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MAJOR ARTICLE

Targeted Genomic Surveillance Unveils Genetic Variations Linked to Regional Malaria Drug Resistance Dynamics in India

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Background. India has made substantial progress in reducing *Plasmodium falciparum* malaria cases and has set a target to eliminate malaria by 2030. Although artemisinin-based combination therapy (ACT) treatment remains effective, tracking regional differences in genetic variants associated with antimalarial resistance is required for effective drug policy implementation.

Methods. We analyzed 238 *P. falciparum* clinical samples from 6 Indian states by sequencing 15 parasite genes associated with reduced drug effectiveness. The method involved nanopore sequencing of target gene amplicons derived from dried blood spots using a highly-sensitive PfMDR15 surveillance panel.

Results. India's historical policy of artesunate-sulfadoxine-pyrimethamine in central India and artemether-lumefantrine in the Northeast has shaped contrasting resistance profiles. In the Northeast, chloroquine resistance persisted at high frequency (*Pfcrt* K76T and CVIET haplotype; *Pfaat1* S258L), alongside quintuple and sextuple *Pfdhfr–Pfdhps* haplotypes conferring complete sulfadoxine–pyrimethamine resistance. Central India showed variable chloroquine resistance (parasites largely retained wild-type *Pfcrt*) and emerging lumefantrine tolerance (*Pfmdr1* Y184F, *Pfaat1* S258L). Interestingly, Delhi (Central India) parasites resembled profiles from the distant Northeast, which borders South East Asia. The detection of *Pfaat1* S258L, previously reported only from Africa and associated with reduced lumefantrine susceptibility, suggests convergent evolution under ACT partner-drug pressure. No WHO-validated *Pfk13* artemisinin resistance mutations were detected, supporting continued efficacy of ACT.

Conclusions. India's resistance landscape is fragmented, with signals of expanding lumefantrine tolerance and importation or evolution of globally relevant mutations. These findings highlight the importance of integrating molecular genomic surveillance into malaria control policy to monitor and protect ACT effectiveness and advance malaria elimination.

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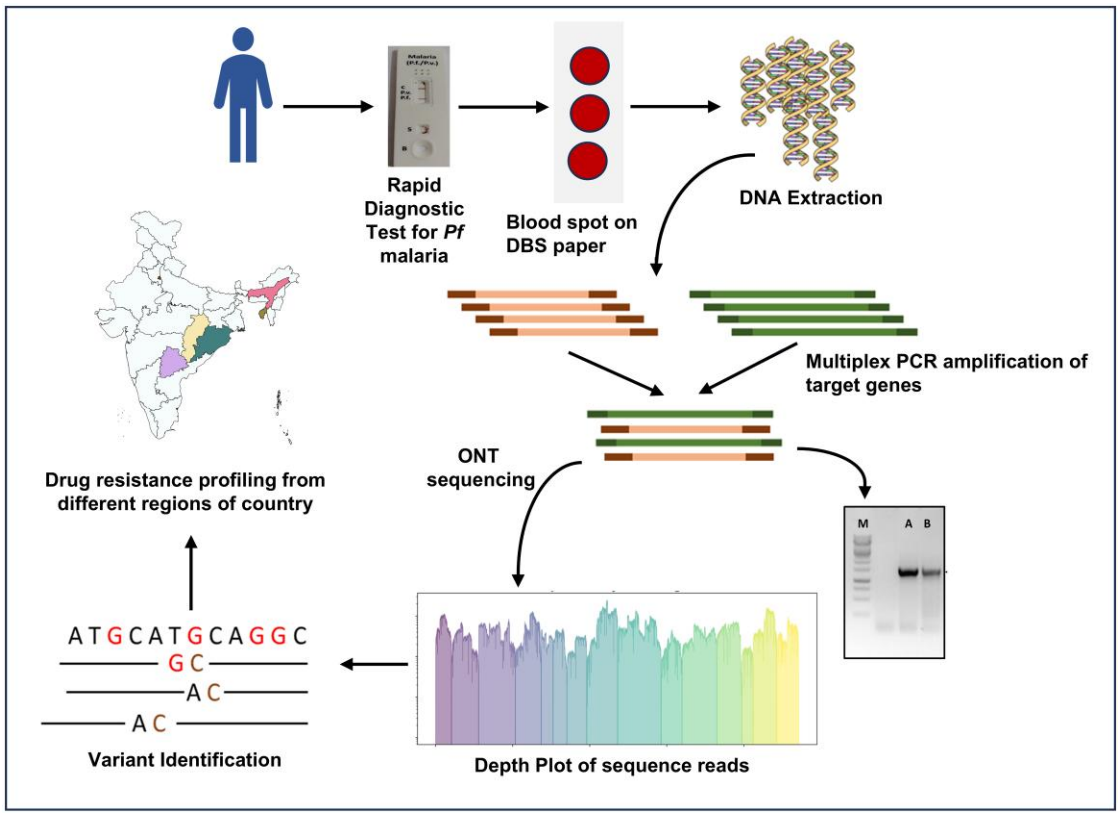
Graphical abstract



Keywords. drug resistance; malaria; Oxford nanopore; *P. falciparum*; pfMDR15.

Malaria caused by *Plasmodium falciparum* (*Pf*) remains a significant public health issue [1], with an estimated 263 million cases and 597 000 deaths in 2023, as per the WHO Malaria report 2024 [2]. The disease predominantly affects tropical and subtropical regions, primarily developing and underdeveloped nations [3]. Antimalarial drug usage is the primary method for effective malaria treatment and control, but this is under continuous threat by the emergence and spread of drug-resistant *Pf* strains. Chloroquine resistance in *Pf* across Central and South America, Africa, and Southeast Asia has rendered the drug ineffective for treating *Pf* malaria, leading to a global shift toward artemisinin-based combination therapy (ACT). Resistance to sulfadoxine–pyrimethamine (SP) has also become prevalent across Africa, South Asia, Southeast Asia, South America, and Oceania. Alarmingly, even artemisinin, the most potent antimalarial available today, has shown decreased effectiveness in Southeast Asia [4, 5]. Resistance to key antimalarial drugs has complicated treatment strategies and necessitates rigorous surveillance to guide global malaria control programs effectively [6]. The way forward to address antimalarial resistance is through the development of new antimalarial drugs, but this is a slow and challenging process. Therefore, the usage of existing antimalarial drugs must be effectively managed.

India has set an ambitious goal to eliminate malaria by 2030 [7], and *Pf* remains the predominant species, accounting for approximately 58.37% of malaria cases in 2024 (149 118/255 500), as reported by the National Centre for Vector Borne Diseases Control program [8]. Achieving this target will require continuous monitoring and proactive management of drug-resistant parasite populations. Notably, resistance to certain antimalarials can be reversible, as demonstrated in Malawi, where chloroquine sensitivity reemerged following the drug's withdrawal. This reversal of resistance has been observed in some settings; however, it is not universal and depends on regional differences in antimalarial drug-use policies, the extent and duration of drug withdrawal, continued use of related drugs, and local transmission dynamics, all of which shape ongoing selection pressure on parasite populations. This highlights the importance of timely and accurate surveillance in adjusting treatment guidelines and interventions [9, 10]. This reversal of resistance occurs when drug pressure is reduced or removed, highlighting the critical role of timely, accurate surveillance in adjusting treatment guidelines and interventions.

Monitoring antimalarial resistance in clinical samples can be performed by analyzing *P. falciparum* genes associated with drug resistance. Whole-genome sequencing can identify resistance patterns in parasites, but it is impractical for routine surveillance [11, 12]. We report here an Oxford Nanopore Technologies (ONT)-based targeted amplicon sequencing method for analysis of complete gene sequences of a panel of 15 *Pf* drug-resistance genes (Table 1) known to affect the efficacy of one or more drugs. This method uses clinical samples collected as dried blood spots (DBS) and addresses several limitations of previous studies that targeted only known genotypes and fewer genes [13, 14]. The findings provide critical insights into India's current antimalarial drug-resistance landscape, supporting improved treatment policies, targeted interventions, and ongoing malaria elimination efforts.

