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Associations between *Plasmodium falciparum* merozoite surface variants and drug-resistant haplotypes in *Plasmodium falciparum* field isolates

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ABSTRACT

Objective: To investigate the associations between *Plasmodium falciparum* merozoite surface protein allelic families (*Pfmsp1*, *Pfmsp2*), infection clonality and drug-resistant haplotypes (*Pfcrt*, *Pfdhfr*, *Pfdhps*, *Pfmdr1* and *Pfkelch13*) in malaria samples collected over 30 years in India.

Methods: Infection clonality was assessed using *Pfmsp1* (K1, MAD20, RO33) and *Pfmsp2* (IC/3D7, FC27) allelic families. Five key drug resistance genes were analysed by PCR and Sanger sequencing. Statistical analyses were performed to evaluate associations between specific *Pfmsp* allelic families, infection clonality and drug-resistant haplotypes.

Results: Significant associations were observed between *Pfmsp* allelic families, infection clonality and resistant haplotypes across four genes (*Pfcrt*, *Pfdhfr*, *Pfdhps* and *Pfmdr1*). For *Pfcrt*, K1 (P=0.026) and IC/3D7 (P<0.001) alleles were significantly associated with wild-type CVMNK haplotype, while FC27 allele was linked to **S**VMN**T** haplotype (P=0.005). Similarly, *Pfmdr1* FC27 allele was significantly associated with N**F**SND haplotype (P=0.034) and IC/3D7 allele with NYSND haplotype (P<0.001). Analyses revealed that both K1 and RO33 alleles were associated with resistant *Pfdhfr* and *Pfdhps* haplotypes, whereas MAD20 was exclusively associated with resistant *Pfdhps* (P<0.001) haplotypes. Additionally, *Pfdhfr* and *Pfdhps* haplotypes exhibited distinct associations with both *Pfmsp2* allelic families.

Conclusions: The findings suggest potential associations between antigenic diversity markers and drug-resistant haplotypes, and some patterns may reflect shared ancestral origins, though the basis of certain associations remains unclear. Further studies with larger datasets are needed to validate these observations and elucidate the genetic and ecological factors underlying these relationships.

KEYWORDS: *Plasmodium falciparum*; Drug resistance; *Pfmsp1*; *Pfmsp2*; India

1. Introduction

Malaria caused by *Plasmodium* spp. remains a significant public health concern, with *Plasmodium (P.) falciparum* (*Pf*) being the most lethal due to its ability to cause severe complications[1,2]. Merozoite surface protein genes (*msp*) *msp1* and *msp2* encode merozoite surface proteins essential for erythrocyte invasion and serve as key markers for assessing parasite genetic diversity and population structure[3]. Their polymorphism provides valuable insights into malaria transmission dynamics and the effectiveness of control measures[4]. In the absence of an effective malaria vaccine, antimalarial drugs

Summary

Question: Are *Pfmsp1* and *Pfmsp2* allelic families and infection clonality associated with drug-resistant haplotypes in Indian *Plasmodium (P.) falciparum* field isolates?

Findings: Molecular analysis of 257 *P. falciparum* mono-infections collected over three decades revealed significant associations between *Pfmsp1/Pfmsp2* allelic families and drug-resistant haplotypes of *Pfcrt*, *Pfmdr1*, *Pfdhfr* and *Pfdhps*. Allelic families, including K1, RO33, MAD20, IC/3D7 and FC27, showed significant associations with either wild-type or mutant haplotypes.

Meaning: Genetic diversity markers *Pfmsp1* and *Pfmsp2* may be associated with drug resistance haplotypes in Indian *P. falciparum* populations, highlighting the need for large-scale studies to validate these associations.

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remain the primary line of defence against malaria. Monitoring drug resistance genes is important for detecting the emergence and spread of resistant strains and informing treatment strategies[5]. In malaria-endemic regions, individuals commonly harbour diverse *Pf* clones and infections are often polyclonal[6]. This high genetic diversity can drive intra-host competition and potentially foster the emergence of more virulent or drug-resistant parasites[6,7].

Though several studies have assessed infection clonality using *Pfmsp1* and *Pfmsp2* alongside drug resistance markers, direct investigation of their associations remains limited, particularly in India[8-10]. Globally, studies have explored the relationship between *msp* allelic families and resistance genes but have not found any strong linkages, suggesting that resistance mutations are not confined to specific *Pfmsp1* or *Pfmsp2* allelic families[7,10,11]. In India, a study from Tripura reported a higher prevalence of resistant alleles in polyclonal infections compared to monoclonal infections, although the difference was not statistically significant[12]. This observation suggests that polyclonal infections may contribute to the persistence and spread of resistance mutations. Together, these findings highlight the importance of investigating antigenic diversity markers with drug resistance genotypes to study resistance dynamics and support malaria control strategies especially in high-transmission settings.

