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Genomic Evidence of Pyrimethamine Resistance and Selective Sweeps in Plasmodium Vivax from Pakistan

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ABSTRACT

Background*: Resistance to antifolate drugs such as pyrimethamine threatens malaria elimination in South and Southeast Asia. Mutations in the dihydrofolate reductase (dhfr) gene of Plasmodium vivax reduce drug efficacy by altering enzyme binding, leading to treatment failure. Despite P. vivax causing most malaria cases in Pakistan, genomic data on antifolate resistance remain scarce. This study examines dhfr polymorphisms in P. vivax isolates from Punjab, Pakistan, using deep amplicon sequencing to assess mutation prevalence, genetic diversity, and signatures of positive selection, providing critical insights into the molecular evolution and regional spread of pyrimethamine-resistant P. vivax lineages.*

Methods: *A total of 38 P. vivax–positive blood samples collected during a cross-sectional survey of malaria-endemic districts were analysed using deep amplicon sequencing of the dhfr locus. After quality filtering, single-nucleotide polymorphisms (SNPs) associated with antifolate resistance were quantified. Haplotype reconstruction and genetic diversity analyses were performed using DnaSP and Network 4.6.1, while neutrality and selection pressures were assessed through Tajima's D and Fay & Wu's H statistics.*

Results: *Three major nonsynonymous dhfr mutations—S58R, S117N, and I173L—were identified, with the S58R/S117N double mutant being most prevalent (26%). Allele frequencies ranged from 2.1% to 100%, indicating differential local selection intensity. Fourteen isolates exhibited strong signals of positive directional selection (Tajima's D = −2.1), consistent with ongoing selective sweeps driven by antifolate exposure. Forty-two unique dhfr haplotypes were detected, nine dominated by resistant alleles (>95% frequency) characteristic of hard sweeps, and five showing multiple coexisting haplotypes suggestive of soft sweeps. The distribution of resistant haplotypes across multiple districts indicates gene flow and regional transmission of resistant lineages.*

Conclusion: *This genomic analysis provides compelling evidence of widespread pyrimethamine resistance and adaptive evolution within P. vivax populations in Pakistan. The predominance of double- and triple-dhfr mutant haplotypes reflects sustained antifolate pressure and the emerging fixation of resistant alleles. Integrating genomic surveillance into national malaria control frameworks is imperative for early detection of resistance trends, refinement of treatment policies, and prevention of further spread of antifolate-resistant P. vivax strains.*

Keywords: *Plasmodium vivax, dihydrofolate reductase (dhfr), pyrimethamine resistance, selective sweep, antifolate, Pakistan*

INTRODUCTION

Malaria continues to be a major global health challenge, responsible for considerable morbidity and mortality in tropical and subtropical regions. Despite sustained control efforts, an estimated 249 million malaria cases and over 600,000 deaths were reported worldwide in 2023, the majority caused by Plasmodium falciparum and Plasmodium vivax (Souleiman, 2024). While P. falciparum predominates in sub-Saharan Africa, P. vivax is the leading cause of malaria outside Africa and is responsible for substantial disease burden across South and Southeast Asia, including Pakistan (Bin, 2013). In Pakistan, malaria transmission is seasonal and unstable, with endemic foci in Balochistan, Sindh, and Khyber Pakhtunkhwa provinces, as well as the Federally Administered Tribal Areas bordering Afghanistan. The national malaria control program recognises P. vivax as the dominant species, accounting for approximately 80% of confirmed cases annually (Khan, 2023) . Although chloroquine remains the frontline treatment for P. vivax malaria in Pakistan, the historical use of antifolate drugs—particularly the combination of sulfadoxine and pyrimethamine (SP)—has played a significant role in malaria management, either as a standalone therapy or in combination with other drugs for P. falciparum infections (Leslie, 2007) . SP targets the folate biosynthesis pathway by inhibiting two key enzymes: dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR). Pyrimethamine, a competitive inhibitor of DHFR, interferes with the conversion of dihydrofolate to tetrahydrofolate, an essential step in DNA synthesis. However, widespread and often unsupervised use of antifolates has exerted strong selective pressure on Plasmodium populations, leading to the emergence of resistant strains.