METHODS

Sample Collection and DNA Extraction

Samples were collected from patients who tested positive for malaria by using a Rapid Diagnostic Test (RDT) kit. The detailed sample collection and extraction process was carried out following the protocol outlined on Malarigen.net [15].

Multiplex PCR Amplification and ONT Sequencing

A detailed step-by-step protocol for PCR amplification of the target genes, including full details of the primer panel used, followed by ONT sequencing and genotype analysis, is described in protocols.io (https://www.protocols.io/blind/6AFE457F4B6C11F0936B0A58A9FEAC02). AmpVarPro pipeline was used for data analysis and genotyping as described in the previous studies [16].

Evaluating the Sensitivity of the PCR-sequencing Method

The performance and sensitivity of the PFMDR15 panel were evaluated using a laboratory-grown 3D7 strain of *P. falciparum*. Ring-stage parasite culture (20% parasitemia) was mixed with whole blood at 50% hematocrit to achieve a final parasitemia of 4%. Further suspensions were made with progressively diluted parasitemia from 4% to 0.01%. The parasite suspensions were used to prepare DBS, from which DNA was extracted. The total DNA isolated from DBS was used for multiplex PCR, followed by ONT sequencing of the PCR products.

Clinical Sample Sequencing

A total of 238 clinical samples were processed from the following Indian states: Chhattisgarh, Telangana, Assam, Tripura, Delhi, and Odisha. Detailed information about each sample is provided in Supplementary Table 1. The samples were confirmed for *Pf* infection by 18S PCR detection using the protocol reported by Snounou et al [17], and the positive samples were subsequently processed for target gene-set analysis as described.

Table 1. Target Genes for Drug-Resistance Genotyping

Gene ID	Gene Name
PF3D7_0417200	Bifunctional dihydrofolate reductase-thymidylate synthase (*dhfr-ts*) [34, 35]
PF3D7_0810800	Hydroxymethyldihydropterin pyrophosphokinase-dihydropteroate synthase (*pppk-dhps*) [35, 36]
PF3D7_0709000	Chloroquine resistance transporter (*crt*) [23, 35]
PF3D7_0629500	Amino acid transporter (*aat1*) [24, 25]
PF3D7_0523000	Multidrug resistance protein 1 (*mdr1*) [26, 35]
PF3D7_0112200	Multidrug resistance-associated protein 1 (*mrp1*) [35, 37]
PF3D7_1447900	Multidrug resistance protein 2 (*mdr2*) [38]
PF3D7_1343700	Kelch protein (*k13*) [35, 39]
PF3D7_0106300	Calcium-transporting ATPase 6 (*atpase6*) [35, 40]
PF3D7_1318100	Ferredoxin (*fd*) [41]
PF3D7_1460900	Apicoplast ribosomal protein S10 (*arps10*) [42]
PF3D7_1362500	3′-5′ Exonuclease (*exo*) [43]
PF3D7_MIT02300	Cytochrome b (*cytb*) [35, 44]
PF3D7_0603300	Dihydroorotate dehydrogenase (*dhodh*) [45]
PF3D7_0511000	Translationally-controlled tumor protein homolog (*tctp*) [35, 46]

List of 15 *P. falciparum* genes associated with antimalarial drug resistance, included in the PfMDR15 multiplex PCR panel for amplification and ONT sequencing. Gene IDs and full names are sourced from the PlasmoDB database (release 66). Abbreviated gene names are provided in parentheses for reference.

RESULTS

Selective Multiplexed Amplification of *P. falciparum* Target Genes

The protocol uses the multiplex overlap amplification technique [18, 19] as described in the previous studies primarily for viruses to amplify the complete genes of different sizes keeping the size of amplicon constant. We designed 37 primer pairs to amplify the 15 genes linked to drug resistance, ensuring each PCR product was approximately 1.5 kb. The PCRs were standardized using genomic DNA isolated from laboratory-cultured *Pf* (3D7 strain). The assay conditions were optimized to ensure successful PCR amplification of all target fragments with the desired quantity and quality for ONT sequencing (Supplementary Figure 1). Next, multiplexed amplification of the target genes was achieved in 2 assays, as pool A and pool B PCRs (Figure *1A*) (see Methodology). The products of the 2 PCR assays were combined for sequencing. The successful PCR amplification and the generation of sufficient sequence read for all target genes are apparent from the depth plot generated in real time by mapping base-called sequencing reads to a synthetic reference sequence created by serially linking the individual sequences of all target genes (Figure *1B*). We refer to the primer panel developed for multiplexed PCR amplification of 15 target *Pf* genes linked to antimalarial resistance as PfMDR15.

Evaluating the Specificity and Sensitivity of the PFMDR15 Panel

We tested the specificity of the PFMDR15 panel by incorporating multiplexed PCR amplification of target genes using either

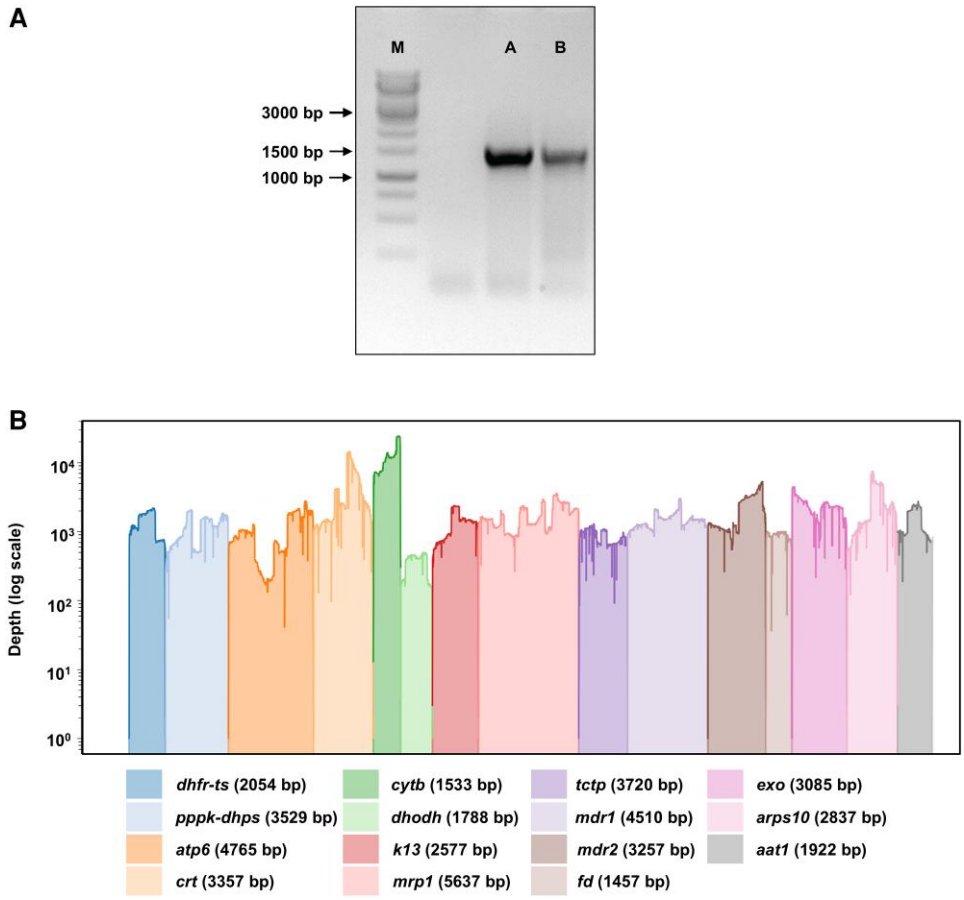


Figure 1. Multiplexed PCR amplification and sequencing of target genes. *A*, Agarose gel electrophoresis confirming successful multiplexed PCR amplification of ~1.5 kb amplicons from the 15 target genes, divided into 2 primer pools (*A* and *B*). Lane M, 1 kb DNA ladder. *B*, Real-time sequencing depth plots for the 15 target genes, generated by mapping base-called ONT reads to a synthetic concatenated reference sequence. Each color represents a specific gene (full gene names given in Supplementary Table 1), with depth indicating uniform coverage across amplicons. Gene sizes (in bp) are indicated in parentheses.