The present study aimed to explore potential associations between drug-resistant genotypes and infection clonality or specific allelic types in *Pf*, an area not previously investigated in the Indian context. We assessed clonality using *Pfmsp1* (K1, RO33, MAD20) and *Pfmsp2* (FC27, IC/3D7) allelic families and genotyped five key drug resistance markers (*Pfcrt, Pfdhfr*, *Pfdhps*, *Pfmdr1* and *Pfkelch13*) to determine whether monoclonal and polyclonal infections, or specific allelic types, differ in their prevalence of resistance-associated genotypes. This analysis aimed to determine whether resistant lineages are specifically associated with monoclonal or polyclonal infections, thereby contributing to an improved understanding of drug resistance dynamics in India.

2. Methods

The study includes a total of 307 malaria rapid diagnostic test (RDT)-positive retrospective samples collected from 14 different states over a 30-year period from 1993 to 2023 as shown in Figure 1 and Table 1. Genomic DNA was extracted from dried blood spots and 18S rRNA PCR assay was performed as described earlier[13,14]. Mixed-species infections with both *P. falciparum* and *P. vivax* were excluded from the study and only *Pf* mono-infections were further analysed. *Pfmsp1* and *Pfmsp2* genes were amplified to determine infection clonality using a standard nested PCR protocol targeting K1, MAD20 and RO33 families of *Pfmsp1* and IC/3D7 and FC27 families of *Pfmsp2*[15]. Five drug resistance genes (*Pfcrt*, *Pfdhfr*, *Pfdhps*, *Pfmdr1* and *Pfkelch13*) were genotyped as described previously[16,17]. Firstly, the presence or absence of specific *Pfmsp1*/*Pfmsp2* (*Pfmsp1/2*) family alleles was determined, followed by an analysis of infection clonality. Monoclonal infections were defined by a single allelic type, while polyclonal infections contained multiple allelic types within a gene. Drug resistance gene amplicons were purified using GeneJet kit and sequenced bidirectionally using Sanger sequencing. Consensus sequences were aligned with 3D7 reference strains (PF3D7_0709000 for *Pfcrt*, PF3D7_0523000 for *Pfmdr1*, PF3D7_0417200 for *Pfdhfr*, PF3D7_0810800 for *Pfdhps* and PF3D7_1343700 for *Pfkelch13*) using BioEdit and MEGA 11 software for identifying resistant mutants[18,19]. Subsequently, the relationship between *Pfmsp1* and *Pfmsp2* genes and five key drug resistance markers was examined using SPSS (IBM, Chicago, IL, USA).



Figure 1. Indian map showing sample collection sites. Sites from which samples were collected are coloured.

3. Results

All 307 malaria RDT-positive samples were further confirmed by 18S rRNA PCR by which 257 *Pf* mono-infections were found (Table 1). A total of 231 samples for *Pfcrt*, 233 for *Pfdhfr*, 231

for *Pfdhps*, 232 for *Pfmdr1* and 211 for *Pfkelch13* were included in final analysis, as they were successfully amplified for both *Pfmsp1* and *Pfmsp2*, along with at least one drug resistance marker. The association between different drug-resistant haplotypes with clonality and the presence of specific *Pfmsp1* and *Pfmsp2* families for each drug resistance gene was analysed. Although *Pfkelch13* was successfully amplified in most isolates, all isolates were found to be wild type and therefore not included in further analysis.

Though no statistically significant differences were observed in *Pfcrt* haplotypes in relation to the clonality of *Pfmsp1/2* infections, significant associations were identified between *Pfcrt* haplotypes and specific *Pfmsp1* and *Pfmsp2* allelic family variants (Table 2). Specifically, for *Pfmsp1*, K1 allele was significantly associated with CVMNK haplotype (73.0%, *P*=0.026), while for *Pfmsp2*, FC27-like strains were significantly associated with **S**VMN**T** haplotype (72.6%, *P*=0.005). In contrast, the proportion of IC/3D7-like strains were significantly associated with CVMNK (89.2%) and CV**IET** (77.8%) haplotypes (*P*<0.001). Similarly, for *Pfmdr1*, although no statistically significant association were seen in *Pfmdr1* haplotype distribution in relation to *Pfmsp1*, significant associations were identified with *Pfmsp2* subfamilies, as shown in Table 2. For *Pfmsp2*, monoclonal infections were significantly associated with strains carrying single mutant N**F**SND haplotype (83.1%, *P*=0.032) and double mutant **YF**SND haplotype (75.0%, *P*=0.032). Additionally, FC27-like strains were also significantly associated with single mutant N**F**SND haplotype (72.5%, *P*=0.034). Conversely, IC/3D7 allele was significantly associated with wild type NYSND haplotype (86.7%) as compared to other haplotypes (*P*<0.001).