In P. falciparum, SP resistance became widespread during the 1990s and early 2000s, largely driven by point mutations in the dhfr and dhps genes (Nair, 2003). These mutations alter the structure of DHFR, reducing its binding affinity for pyrimethamine and allowing the parasite to survive therapeutic drug concentrations. Over time, stepwise accumulation of mutations—such as N51I, C59R, S108N, and I164L—resulted in high-grade pyrimethamine resistance and treatment failure (Lozovsky, 2009) . Although P. vivax is less studied than P. falciparum due to challenges in continuous in vitro culture and genomic manipulation, increasing molecular evidence shows that P. vivax has independently evolved antifolate resistance through similar mechanisms. The dhfr gene of P. vivax exhibits several single-nucleotide polymorphisms (SNPs) that reduce susceptibility to pyrimethamine, including mutations at positions F57L/I, S58R, T61M, S117N/T, and I173L (Hawkins, 2008; Kaur, 2006) . Each of these substitutions affects the enzyme's active site, decreasing its affinity for pyrimethamine while maintaining catalytic efficiency for folate reduction. The sequential accumulation of these substitutions produces a spectrum of resistance phenotypes, ranging from mild to high-level resistance. Molecular epidemiological studies across Asia and Oceania have revealed that certain dhfr haplotypes, such as the double mutant S58R/S117N and the triple mutant F57L/S58R/S117N, have become predominant under sustained antifolate pressure. In Thailand, Cambodia, and Indonesia, these alleles have reached near fixation, demonstrating strong positive selection and reduced genetic diversity in the dhfr locus—a hallmark of selective sweeps (Nair, 2003; Hastings, 2005). Phylogenetic and population genetic analyses indicate that these resistant haplotypes have evolved both independently and through recombination, resulting in convergent evolution across geographically distant P. vivax populations (Ngwana-Joseph, 2024).

In Pakistan, where antifolate drugs have been widely used for decades in both formal and informal healthcare systems, the molecular basis of P. vivax antifolate resistance remains insufficiently characterised. Early molecular surveys relied primarily on PCR–restriction fragment length polymorphism (RFLP) or Sanger sequencing, which limited their ability to detect low-frequency variants and mixed infections (Hublin, 2022). For example, (Khan, 2023) reported sporadic dhfr mutations but

lacked population-level resolution to evaluate selection dynamics or haplotype diversity. These limitations have hindered the identification of emerging resistant alleles and the understanding of evolutionary mechanisms shaping resistance within local parasite populations. The evolutionary dynamics of antifolate resistance in P. vivax are further complicated by its biological and ecological characteristics. Unlike P. falciparum, P. vivax can form dormant liver-stage hypnozoites that reactivate and cause relapses weeks or months after the primary infection, sustaining transmission even in low-transmission settings (Schäfer, 2021) . Moreover, P. vivax populations exhibit higher levels of genetic diversity and recombination, increasing their capacity for adaptation under drug pressure. This diversity can mask selective sweeps and allow multiple resistant lineages to coexist—a phenomenon known as "soft selective sweeps" (Nair, 2003) . Selective sweeps represent genomic footprints of adaptation, wherein beneficial mutations rise in frequency and reduce genetic variation in surrounding loci. "Hard sweeps" occur when a single advantageous mutation rapidly reaches fixation, while "soft sweeps" arise when several resistance alleles emerge independently or through recombination and simultaneously increase in frequency (Messer, 2013) . Identifying whether resistance in P. vivax is driven by hard or soft sweeps is critical to understanding its evolutionary trajectory and predicting future trends in drug efficacy.

With the advent of high-throughput sequencing, particularly deep amplicon and next-generation sequencing (NGS) technologies, it is now possible to detect low-frequency alleles, characterise haplotype structures, and quantify genetic diversity with unprecedented resolution. Deep sequencing of the dhfr locus allows the identification of minor resistant variants that would otherwise escape detection in standard genotyping assays, providing insight into ongoing selection and the potential for resistance expansion (Kunasol, 2022) . Moreover, coupling allele frequency data with neutrality tests such as Tajima's D and Fay & Wu's H enables the detection of genomic regions under selection pressure, revealing whether antifolate resistance is evolving through selective sweeps or neutral drift. In Pakistan, the continued use of SP and related antifolates in malaria treatment and prophylaxis—often without strict diagnostic confirmation—has created an environment conducive to selection for resistant P. vivax genotypes. This situation is exacerbated by cross-border population movement from Afghanistan and Iran, where antifolate-resistant malaria strains have been reported (Khan, 2023). The interplay of drug pressure, gene flow, and local ecological variation provides a unique context for studying resistance evolution in Pakistani P. vivax populations.