Pf or human genomic DNA as template. The primer panel specifically amplified the target genes (as approximately 1500 bp amplicons) only when *Pf* genomic DNA was used as the template (Supplementary Figure 2*A*). The integrity of the human DNA was established by obtaining the expected approximately 250 bp PCR product from the human RAGE gene locus. From this experiment, it was clear that the PFMDR15 panel can be used for multiplex PCR amplification of *Pf* target genes from total DNA isolated from blood, without interference from the human DNA background. Given that parasitemia in malaria clinical samples can range widely from below 1% to above 5% and considering the overwhelming excess of human DNA relative to parasite DNA, we evaluated the minimum parasitemia levels at which multiplex PCR amplification remains effective.

To assess this, mock-infected blood samples were prepared by mixing *Pf* cultures with healthy human whole blood to achieve parasitemia levels ranging from 0.01% to 4%. These samples were then spotted onto dried blood spot (DBS) paper to mimic clinical sample collection and processed further (see Methodology). DNA extracted from the spots was used for multiplexed PCR amplification using the PFMDR15 panel. The PCR products were analyzed by gel electrophoresis (Supplementary Figure 2*B*) and by ONT sequencing (Supplementary Figure 2*C* and 2*D*). PCR amplification and sequence data with sufficient quality for genotyping were obtained even at 0.01% parasitemia. These results demonstrate the assay's high sensitivity, capable of detecting as few as 1 parasite in 10 000 red blood cells and providing sufficient data for the reliable identification of mutations associated with drug resistance, which is essential for the analysis of clinical samples.

Sampling Strategy and Evaluation of Positivity

We collected clinical samples from 6 Indian states: Assam, Chhattisgarh, Delhi, Odisha, Telangana, and Tripura, with assistance from the Indian Council of Medical Research network (Figure 2*A*). Data from the National Vector Borne Disease Control Program, India, shows that the number of *Pf* malaria cases reported has not changed much from 2019 to 2024 in

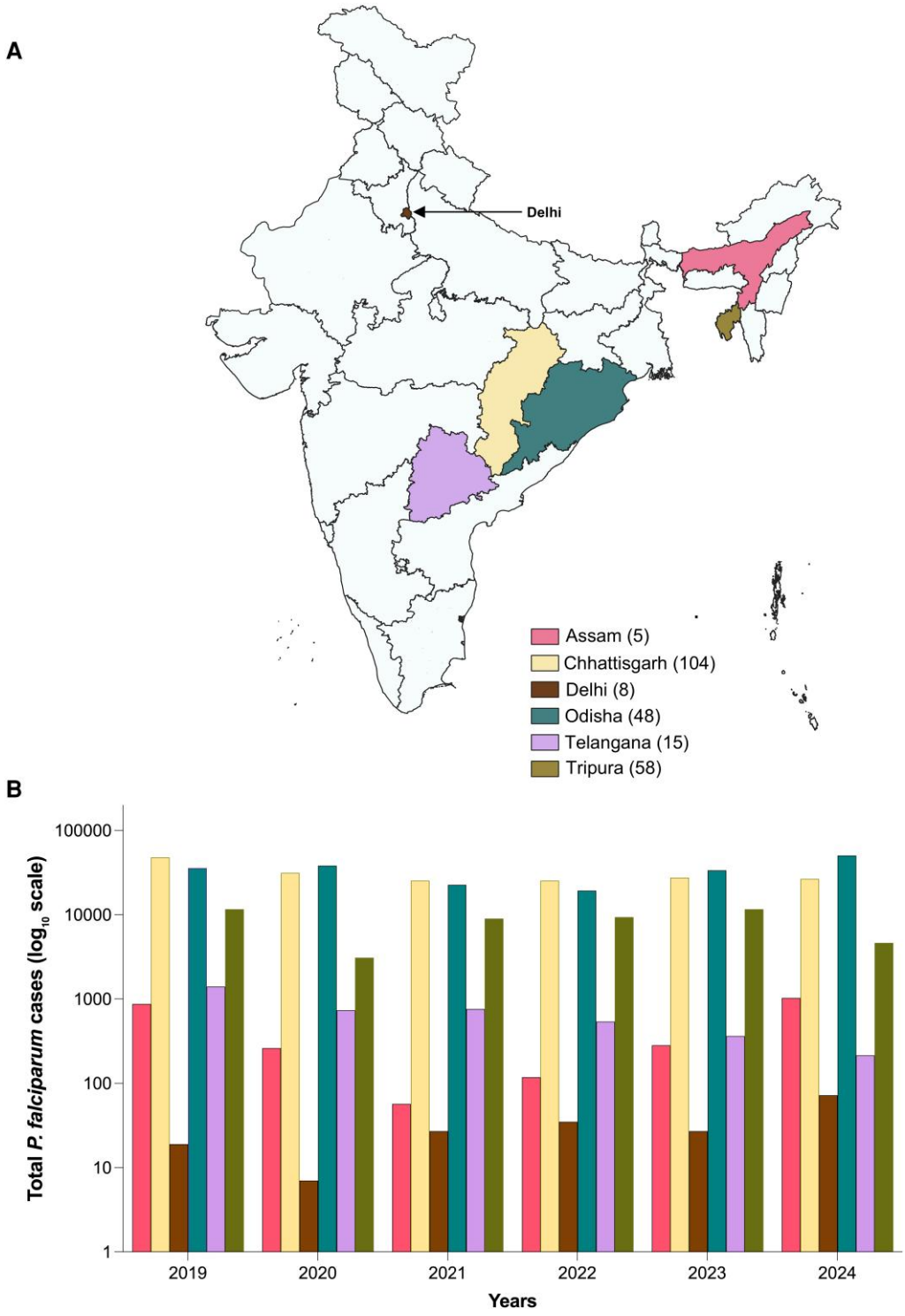


Figure 2. Geographical distribution of coverage. *Pf* clinical samples and temporal trends in malaria cases across Indian states. *A*, Map of India highlighting sample collection sites in 6 states (Tripura, Assam, Delhi, Odisha, Telangana, Chhattisgarh), with sample numbers per state indicated. Samples were collected via DBS from RDT-positive patients through the ICMR network. *B*, Annual *Pf* case trends (2019–2024) in states from where samples were collected, based on national program data [8]. Colors match states in (*A*).

the states from where samples were collected for the study (Figure *2B*). Sequencing was performed on 238 samples from these states using the protocol described in the method section. The details of samples processed, 18S rRNA PCR positivity and sequencing carried out are given in Supplementary Table 1. The gene-wise sequencing depth for all analyzed samples is shown

in Supplementary Figure 3, indicating that sufficient data were available for reliably genotyping the target genes. Data analysis was carried out using the custom data analysis pipeline published in the previous study [16], which used standard ONT-recommended data analysis tools (see Methodology) to facilitate rapid, automated analysis. The information on validated drug resistance markers was obtained from *the malariagen.net* Pf6 dataset for global surveillance [14] and from other published studies, as shown in Supplementary Table 2. Genetic variations were mapped to sequences generated from the samples for the target genes by comparing them with the 3D7 strain as the reference.

