A / G1823A / G1829A / A1875C	5	9.26	MohmundAgen1, MohmundAgen2, MohmundAgen3, KhybAgen3, BajAgen5
C-4	G1310C / T1575G / G1666A / G1673A / G1746A / G1823A / G1829A / A1875C	5	9.26	FRDIKhan1, FRDIKhan2, KurramAgen1, KurramAgen2, KurramAgen3
C-5	T1575G / T1613G / G1642T / G1666A / G1746A / G1823A / G1829A	4	7.41	FRKohat1, FRKohat2, FRKohat3, FRKohat4
C-6	T1575G / T1613G / G1666A / G1746A	4	7.41	MohmundAgen4, MohmundAgen5, MohmundAgen6, KhybAgen6
C-7	G1310C / T1575G / T1613G / G1666A / G1673A / G1746A / G1823A / G1829A / A1875C	4	7.41	WaziristanAgen1, WaziristanAgen2, WaziristanAgen3, WaziristanAgen4
C-8	G1310C / T1575G / T1613G / G1666A / G1673A / G1746A / G1829A	3	5.56	FRPesh6, FRDIKhan5, FRPesh5
C-9	G1579T / A1605C / A1607T / T1613G / G1642T / G1666A / G1673A / A1726T / G1743A / G1746A / G1823A / G1829A / A1875C / A1889T	2	3.70	BajAgen4, BajAgen6
C-10	G1310C / T1575G / A1605C / G1666A / G1673A / G1743A /	2	3.70	WaziristanAgen5, WaziristanAgen6

	G1746A / G1823A / G1829A / A1875C			
C-11	G1310C / T1575G / T1613G / G1666A / G1746A	2	3.70	FRPesh3, FRPesh4
C-12	T1613G / G1666A / G1746A	1	1.85	KhybAgen5
C-13	G1579T / A1605C / T1613G / G1642T / G1666A / G1673A / G1743A / G1746A / G1823A / G1829A / A1875C	1	1.85	BajAgen2
C-14	G1310C / A1605C / G1666A / G1673A / G1743A / G1746A / G1823A / G1829A / A1875C	1	1.85	KurramAgen4
C-15	G1310C / G1579T / A1605C / T1613G / G1666A / G1673A / G1746A / G1823A / G1829A / A1875C	1	1.85	BajAgen1
C-16	G1310C / T1613G / G1666A / G1746A / G1829A	1	1.85	KhybAgen4
C-17	T1575G / T1613G / G1666A / G1746A / G1829A	1	1.85	BajAgen3