Despite this significance, comprehensive genomic studies of antifolate resistance in P. vivax from Pakistan remain scarce. There is an urgent need to generate baseline genomic data to inform national drug policies and integrate molecular surveillance into malaria control frameworks. Understanding the structure of dhfr polymorphism, the distribution of resistance alleles, and the presence of selective sweeps will provide valuable insights into local transmission dynamics and the potential spread of resistant lineages. The present study addresses this gap by applying deep amplicon sequencing to analyse the dhfr locus in P. vivax isolates from malaria-endemic regions of Punjab, Pakistan. By quantifying allele frequencies, reconstructing haplotypes, and evaluating neutrality indices, this work aims to elucidate the evolutionary mechanisms driving antifolate resistance and assess whether selective sweeps—either hard or soft—are shaping the genetic architecture of dhfr in regional parasite populations. Findings from this research will contribute to the growing body of knowledge on P. vivax population genetics and resistance evolution, and may inform evidence-based strategies for the containment of drug-resistant malaria in Pakistan and neighbouring regions.

MATERIALS AND METHODS

Study Design and Sample Collection

A cross-sectional study was conducted between July and October 2023 in malaria-endemic districts of Punjab, Pakistan, including Lahore, Dera Ghazi Khan, and Gujranwala. Peripheral blood samples were

collected from symptomatic patients attending basic health units and diagnostic centres. Ethical approval was obtained from the Departmental Ethical Review Committee (Ref. FOST/DERC/2024/01), and written informed consent was secured from all participants before sample collection. Malaria infection was initially confirmed by microscopy and rapid diagnostic tests (RDTs), and only *Plasmodium vivax* mono-infections were included for molecular analysis. Confirmed positive samples were preserved at −20 °C until further processing.

DNA Extraction and Target Amplification

Genomic DNA was extracted from 200 µL of whole blood using the TIANamp Blood DNA Kit (Tiangen, China), following the manufacturer's protocol. A 700 bp fragment of the *dihydrofolate reductase (dhfr)* gene encompassing key resistance-associated codons was amplified using custom Illumina-barcoded primers. Polymerase chain reaction (PCR) products were purified with AMPure XP magnetic beads and quantified spectrophotometrically to ensure optimal library balance.

Deep Amplicon Sequencing

Purified amplicons were pooled and subjected to Illumina MiSeq sequencing using a 2 × 250 bp paired-end protocol. Raw reads were quality-filtered, demultiplexed, and aligned against the *P. vivax dhfr* reference sequence (GenBank accession XM_001615397) using the QIIME 2 and Mothur pipelines. Variant detection and allele frequency estimation were performed with VCFtools, and low-quality variants (depth < 30× or Q < 30) were excluded from downstream analyses.

Haplotype and Diversity Analysis

Distinct *dhfr* haplotypes were reconstructed in DnaSP v6 and visualised using Network 4.6.1. Genetic diversity indices—haplotype diversity (Hd) and nucleotide diversity (π)—were calculated for each population. Neutrality and selection pressure were assessed through Tajima's D and Fay & Wu's H statistics to detect signatures of directional selection and selective sweeps.

Statistical Analysis

Allele frequencies were expressed as percentages of total reads per locus. Selection was considered significant when Tajima's $D < -1.5$ or $p < 0.05$. All statistical computations and visualisations were performed using R v4.2.2.