Detection of *Pfdhfr* and *Pfdhps* Mutations

Resistance to SP drug combination was inferred by the presence of validated mutations in the dihydrofolate reductase (*Pfdhfr*) and dihydropteroate synthase (*Pfdhps*) genes [20, 21]. Figure 3*A* and 3*B* illustrate the mutations identified in both proteins and their frequencies. The S108N mutation in DHFR, associated with resistance to pyrimethamine, was present in all samples from Assam and Delhi and in nearly all samples from Tripura (98%/58%). Whereas, it was found at lower frequencies in samples from Chhattisgarh (20%/104%), Odisha (27%/48%), and Telangana (40%/15%). The A437G mutation in DHPS, linked to sulfadoxine resistance, was widespread in samples from the Northeast (93%/58% in Tripura) and central India (98%/48% in Odisha, 75%/8% in Delhi and 46%/104% in Chhattisgarh). Four different haplotypes conferring resistance to the 2 drugs have been described for *Pfdhfr* and *Pfdhps* [20, 21]; IRN = *pfdhfr* N51I-C59R-S108N commonly known as triplet mutation known to cause partial SP resistance; IRNL = *pfdhfr* N51I-C59R-S108N-I164L and IRN-G = *pfdhfr* N51I-C59R-S108N and *pfdhps* A437G commonly known as quadruple; IRN-GE = *pfdhfr* N51I-C59R-S108N and *pfdhps* A437G-K540E and IRN-GG = *pfdhfr* N51I-C59R-S108N and *pfdhps* A437G-A581G commonly known as quintuple; IRN-GEG = *pfdhfr* N51I-C59R-S108N and *pfdhps* A437G-K540E-A581G commonly known as sextuple known to cause complete resistance to SP. The IRN (triplet) haplotype, which confers partial resistance to SP treatment, was present in only one sample from Tripura and absent from all other locations. The IRNL and IRN-G (quadruple) haplotype was observed in about 9%/58% of Tripura and 80%/5% of Assam samples, while the fully resistant IRN-GE and IRN-GG (quintuple) and IRN-GEG (sextuple) haplotypes were exclusively identified in Tripura at 17%/58% and 3%/58% frequencies, respectively. Thus, resistance to SP is not a concern, except in Tripura, where the K540E mutation is common (Figure 3*C*). Despite the presence of individual mutations, only the sensitive haplotype was found in samples from central India (Delhi, Chhattisgarh, Odisha, and Telangana).

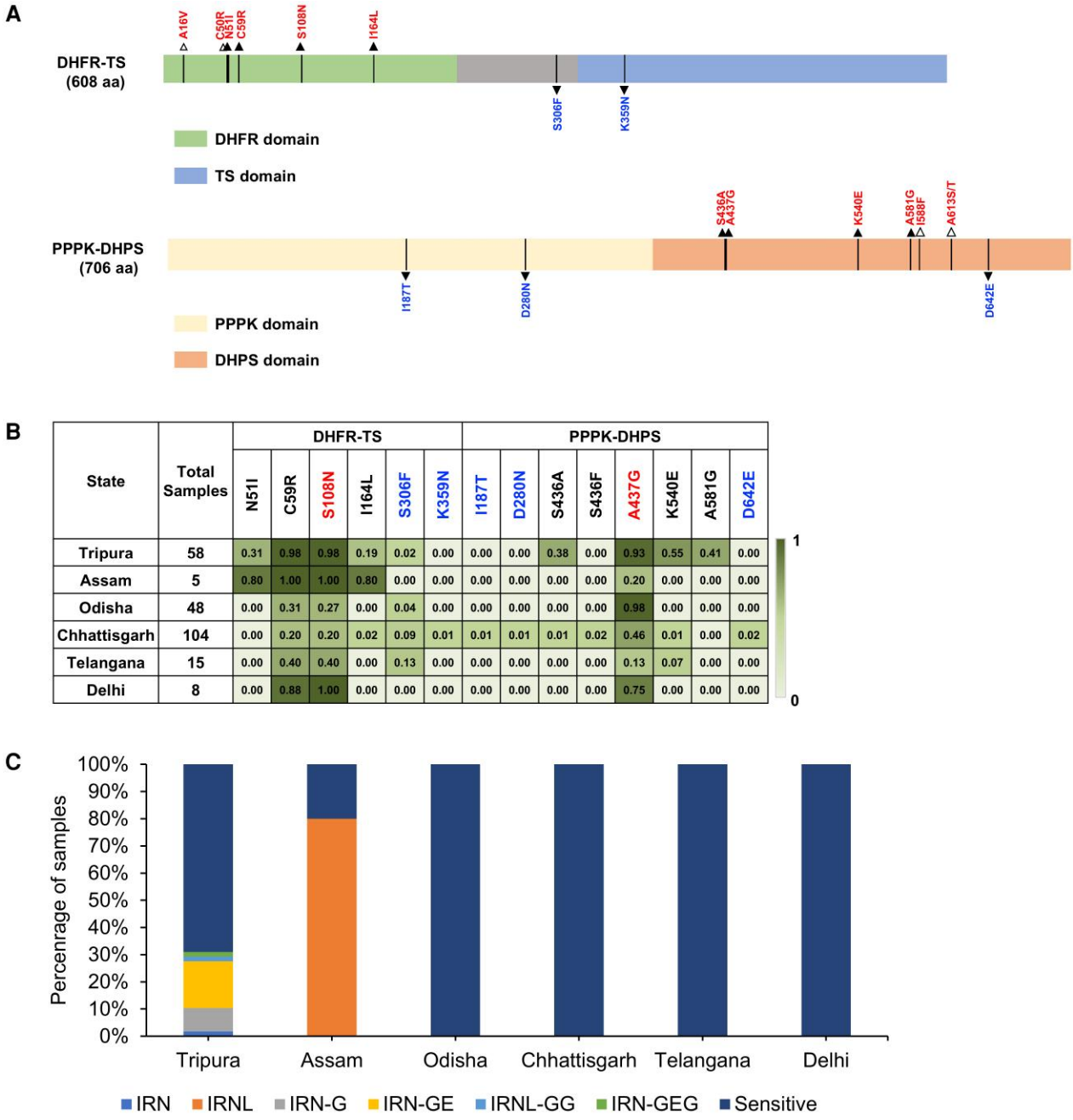
Regional Differences in *Pfcrt* and *Pfaat1* Mutations

Chloroquine resistance was inferred from the mutational pattern in 2 genes: *Pfcrt* and the recently reported *Pfaat1*, which has been identified as a potential marker of reduced chloroquine and lumefantrine efficacy. Several *Pfcrt* mutations and all 3 reported pfaat1 mutations were detected in the samples (Figure 4*A*). Mutation frequency analysis (Figure 4*B*) showed that the K76T mutation in the CRT protein, a confirmed marker for chloroquine resistance, was seen in all samples from Assam, 88%/8% of samples from Delhi, 43%/58% of samples from Tripura and 13%/48% of samples from Odisha. Interestingly, Telangana and Chhattisgarh samples did not possess this mutation. Studies in Africa and India have reported that, even after chloroquine's withdrawal as the frontline malaria drug, the K76T mutation in CRT can persist in the parasite population [9, 10]. This is also reflected in this study as the K76T mutation is prevalent in Delhi, Assam, and Tripura samples. Haplotype analysis of *Pfcrt* also revealed distinct patterns between the Northeast and other regions. The chloroquine-sensitive CVMNK haplotype is converted to the chloroquine-resistant CVIET haplotype due to an indel mutation affecting residues M74 and N75. Notably, the CVIET haplotype was found in 80%/5% of Assam samples, 36%/58% in Tripura, 12%/8% in Delhi and 10%/48% in Odisha, while in Chhattisgarh and Telangana, only the sensitive haplotype CVMNK was present (Figure 4*C*). The SVMNT haplotype was found only in Delhi (about 62%/8%), and the CVMNT haplotype was found in samples from Tripura, Assam, and Odisha at a lower frequency [22, 23]. These findings underscore regional variations in chloroquine resistance across India.