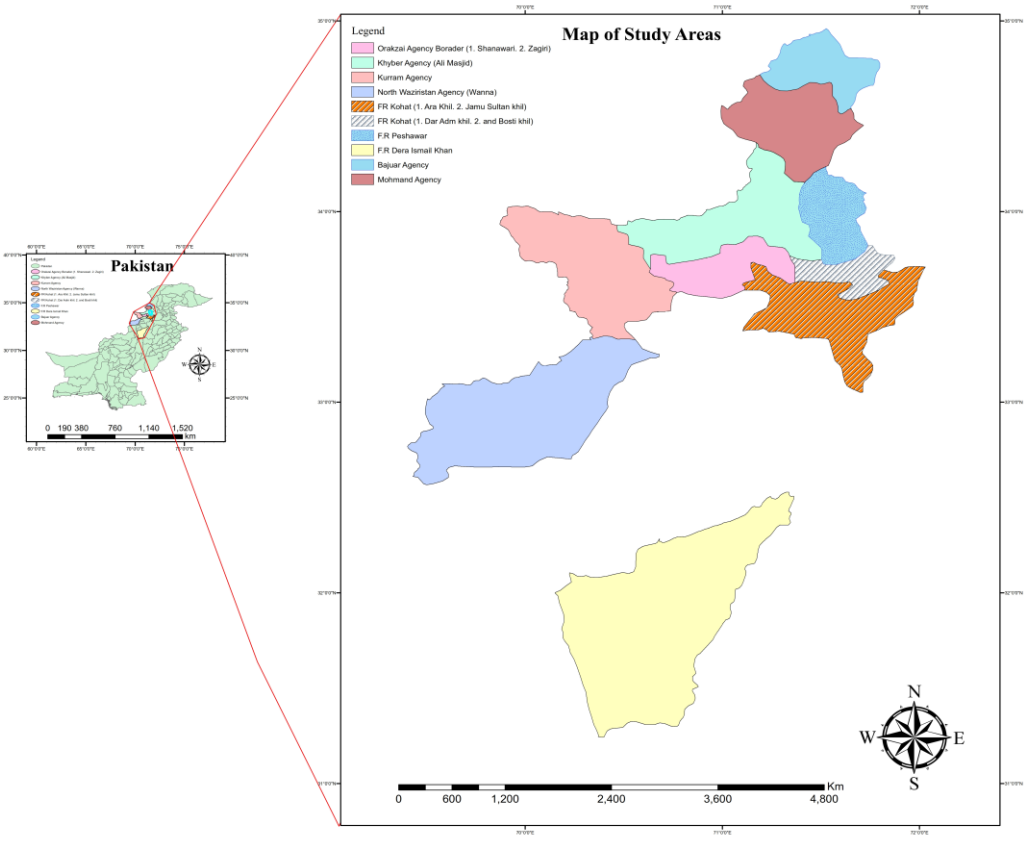

Figure 1: Geographic location of the sample collection sites in the different agencies and Federal Administration Trible Areas (FATA) of Khyber Pakhtunkhwa