RESULTS

Mutation Frequencies and Distribution

Analysis of 38 *Plasmodium vivax* isolates revealed multiple single-nucleotide polymorphisms (SNPs) in the *dihydrofolate reductase (dhfr)* gene that are known to confer pyrimethamine resistance. Three key nonsynonymous mutations were identified at codons S58R**,** S117N, and I173L, while no substitutions were detected at codons F57L/I or T61M, which have been reported in some Southeast Asian populations. The S58R/S117N double mutant was the most prevalent genotype, observed in 10 isolates (26.3%), followed by single S117N mutations in eight isolates (21%) and single S58R in one isolate (2.6%). The I173L substitution occurred in three isolates (7.8%) and was frequently found in combination with S117N, suggesting additive resistance effects. Allele frequencies across all samples ranged from 2.1% to 100%**,** indicating marked variability in resistance allele prevalence among different districts. Several isolates demonstrated near fixation (>95%) of resistant alleles, consistent with strong localised selection pressure. The high prevalence of the S58R/S117N combination aligns with patterns observed in South and Southeast Asia, where this double mutant is strongly associated with high-level pyrimethamine resistance and reduced drug efficacy (Shaukat, 2018).

Table 1: Relative allele frequencies of dhfr pyrimethamine-resistance mutations across isolates.

Codon Position	Amino Acid Substitution	Type of Mutation	No. of Isolates (n = 38)	Frequency (%)	Resistance Level / Interpretation
57	F57L / F57I	Nonsynonymous (absent in this study)	0	0.0	Not detected
58	S58R	Nonsynonymous	11	28.9	Moderate resistance; frequently combined with S117N
61	T61M	Nonsynonymous (absent in this study)	0	0.0	Not detected
117	S117N	Nonsynonymous	18	47.4	Key pyrimethamine-resistance mutation
173	I173L	Nonsynonymous	3	7.9	Associated with enhanced resistance when combined with S117N
58 + 117	S58R/S117N (Double mutant)	Combined haplotype	10	26.3	High-level pyrimethamine resistance
58 + 117 + 173	S58R/S117N/I173L (Triple mutant)	Combined haplotype	2	5.3	Very high-level resistance (rare)

Notes

- Frequencies are based on read depth–weighted allele proportions across all isolates.
- Mutations at codons F57L/I and T61M were not detected in this dataset.
- Double and triple mutant haplotypes were inferred from the co-occurrence of nonsynonymous substitutions within individual isolates.

Genetic Diversity and Neutrality

Population genetic analyses revealed substantial variation in genetic diversity among isolates. Haplotype diversity (Hd) ranged from 0.159 to 0.822, suggesting the coexistence of both clonal and heterogeneous populations across sampling sites. A total of 33 polymorphic sites were identified within the *dhfr* locus, reflecting moderate genetic differentiation among parasite populations. Neutrality tests supported the presence of positive directional selection. Fourteen isolates exhibited significantly negative Tajima's D values (ranging from −2.1 to 1.8), coupled with negative Fay & Wu's H scores, consistent with an excess of low-frequency polymorphisms. These results indicate recent selective sweeps likely driven by antifolate drug pressure in the study regions.

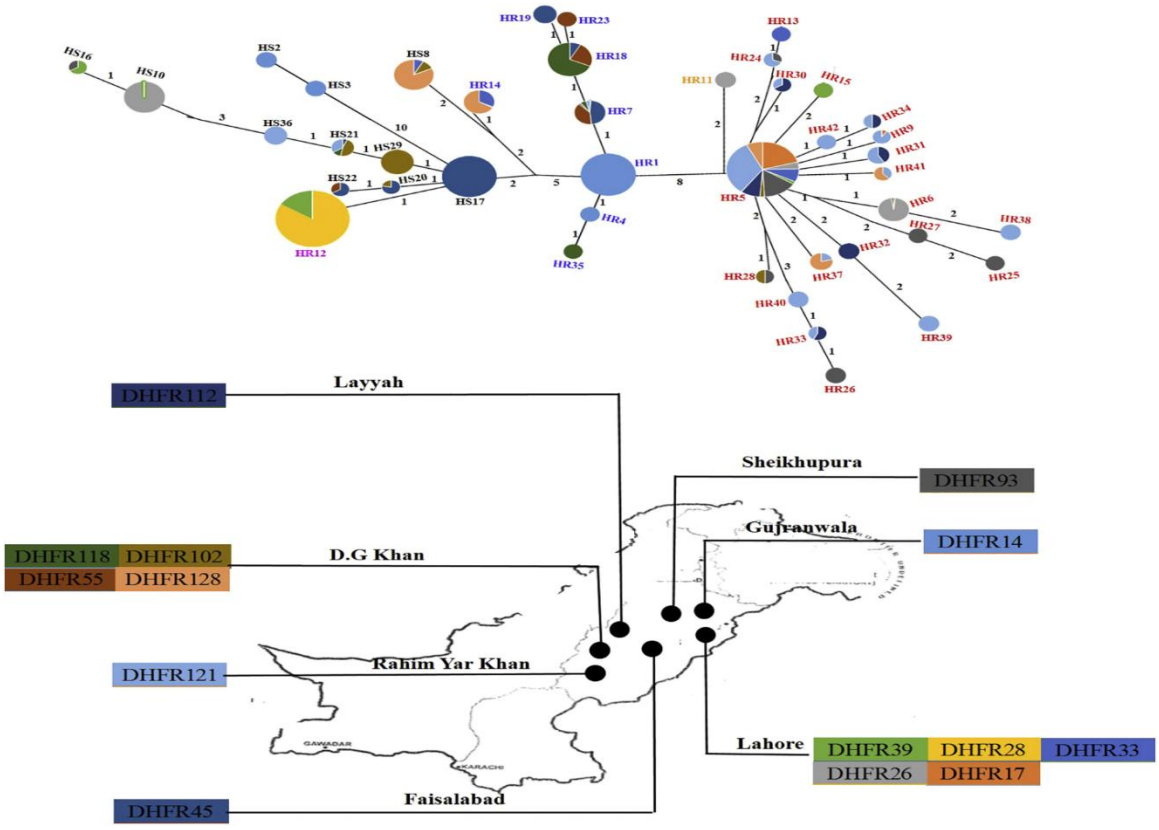
Haplotype Structure and Selective Sweeps