Three distinct mutations in the *Pfaat1* gene have been recently described, associated with resistance to chloroquine and lumefantrine [24, 25]. One of them, the S258L mutation, is linked to both chloroquine and lumefantrine resistance and is seen in all Delhi samples (65%/43%), 44%/48% in Odisha, and at a lower level in Chhattisgarh (9%/104%; Figure 4*D*). The other 2 mutations, F313S and K541N, which are associated with reduced chloroquine efficacy, were found in 23% of Tripura samples and all Assam samples, but were absent in central India samples. Analysis of sample-level occurrence of the *Pfaat1* gene mutations revealed some interesting patterns (Supplementary Figure 4). The F313S and K541N mutations always co-occurred but never in combination with the S258L mutation. This suggests that there must be a fitness cost to harboring all 3 mutations. This pattern may also reflect differences in selection pressure for the evolution of these mutations, given that S258L reduces sensitivity to both chloroquine and lumefantrine.

Prevalence of *Pfmdr1* Mutations and Their Haplotypes

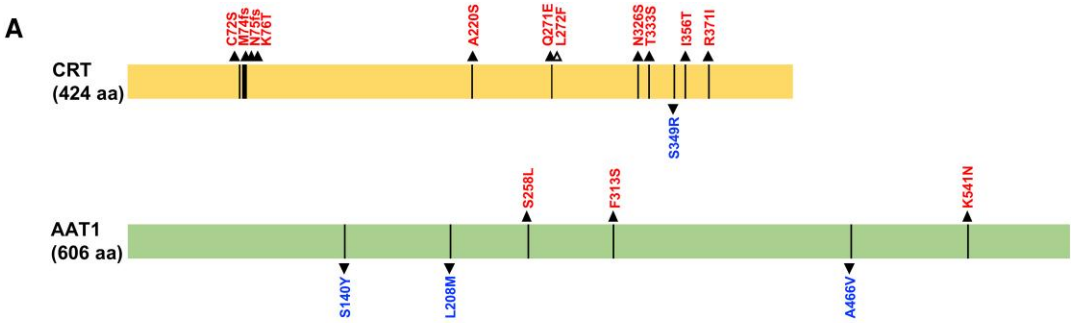
Genetic variation in the *Pfmdr1* gene is associated with resistance to mefloquine, chloroquine, and lumefantrine [17–19].



State	Total Samples	DHFR-TS						PPPK-DHPS							
		N51I	C59R	S108N	I164L	S306F	K359N	I187T	D280N	S436A	S436F	A437G	K540E	A581G	D642E
Tripura	58	0.31	0.98	0.98	0.19	0.02	0.00	0.00	0.00	0.38	0.00	0.93	0.55	0.41	0.00
Assam	5	0.80	1.00	1.00	0.80	0.00	0.00	0.00	0.00	0.00	0.00	0.20	0.00	0.00	0.00
Odisha	48	0.00	0.31	0.27	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.98	0.00	0.00	0.00
Chhattisgarh	104	0.00	0.20	0.20	0.02	0.09	0.01	0.01	0.01	0.01	0.02	0.46	0.01	0.00	0.02
Telangana	15	0.00	0.40	0.40	0.00	0.13	0.00	0.00	0.00	0.00	0.00	0.13	0.07	0.00	0.00
Delhi	8	0.00	0.88	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.75	0.00	0.00	0.00

Figure 3. Mutation profiling and haplotype analysis for DHFR and DHPS. *A*, Schematic of DHFR (top) and DHPS (bottom) proteins, with positions of validated resistance-associated mutations shown above the scheme (red text); filled triangles indicate mutations detected in this study. Novel variants detected in this study are shown below the scheme (blue text). *B*, Heatmap of mutation frequencies by state (columns: mutations; rows: states; color scale: prevalence % as fraction). Validated mutations (red) confer pyrimethamine (S108N) or sulfadoxine (A437G) resistance. Novel mutations shown in blue. *C*, Stacked bar plot of DHFR-DHPS haplotypes by state (x-axis: states; y-axis: %). Haplotypes: IRN = *pfdhfr* N51I-C59R-S108N in DHFR; partial resistance commonly known as triplet mutation; IRNL = *pfdhfr* N51I-C59R-S108N-I164L and IRN-G = *pfdhfr* N51I-C59R-S108N and *pfdhps* A437G commonly known as quadruple; IRN-GE = *pfdhfr* N51I-C59R-S108N and *pfdhps* A437G-K540E and IRN-GG = *pfdhfr* N51I-C59R-S108N and *pfdhps* A437G-A581G commonly known as quintuple; IRN-GEG = *pfdhfr* N51I-C59R-S108N and *pfdhps* A437G-K540E-A581G commonly known as sextuple known to cause full resistance to SP).

Three resistance-associated mutations reported in *Pfmdr1* were detected in the samples, along with 21 new mutations (Figure 5*A*). N86Y, Y184F, and F1226Y are key mutations that confer resistance to chloroquine and lumefantrine. Notably, the Y184F mutation linked to lumefantrine resistance was observed in all samples from Delhi, 60% in Telangana, 34% in Tripura, 25% in Odisha, and 23% in Chhattisgarh. The N86Y mutation, linked to chloroquine and mefloquine resistance,



B

State	Total Samples	CRT													
		C72S	C72R	M74fs	N75fs	K76T	A220S	Q271E	N326D	N326S	T333S	S349R	I356L	I356T	R371I
Tripura	58	0.00	0.00	0.40	0.38	0.43	0.43	0.34	0.00	0.05	0.00	0.00	0.00	0.10	0.45
Assam	5	0.00	0.00	1.00	0.80	1.00	0.80	0.60	0.00	1.00	0.00	0.00	0.00	0.80	1.00
Odisha	48	0.00	0.00	0.13	0.10	0.13	0.13	0.00	0.00	0.13	0.00	0.00	0.00	0.13	0.13
Chhattisgarh	104	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01
Telangana	15	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Delhi	8	0.63	0.13	0.13	0.13	0.88	0.88	0.00	0.75	0.13	0.38	0.25	0.75	0.13	0.13

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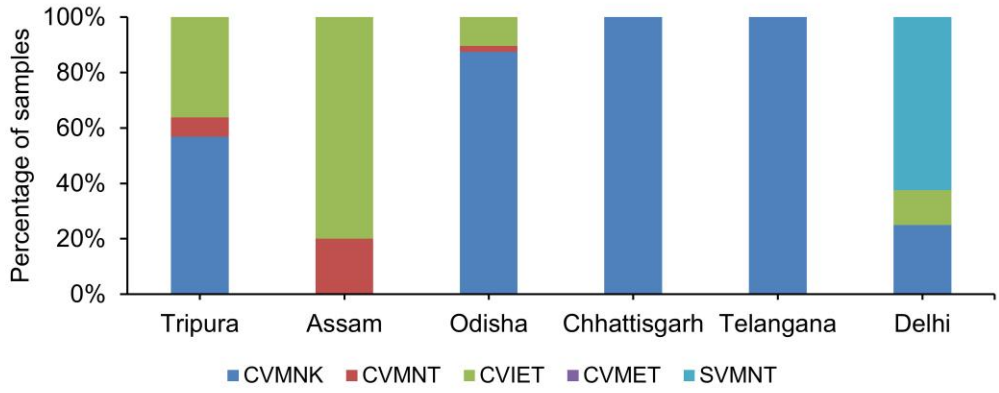