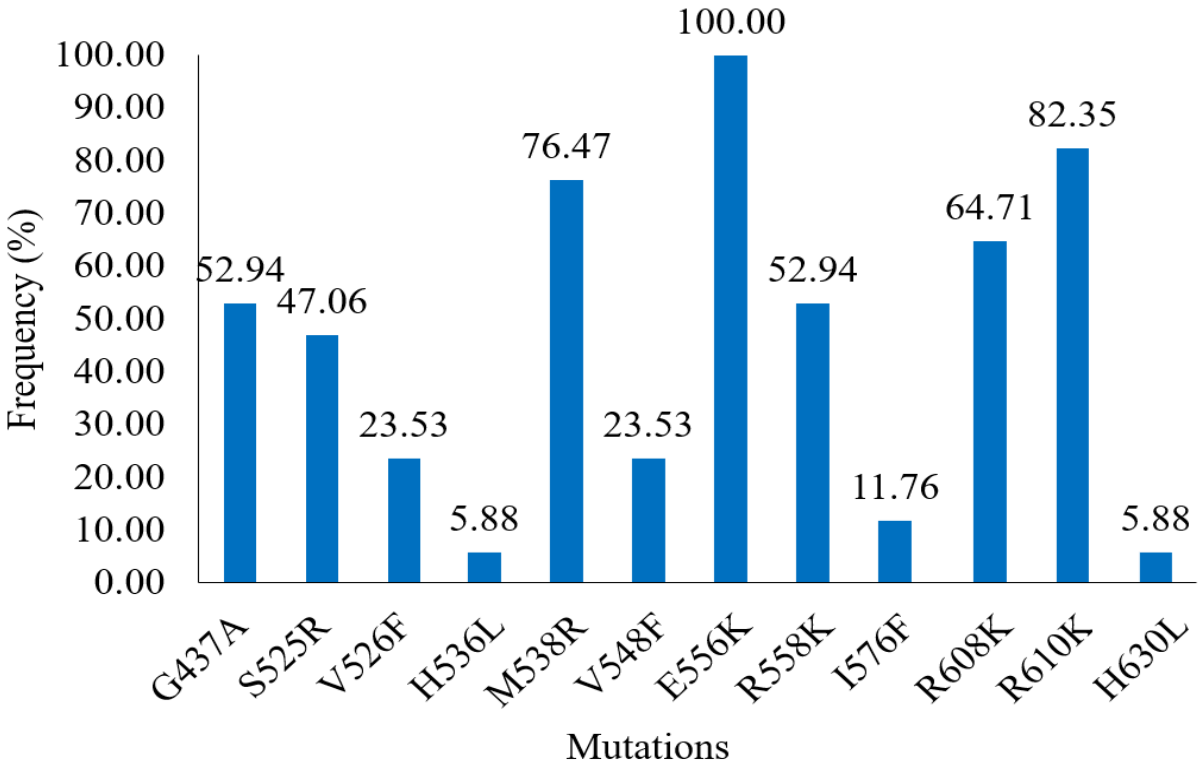

Figure 2: Overall mutation frequency in DHPS gene product of Plasmodium falciparum collected from tribal areas. Variants A1605C (P535P), G1743A (A581A), G1746A (K582K), and A1875T (T625T) are not included here due to their silent (synonymous) nature

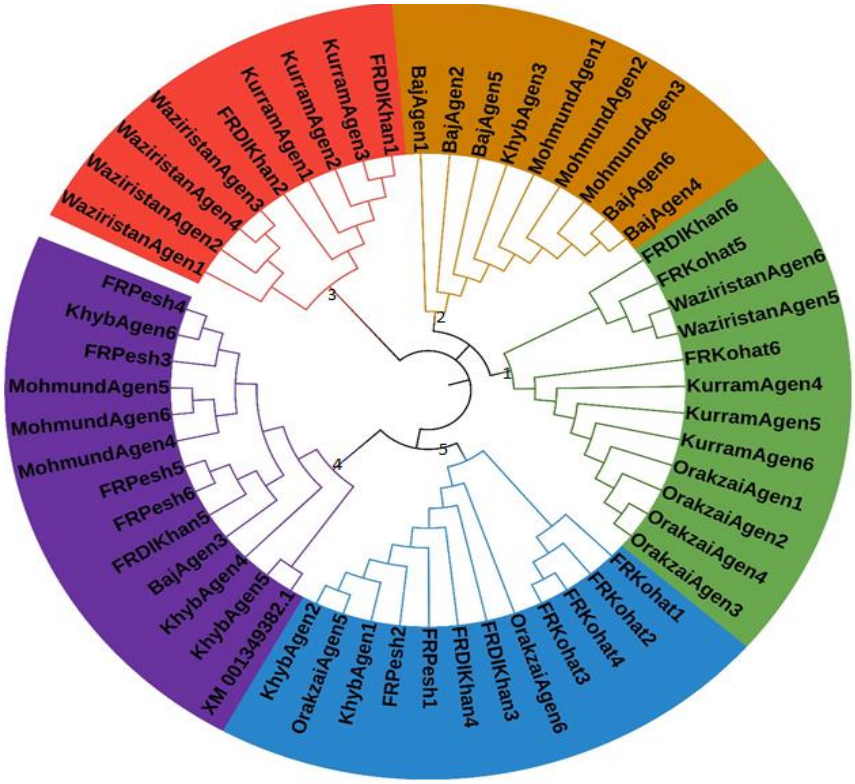

Figure 3: Phylogenetic relationship of the collected isolates of Plasmodium falciparum collected from tribal areas of Pakistan