Haplotype reconstruction identified 42 distinct dhfr haplotypes among the 38 isolates analysed. Of these, nine isolates displayed dominant resistant haplotypes with frequencies exceeding 95%, consistent with hard selective sweeps indicative of recent fixation events. Conversely, five isolates exhibited multiple coexisting haplotypes without a single dominant genotype, reflecting soft selective sweeps arising from either recurrent mutation or genetic recombination. Phylogenetic network analysis further illustrated the

evolutionary relationships among haplotypes. Haplotypes carrying the S58R/S117N double mutation clustered independently from susceptible types, suggesting convergent evolution of resistance under similar selective pressures. A particularly notable resistant haplotype, designated HR5, occupied a central position in the median-joining network and was distributed widely across multiple districts, including Lahore, Dera Ghazi Khan, and Gujranwala. The widespread presence of HR5 suggests that this lineage possesses a strong adaptive advantage and may represent a highly transmissible resistant strain under continuous antifolate selection.

These results collectively reveal that *P. vivax* populations in Punjab harbour significant *dhfr* polymorphism, driven by both local selection and regional gene flow. The coexistence of hard and soft selective sweeps demonstrates ongoing adaptive evolution in response to antifolate pressure and underscores the dynamic nature of drug resistance in *P. vivax* populations.

Figure 1: Median-joining haplotype network of dhfr variants.



DISCUSSION

This study provides strong genomic evidence of widespread pyrimethamine resistance and adaptive evolution in Plasmodium vivax populations circulating in Pakistan. The detection of multiple nonsynonymous mutations in the dihydrofolate reductase (dhfr) gene, particularly the S58R and S117N substitutions, underscores a persistent antifolate selection pressure within regional parasite populations. The predominance of these mutations parallels findings from endemic areas across Southeast Asia—such as Thailand, Myanmar, and Indonesia—where double and triple dhfr mutants have become fixed following prolonged sulfadoxine–pyrimethamine (SP) use (Kuesap, 2022; Mita, 2014) . These patterns collectively suggest that P. vivax in Pakistan is undergoing a similar trajectory of antifolate resistance

evolution driven by extensive historical and ongoing drug exposure. The co-occurrence of hard and soft selective sweeps observed in this dataset reflects complex evolutionary dynamics acting upon P. vivax dhfr. Hard sweeps arise when a single advantageous mutation rapidly increases in frequency and becomes fixed, resulting in reduced genetic diversity around the selected locus (Nair, 2003) . Conversely, soft sweeps emerge when multiple resistance alleles arise independently or through recombination and concurrently expand in response to drug pressure (McCollum, 2012). The presence of both sweep types in Pakistani isolates indicates that antifolate resistance is being maintained through a combination of recent fixation events and ongoing introduction or recombination of resistance alleles. Such mixed patterns are characteristic of highly recombining populations under variable drug selection, a hallmark of P. vivax evolutionary ecology (Hupalo, 2016).