D

State	Total Samples	AAT1					
		S140Y	L208M	S258L	F313S	A466V	K541N
Tripura	43	0.00	0.00	0.65	0.23	0.00	0.23
Assam	5	0.00	0.00	0.00	1.00	0.00	1.00
Odisha	48	0.00	0.00	0.44	0.00	0.00	0.00
Chhattisgarh	104	0.01	0.02	0.09	0.00	0.01	0.00
Telangana	15	0.00	0.00	0.00	0.00	0.00	0.00
New Delhi	8	0.00	0.00	1.00	0.00	0.00	0.00

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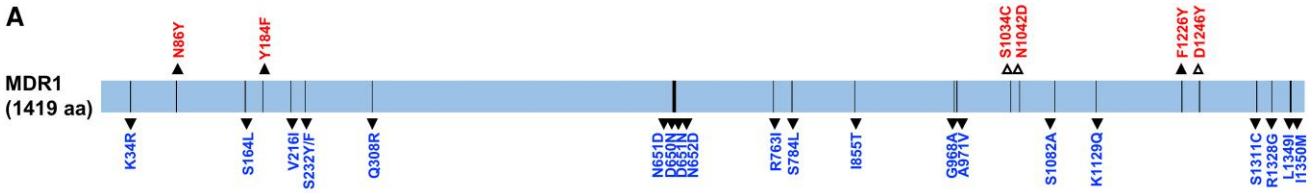
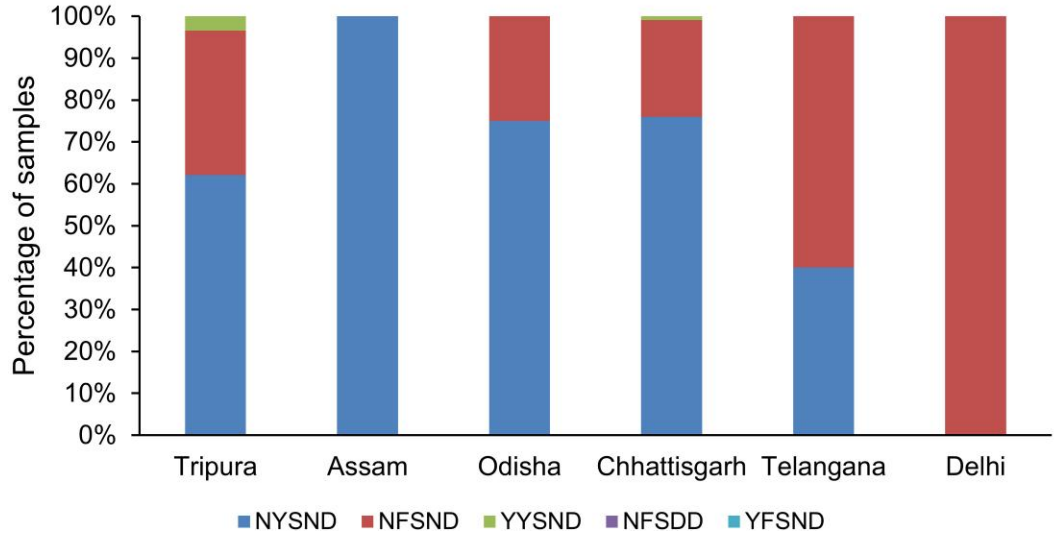
0

Figure 4. Mutation profiling and haplotype analysis for CRT and AAT1. *A*, Schematic of CRT (top) and AAT1 (bottom) proteins, with positions of validated resistance mutations shown above the scheme (red text); filled triangles indicate mutations detected in this study. Novel variants (blue text) are shown below the scheme. *B*, Heatmap of *Pfcrt* mutation prevalence by state. *C*, Stacked bar plot of *Pfcrt* haplotypes by state (x-axis: states; y-axis: %). *D*, Heatmap of *Pfaat1*1 mutation prevalence by state. Annotation of heatmaps (*B* and *D*) as in Figure 3.



B

Gene Name	Mutation	Tripura (N=58)	Assam (N=5)	Odisha (N=48)	Chhattisgarh (N=104)	Telangana (N=15)	Delhi (N=8)
MDR1	K34R	0.00	0.00	0.02	0.00	0.33	0.00
	N86Y	0.03	0.00	0.00	0.01	0.00	0.00
	S164L	0.00	0.00	0.00	0.01	0.00	0.00
	Y184F	0.34	0.00	0.25	0.23	0.60	1.00
	V216I	0.02	0.00	0.02	0.01	0.00	0.00
	S232Y	0.00	0.00	0.02	0.00	0.00	0.25
	S232F	0.00	0.00	0.00	0.00	0.00	0.13
	Q308R	0.00	0.00	0.00	0.01	0.00	0.00
	N649D	0.00	0.00	0.04	0.02	0.00	0.00
	D650N	0.00	0.00	0.00	0.00	0.33	0.00
	D651N	0.00	0.00	0.02	0.01	0.00	0.00
	N652D	0.00	0.00	0.00	0.00	0.13	0.00
	R763I	0.00	0.00	0.02	0.00	0.00	0.00
	S784L	0.02	0.80	0.00	0.00	0.00	0.00
	I855T	0.00	0.00	0.00	0.00	0.00	0.00
	G968A	0.02	0.00	0.00	0.05	0.00	0.38
	A971V	0.02	0.00	0.00	0.00	0.00	0.00
	S1082A	0.07	0.00	0.00	0.00	0.00	0.00
	K1129Q	0.00	0.00	0.02	0.01	0.00	0.00
	F1226Y	0.00	0.00	0.00	0.01	0.00	0.00
	S1311C	0.00	0.00	0.00	0.01	0.00	0.00
	R1328G	0.00	0.00	0.00	0.03	0.00	0.00
	L1349I	0.00	0.00	0.04	0.00	0.00	0.00
	I1350M	0.00	0.00	0.04	0.00	0.00	0.00



C

Figure 5. Mutation profiling and haplotype analysis for MDR1. *A*, Schematic of MDR1 protein, with positions of validated resistance mutations (red; detected mutations shown by filled triangles) and novel variants identified in this study (blue; shown below the scheme). *B*, Heatmap of mutation frequencies by state. Validated mutations (red) for CQ resistance (N86Y), LF resistance (Y184F), or resistance to both (F1226Y). *C*, Stacked bar plot of *Pfmdr1* haplotypes by state (x-axis: states; y-axis: %). Wild-type NYSND (drug sensitive, blue); others are resistant haplotypes shown in different colors.

and the F1226Y mutation, linked to reduced susceptibility to lumefantrine and quinine, were found at low frequencies in some regions (Figure 5*B*). From sample-level analysis of mutations, it is apparent that the F1226Y mutation is sporadic (present in only one sample), whereas the N86Y and Y184F mutations are more common (Supplementary Figure 5). The N86Y and Y184F mutations did not co-occur in any of the samples, suggesting that there may be a fitness cost to carrying both mutations and that they evolve independently under selection imposed by the 2 drugs. Only 2 of the 4 different resistance haplotypes of MDR1 protein [26], NFSND and YYSND, were present in the samples (Figure 5*C*), and the drug-sensitive wild-type haplotype NYSND was present in most of the samples. NFSND haplotype was widely distributed, with 100% prevalence in Delhi, 35% in Tripura, 60% in Telangana, 25% in Odisha and 23% in Chhattisgarh. In contrast, the YYSND haplotype was observed at low frequencies in Tripura and Chhattisgarh.

Tolerance to lumefantrine can emerge due to mutations in the *Pfaat1* and *Pfmdr1* genes. Data from this study suggest that in Delhi, Odisha, and Tripura, mutations in both genes are likely to contribute to lumefantrine tolerance; 32% of the Tripura samples (9/28) and 27% of the Odisha samples (6/22), which have the S258L mutation in AAT1 also contain the Y184F mutation in MDR1; in Delhi, both mutations were seen in 100% of samples (Figures 4 and 5). In Chhattisgarh and Telangana, the presence of only MDR1 mutations may be contributing to lumefantrine tolerance. This is likely a reflection of differences in the recommended antimalarial drugs used to treat *Pf* malaria in these regions (AL in the Northeast and AS + SP in central India).