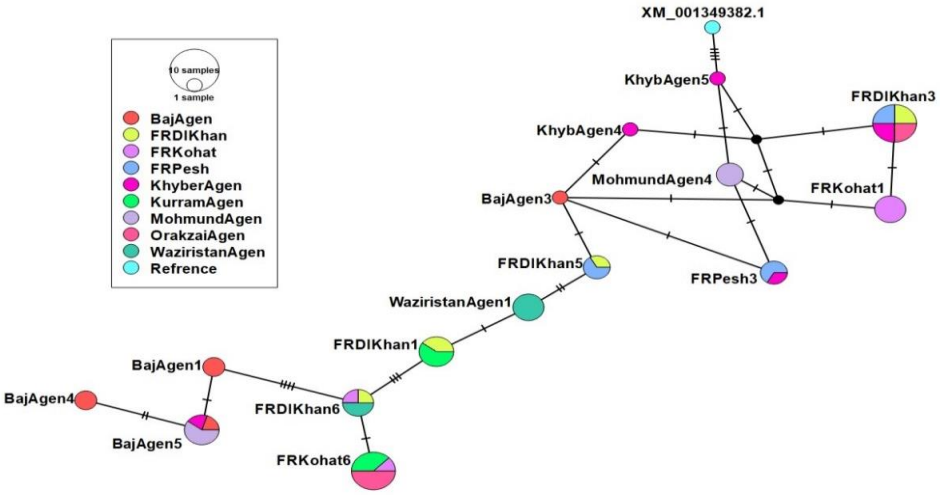

Figure 4: The neighbor-joining network tree of 54 P. falciparum dihydropteroate synthetase (DHPS) haplotypes from nine geographical tribal areas of Khyber Pakhtunkhwa region was constructed using the Neighbor-Joining method within Network 4.6.1 software by Fluxus Technology Ltd. Unnecessary median vectors and links were eliminated via star contractions (need reference). The size of each circle corresponds to the haplotypes, scaled to the frequency of sequences derived from various populations of the tribal areas of Khyber Pakhtunkhwa Pakistan.

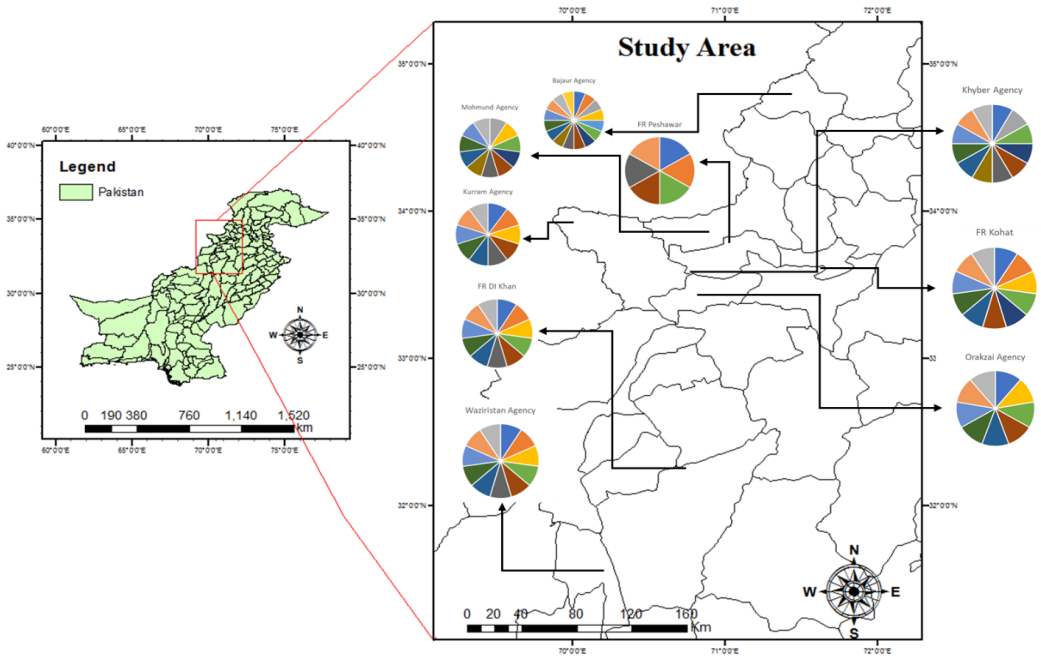

Figure 5: As shown in the map, the colors within the pie chart circles represent the frequency of haplotypes and their distribution throughout the populations. Length of lines linking haplotypes corresponding to number of nucleotide changes indicates number of mutations between neighboring sequence nodes along connecting branches. Each haplotype's carried mutation is distinguished by color as follows.