The high haplotype diversity observed among resistant isolates likely reflects geographic and pharmacological heterogeneity in drug exposure. In Pakistan, malaria treatment practices vary considerably between districts and healthcare settings, with SP sometimes used empirically for undiagnosed febrile illnesses or in mixed infections involving P. falciparum (Khan, 2023) . This inconsistent drug pressure may have generated a patchwork of local selection regimes, facilitating the coexistence of multiple dhfr variants and recombination between resistant and susceptible genotypes. Moreover, the presence of dominant resistant haplotypes across several districts, including Lahore, Gujranwala, and Dera Ghazi Khan, indicates gene flow between regions and suggests that human mobility contributes to the spread of resistant alleles. Subclinical or asymptomatic carriage of P. vivax—a well-documented phenomenon—further enhances the potential for regional dissemination of resistance (Zhao, 2020). Compared with P. falciparum, P. vivax displays greater population-level genetic diversity and recombination potential, partly due to its ability to relapse via dormant hypnozoite stages and infect Duffy-positive as well as some Duffy-negative hosts (Ord, 2008) . This inherent genomic plasticity enables P. vivax to adapt rapidly to environmental and therapeutic pressures. Consequently, antifolate resistance in P. vivax may not follow the same linear fixation pattern observed in P. falciparum, but instead proceed through a mosaic of partial sweeps and recombination-driven diversification. The predominance of S58R/S117N double mutants, along with sporadic detection of I173L, indicates an intermediate evolutionary stage—where moderate resistance alleles have become common while additional substitutions required for high-level resistance (such as F57L/I or T61M) remain rare. Similar transitional resistance profiles have been reported in early-stage resistance zones of Vietnam and India (Phu, 2022).

The neutrality analyses conducted in this study further support the hypothesis of ongoing directional selection rather than neutral genetic drift at the dhfr locus. Strongly negative Tajima's D values (−2.1) across multiple isolates suggest an excess of low-frequency polymorphisms, consistent with recent selective sweeps following antifolate exposure. This statistical signal aligns with previous population genetic studies demonstrating that antifolate drug pressure can produce distinct signatures of selection in Plasmodium populations (Nair, 2003). In Pakistan, sustained SP use within public health programs and private markets, combined with incomplete adherence and self-medication, likely maintains this selective pressure and contributes to the persistence of resistant dhfr alleles. The fixation of specific resistant haplotypes across multiple geographic areas also suggests that P. vivax resistance evolution in Pakistan involves both local adaptation and regional dissemination. Migration and cross-border movement between Pakistan, Afghanistan, and Iran—regions sharing similar malaria ecologies and treatment practices—may further accelerate the spread of resistant lineages (Khan, 2023). This scenario underscores the importance of coordinated cross-border molecular surveillance and data sharing to track resistant haplotypes and predict their regional trajectories. From an evolutionary perspective, the coexistence of multiple resistance haplotypes implies that P. vivax populations are experiencing episodic rather than uniform drug pressure. While hard sweeps may represent strong local drug selection or a single successful resistant lineage, soft sweeps indicate recurrent mutation and gene flow across subpopulations. The interplay of these forces

creates a dynamic resistance landscape that can shift rapidly with changing treatment practices or drug availability. Consequently, antifolate resistance in P. vivax should be viewed not as a static endpoint but as an ongoing evolutionary process responsive to both pharmacological and ecological pressures.