Detection of Additional Drug Resistance Mutations

Beyond the 5 significant resistance genes, genetic variations were detected in 10 additional *P. falciparum* genes. Among these, *Pfmrp1* and *Pfmdr2* carried several mutations that were previously linked to drug resistance (Figure 6). In *Pfmrp1*, mutations H191Y, S437A, and I876V associated with resistance to chloroquine, mefloquine, artemisinin, and lumefantrine were highly prevalent across all regions (30–100%). The F1390I mutation appeared only in a few samples from Tripura and Delhi. The F423Y mutation in the *Pfmdr2* gene, which is also reported to cause pyrimethamine resistance, was found at very high frequency (79%/104% in Chhattisgarh, 95%/58% in Tripura and in all samples from all other states). While this mutation, by itself, is linked to pyrimethamine resistance, its relevance when combined with *Pfdhfr* mutations needs to be further investigated. It appears to be almost fixed in the parasite populations of Assam, Odisha, Telangana, and Delhi, with similarly high frequencies (95% and 79%) in Tripura and Chhattisgarh.

No validated artemisinin-resistance mutations were detected in the *Pfk13* gene; however, 4 uncharacterized variants were observed. The K189T mutation was most common, occurring at frequencies of 21% in Tripura, 47% in Telangana, 17% in Odisha, and 10% in both Chhattisgarh and Delhi, and its role in resistance, if any, remains unknown. In *Pfatpase6*, 3 reported artemisinin-associated mutations E431K, A623E, and N683K were detected. E431K occurred at 13–17% in samples from Chhattisgarh, Odisha, and Telangana. A623E was detected at low frequency, while N683K reached 80% in Assam but remained below 10% elsewhere. Several novel Pfatpase6 mutations, including H243Y and K776N, were common in Telangana. Other genes (*Pffd*, *Pfarps10*, *Pfexo*, *Pftctp*, *Pfdhodh*, and *Pfcytb*) carried only novel variants with no known resistance associations (see Supplementary Material - Result section).

Regional Patterns in Genetic Variation

Comparison of mutation profiles revealed distinct regional patterns (Supplementary Figure 6). *Pfcrt* and *Pfaat1* mutations were concentrated in samples from the Northeast, Odisha, and Delhi, highlighting possible differences in chloroquine susceptibility between these regions and Telangana and Chhattisgarh. *Pfdhfr* and *Pfdhps* mutations were also more common in the Northeast. Drug-resistance mutations in *Pfmrp1* and *Pfmdr2* were widespread across all states, with *Pfmdr2* F423Y nearly fixed in the population (present in >90% of samples). Several additional variants, including *pfmdr2* S208N and *pfexo* K614N, were also commonly observed across multiple regions. Although these positions differ from the 3D7 reference genome, comparison with orthologous sequences from primate-infecting *Plasmodium* (eg, *P. reichenowi)* indicated that the amino acids observed in our samples are shared with primate parasites and therefore likely represent the ancestral state. At the same time, the corresponding residues in 3D7 are already a variant. New haplotypes were identified, including combinations of known and novel mutations in *Pfcrt* and *Pfmrp1*, warranting further evaluation of their role in drug resistance (see Supplementary Material - Result section).

DISCUSSION

This study provides a comprehensive molecular snapshot of *Pf* drug resistance markers across multiple sites in India, generating evidence that is directly relevant to national malaria control and elimination efforts. An important outcome of this study is the demonstration of a robust, easy-to-implement multiplexed amplicon sequencing protocol for genotyping the full-length coding regions of a set of genes linked to antimalarial resistance. By combining a clinical sampling strategy with the established molecular surveillance technique, we were able to track the prevalence and dynamics of key resistance-associated mutations, including those in *Pfcrt*, *Pfmdr1*, and *kelch13*.

The persistence of the *Pfcrt* K76T mutation in several sites confirms the entrenched nature of chloroquine resistance in *Pf*,

Gene Name	Mutation	Tripura (n=58)	Assam (n=5)	Odisha (n=48)	Chhattisgarh (n=104)	Telangana (n=15)	Delhi (n=8)
ATPase6	I89T	0.00	0.00	0.00	0.00	0.00	0.50
	H243Y	0.00	0.00	0.02	0.04	0.47	0.00
	L402V	0.03	0.00	0.08	0.03	0.00	0.00
	E431K	0.00	0.00	0.15	0.17	0.13	0.00
	A438D	0.00	0.00	0.00	0.00	0.00	0.13
	P440S	0.00	0.00	0.00	0.02	0.00	0.00
	N569K	0.02	0.00	0.06	0.08	0.13	0.00
	P572S	0.00	0.00	0.00	0.02	0.00	0.00
	K578R	0.00	0.00	0.00	0.02	0.00	0.00
	D607G	0.00	0.00	0.02	0.00	0.00	0.00
	A613S	0.00	0.00	0.02	0.00	0.00	0.00
	A613P	0.00	0.00	0.02	0.00	0.00	0.00
	A623E	0.00	0.00	0.00	0.01	0.07	0.00
	A630D	0.00	0.00	0.00	0.01	0.00	0.00
	E643Q	0.00	0.00	0.00	0.01	0.00	0.00
	K649I	0.00	0.00	0.04	0.00	0.00	0.00
	N683K	0.07	0.80	0.04	0.01	0.00	0.00
	I723V	0.00	0.00	0.00	0.01	0.00	0.00
	P737L	0.00	0.00	0.02	0.00	0.00	0.00
	K776N	0.00	0.00	0.00	0.00	0.33	0.00
	A985T	0.00	0.00	0.00	0.01	0.00	0.00
	I1149V	0.00	0.00	0.00	0.01	0.00	0.00
MRP1	R151H	0.00	0.00	0.00	0.00	0.00	0.13
	H183D	0.00	0.00	0.02	0.00	0.00	0.00
	H191Y	0.55	1.00	0.75	0.55	0.87	0.88
	K202E	0.00	0.00	0.00	0.00	0.00	0.25
	N325S	0.05	0.00	0.06	0.07	0.00	0.00
	S437A	0.55	1.00	0.75	0.52	0.87	0.88
	N529K	0.00	0.00	0.00	0.00	0.00	0.13
	F572L	0.00	0.00	0.04	0.00	0.00	0.00
	L593F	0.00	0.00	0.00	0.00	0.00	0.13
	H785N	0.43	1.00	0.60	0.39	0.73	0.13
	I876V	0.53	1.00	0.42	0.46	0.73	0.25
	Q929E	0.00	0.00	0.00	0.00	0.00	0.00
	T1007M	0.47	1.00	0.65	0.38	0.73	0.25
	E1338A	0.00	0.00	0.02	0.00	0.00	0.00
	Q1340P	0.00	0.00	0.25	0.09	0.00	0.38
	Q1340H	0.00	0.00	0.25	0.11	0.00	0.50
	L1342M	0.00	0.00	0.00	0.00	0.00	0.25
	A1357E	0.00	0.00	0.00	0.00	0.33	0.00
	F1390I	0.10	0.00	0.00	0.00	0.00	0.38
	A1782V	0.00	0.00	0.00	0.02	0.00	0.00
FD	I71V	0.02	0.00	0.00	0.00	0.00	0.00
	E125D	0.02	0.00	0.04	0.08	0.00	0.00
K13	E23Q	0.00	0.00	0.02	0.00	0.00	0.00
	T126N	0.00	0.00	0.04	0.02	0.00	0.00
	K189T	0.21	0.00	0.17	0.10	0.47	0.13
	K266R	0.00	0.00	0.02	0.01	0.00	0.00
	S711T	0.02	0.00	0.00	0.00	0.00	0.00
EXO	S6I	0.00	0.00	0.04	0.01	0.00	0.00
	E12G	0.00	0.00	0.02	0.04	0.00	0.00
	C61Y	0.02	0.00	0.00	0.00	0.00	0.00
	L149M	0.00	0.00	0.02	0.00	0.00	0.00
	V362G	0.00	0.20	0.00	0.00	0.00	0.00
	K398M	0.00	0.00	0.02	0.00	0.00	0.00
	V410I	0.07	0.00	0.00	0.00	0.00	0.00
	L453F	0.00	0.00	0.04	0.00	0.00	0.00
	V480L	0.28	0.00	0.25	0.14	0.13	0.00
	I557L	0.00	0.00	0.00	0.01	0.00	0.00
	K614N	1.00	1.00	1.00	0.86	1.00	1.00
	L700I	0.02	0.20	0.00	0.00	0.00	0.00

Figure 6. Regional frequencies of mutations in additional *Pf* drug-resistance genes. Heatmap of prevalence (color scale: % as fraction) for mutations in *Pfatpase6*, *Pffd*, *Pfarps10*, *Pfmrp1*, *Pfmdr2*, *Pfexo*, and *Pfk13* (rows: mutations; columns: states). Validated mutations (red text) are associated with resistance (H191Y/S437A/I876V in MRP1 for CQ/mefloquine/artemisinin/LF); blue, novel mutations. High-frequency variants are seen in all states (eg, *Pfmdr2* F423Y >79%; resistance for pyrimethamine).

Gene Name	Mutation	Tripura (n=58)	Assam (n=5)	Odisha (n=48)	Chhattisgarh (n=104)	Telangana (n=15)	Delhi (n=8)
MDR2	L133F	0.00	0.00	0.02	0.00	0.00	0.00
	L143V	0.00	0.00	0.00	0.01	0.00	0.00
	D198E	0.02	0.00	0.00	0.00	0.00	0.00
	S208N	0.98	1.00	0.98	0.86	1.00	1.00
	N276K	0.02	0.00	0.00	0.03	0.00	0.00
	D288N	0.09	0.00	0.00	0.01	0.00	0.00
	G299D	0.60	0.00	0.67	0.61	1.00	0.13
	K306N	0.02	0.00	0.00	0.01	0.00	0.00
	I362V	0.00	0.00	0.00	0.01	0.00	0.00
	F423Y	0.95	1.00	1.00	0.79	1.00	1.00
	I492V	0.36	0.00	0.13	0.24	0.00	0.00
	I495N	0.00	0.00	0.00	0.01	0.00	0.00
	I563T	0.00	0.00	0.04	0.02	0.00	0.00
	I563M	0.05	0.00	0.00	0.00	0.00	0.00
	H692Y	0.00	0.00	0.02	0.00	0.00	0.00
	H701D	0.00	0.00	0.04	0.00	0.00	0.00
	L953I	0.00	0.00	0.04	0.00	0.00	0.00
	N980K	0.00	0.00	0.08	0.02	0.00	0.00
	D990E	0.02	0.00	0.06	0.06	0.27	0.00
	D1014N	0.00	0.00	0.04	0.00	0.00	0.00
	D1017N	0.02	0.00	0.00	0.00	0.00	0.00
ArpS10	A107S	0.00	0.00	0.00	0.01	0.00	0.00
	S157T	0.02	0.00	0.00	0.00	0.00	0.00
	K249Q	0.09	0.00	0.00	0.01	0.00	0.00

Figure 6. Continued

despite the drug's withdrawal from *Pf* case management over a decade ago [27, 28]. This is consistent with observations from other endemic regions in Asia and Africa, where the slow reversion to wild-type alleles likely reflects ongoing selection pressure from chloroquine use against *P. vivax*, cross-species exposure, or compensatory fitness advantages of resistant haplotypes. Notably, the high prevalence of the CVIET haplotype mirrors reports from Myanmar and parts of Southeast Asia, indicating continued stability of this resistance lineage in the region [29].

Mutations in *Pfmdr1*, particularly N86Y and Y184F, were also detected at moderate to high frequencies. These alleles are known to influence susceptibility to multiple partner drugs used in artemisinin-based combination therapies (ACTs), including lumefantrine and amodiaquine [30]. The pattern observed here of coexistence of *Pfcrt* mutant haplotypes with *Pfmdr1* variants warrants careful monitoring, as certain combinations have been linked to reduced ACT efficacy in African and Southeast Asian settings [29]. Particularly noteworthy is the first documentation of *pfaat1* S258L in India. Previously reported only in Africa, this mutation is implicated in reduced CQ and lumefantrine activity. African data show that S258L reached near fixation in Rwanda by 2018 despite long-standing CQ withdrawal, probably because AL continued to favor S258L without imposing a severe fitness cost [24, 25].

Our data on *kelch13* revealed no confirmed artemisinin resistance–associated mutations of the WHO-validated set, aligning with therapeutic efficacy and genomic study results from India that continue to show high ACT cure rates [31, 32]. These high cure rates also reflect the generally low to moderate prevalence of highly SP-resistant genotypes seen in this study. Nevertheless, the detection of nonvalidated or region-specific polymorphisms underscores the need for vigilant genomic surveillance, as emergent variants could become relevant if selection pressures shift. Given reports of partial artemisinin resistance spreading westward from the Greater Mekong Subregion [33], maintaining baseline data on *kelch13* variation is critical for early detection of any threat to ACT efficacy.

The temporal analysis did not indicate a uniform increase or decrease in resistance allele frequencies across sites, suggesting that local drug use patterns, transmission intensity, and parasite population structure play significant roles in shaping resistance dynamics. Importantly, these site-specific differences emphasize the value of geographically targeted surveillance rather than assuming national uniformity. However, the interpretation of these findings is constrained by small sample sizes at individual sites, the absence of longitudinal trends, and the lack of phenotype confirmation. Our findings should be interpreted as regionally representative rather than nationally exhaustive. Future studies will be required to extend surveillance to additional states.

From a public health perspective, our findings reinforce 3 priorities for India's malaria elimination agenda: (1) Sustain molecular surveillance at sentinel sites to capture emerging trends in resistance markers before they manifest clinically. (2) Integrate genotyping with therapeutic efficacy studies to ensure molecular findings are grounded in phenotypic outcomes.

(3) Coordinate drug policy with resistance data, especially for ACT partner drugs, to preempt efficacy loss, especially in the case of sulfadoxine–pyrimethamine and lumefantrine. This multisite molecular surveillance effort strengthens the evidence base for strategic malaria drug policy in India. While artemisinin-based combinations remain effective, the persistence of chloroquine-resistant *Pfcrt* haplotypes and the presence of *Pfmdr1* variants highlight the complex interplay of historical and ongoing drug pressures. Regular, standardized molecular monitoring, paired with rapid data sharing between research laboratories, control programs, and policy-makers, will be essential to safeguard treatment efficacy and sustain progress toward the 2030 malaria elimination goal.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors declare no potential conflicts of interest.

Software and data availability.

1. Data analysis pipeline used in this study can be accessed using https://github.com/ajinkyakhilari/AmpVarPro.git.
2. Protocol details can be accessed from protocols.io https://www.protocols.io/blind/6AFE457F4B6C11F0936B0A58A9FEAC02.
3. All raw sequences and associated data have been deposited in the NCBI database under accession PRJNA1338190.

Ethical approval. The protocols for collecting samples from malaria patients and conducting experiments were approved by the ICMR-VCRC Ethical Committee (Regn. No. ECR 681/Inst/Py/2014/RR-21).

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