The absence of certain high-resistance mutations (F57L/I, T61M) observed in Southeast Asian populations suggests that Pakistani P. vivax populations are at an intermediate stage of antifolate resistance evolution. This transitional state offers an opportunity for intervention through rational drug policy and controlled use of antifolates. Phasing out SP monotherapy, improving diagnostic precision to reduce unnecessary antifolate exposure, and monitoring resistance markers through genomic surveillance could delay or prevent fixation of high-level resistant haplotypes. These findings also highlight the need for integrating genomic data with epidemiological and pharmacological surveillance. By correlating resistance genotypes with drug-use patterns, health authorities can identify emerging hotspots of antifolate resistance and adapt national treatment guidelines accordingly. Routine molecular monitoring of dhfr and associated genes (e.g., dhps, pvdhfr-ts) would further enhance the understanding of multifactorial resistance mechanisms. This study demonstrates that P. vivax in Pakistan exhibits substantial dhfr polymorphism, strong signatures of positive selection, and complex patterns of hard and soft selective sweeps. These results are consistent with sustained antifolate selection pressure and regional transmission of resistant haplotypes. Continuous genomic monitoring, coupled with rational drug policy and regional collaboration, is essential to prevent further spread of pyrimethamine-resistant P. vivax and to safeguard the effectiveness of current malaria treatment strategies in Pakistan and beyond.

LIMITATIONS

This study has several important limitations that should be considered when interpreting the findings. First, its cross-sectional design provides a snapshot of Plasmodium vivax antifolate resistance at a single time point and does not capture temporal changes in allele frequencies or the evolution of resistance over successive transmission seasons. A longitudinal approach would better reveal how dhfr haplotypes fluctuate under ongoing drug pressure.

Second, the sample size was moderate and geographically confined to malaria-endemic districts of Punjab. While representative of regional transmission, it may not fully reflect the genetic diversity of P. vivax populations across Pakistan, particularly in hyperendemic zones such as Balochistan and Khyber Pakhtunkhwa. Broader geographic sampling would strengthen the generalizability of these findings.

Third, this investigation focused exclusively on the dhfr locus. Other antifolate-related genes, such as dhps and potential compensatory loci, were not analysed, limiting insights into the full molecular basis of resistance. Additionally, clinical outcome data were not collected; thus, correlations between genotypes and therapeutic efficacy could not be established.

Although deep amplicon sequencing offers high sensitivity, it may still be influenced by PCR bias or sequencing artifacts. Future research should integrate multi-gene genomic approaches, drug efficacy trials, and longitudinal sampling to provide a comprehensive understanding of antifolate resistance evolution and its clinical implications in P. vivax malaria.

CONCLUSION

This study provides molecular and population-genetic evidence of extensive dihydrofolate reductase (dhfr) polymorphism and strong selective sweeps in Plasmodium vivax populations circulating in Pakistan. Through deep amplicon sequencing, multiple nonsynonymous mutations associated with pyrimethamine resistance were identified, with the S58R/S117N double mutant emerging as the most prevalent. The frequent occurrence of double and triple mutant haplotypes indicates high-level antifolate resistance sustained by persistent drug selection pressure within regional parasite populations. Patterns of both hard

and soft selective sweeps highlight complex evolutionary dynamics, reflecting recurrent mutation, recombination, and possible gene flow between endemic districts. These findings suggest that P. vivax populations in Pakistan are undergoing an adaptive response to long-term antifolate exposure and may represent an intermediate stage in the fixation of fully resistant alleles.

The results underscore the urgent need to integrate genomic surveillance into national malaria control and elimination programs. Regular molecular monitoring of dhfr and related resistance genes, coupled with rational drug-use policies, can help detect emerging resistant lineages before widespread dissemination occurs. Strengthening molecular diagnostics and expanding regional data sharing will be critical for guiding treatment strategies, refining drug policy, and preventing the further spread of antifolate-resistant P. vivax across Pakistan and neighbouring regions.

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Ethical Approval

Approved by the Departmental Ethical Review Committee, Faculty of Science and Technology, University of Central Punjab (Ref: FOST/DERC/2024/01).

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Declaration of Competing Interests

None declared.

Ethics approval and consent to participate

We confirm that this research was conducted in line with the journal's guidelines on the protection of research participants, and participation in the study was informed and voluntary. The Departmental Ethical Review Committee of the faculty of Science & Technology, University of Central Punjab, Lahore, Pakistan, has approved this study and the instruments used in this study for the data collection. The participation in this study was voluntary, and informed consent was obtained before filling out the questionnaire.

Consent for publication: yes

Availability of data and material: yes

Conflict of interest: None, declared

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Author contribution statement

Sobia Nosheen Nadeem led the data curation, formal analysis, investigation, and methodology. Dr Aatif Amin contributed to the Project administration, Validation and supervision. Conceptualisation, Writing, review & editing were done by Ayaz Shaukat.

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