Table 1. State-wise distribution of malaria positive samples and confirmed *Pf* mono-infections.

S.No.	States	No. of malaria RDT-positive and 18S rRNA PCR-confirmed samples	*Pf* mono-infections	Mixed-species infections
1	Assam	13	12	1
2	Chhattisgarh	25	19	6
3	Delhi	63	59	4
4	Goa	53	49	4
5	Gujarat	16	15	1
6	Karnataka	9	5	4
7	Madhya Pradesh	17	6	11
8	Maharashtra	10	10	0
9	Meghalaya	14	12	2
10	Mizoram	3	3	0
11	Odisha	26	19	7
12	Rajasthan	20	16	4
13	Tamil Nadu	14	11	3
14	Uttar Pradesh	24	21	3
	Total	307	257	50

Pf: Plasmodium falciparum.

Two major limitations were encountered for assessing the link between specific *Pfdhps* and *Pfdhfr* haplotypes and *Pfmsp1/2* family groups. First, the presence of numerous haplotypes for these genes, unlike the limited number observed in *Pfcrt* or *Pfmdr1*. Second, insufficient sample sizes for each specific haplotype, limiting the statistical power of the analysis. To address these limitations, samples were grouped into single, double or triple mutant categories based on the total number of non-synonymous mutations observed per sample at amino acid positions 16, 50, 51, 59, 108 and 164 in *Pfdhfr* and 436, 437, 540, 581 and 613 in *Pfdhps*. The distribution of *Pfdhfr* haplotypes in relation to *Pfmsp1*/2 allelic families and clonality of infections is summarized in Table 3. For both *Pfmsp1* and *Pfmsp2* genes, the proportion of monoclonal infections were significantly more frequent in isolates carrying drug-resistant double mutant haplotypes [72.2% for *Pfmsp1* (*P*=0.048) and 81.2% for *Pfmsp2* (*P*=0.044)]. A more granular analysis revealed significant associations between *Pfdhfr* haplotypes and allelic families RO33, K1, IC/3D7 and FC27. The proportion of RO33-like infections was frequently seen in double mutants (38.3%), whereas the lowest frequency was seen in single mutants (10.5%), and this difference was statistically significant (*P*=0.014). K1-like and IC/3D7-like infections were frequently seen in triple mutants, at similar frequencies of 81.8% (*P*<0.001 for both allelic families). Finally, FC27-like isolates were more frequent in double (75.2%) and triple mutants (72.7%, *P*=0.006). Distribution of *Pfdhps* haplotypes in relation to *Pfmsp1/2* allelic families and clonality of infections revealed distinct and statistically significant associations (Table 3). These findings highlight the significant associations between *Pfdhps* haplotypes (single, double and triple mutants) and specific allelic families, including RO33, MAD20, K1, IC/3D7 and FC27. For both *Pfmsp1* and *Pfmsp2* genes, monoclonal infections were prevalent in isolates with drug-resistant single-mutant haplotypes, although this trend was not statistically significant. RO33-like infections were significantly associated with single-mutant haplotypes (66.7%), while wild-type haplotypes were frequently observed in the absence of RO33 (79.0%, *P*<0.001). MAD20-like infections were predominantly observed in triple-mutant haplotypes (81.8%), with significantly low frequencies in single-mutant haplotypes (22.2%) (*P*<0.001). Similarly, K1-like infections were frequently associated with double-mutant haplotypes (75.0%, *P*=0.041). The proportion of IC/3D7-like infections were frequently seen with triple-mutant haplotypes (81.8%, *P*<0.001) whereas their lowest frequency was seen in single mutant haplotypes (27.8%) and the difference was statistically significant (*P*<0.001). Conversely, FC27-like isolates were significantly more common in single-mutant haplotypes (85.2%, *P*=0.010).

Table 2. Association between the *Pfmsp1/2* allelic families and clonality of *Pf* infections with *Pfcrt* and *Pfmdr1* haplotypes.

Variable		*Pfcrt* haplotypes, *n* (%)			*P* value	*Pfmdr1* haplotype, *n* (%)				*P* value
		CVMNK	**S**VMN**T**	CV**IET**		NYSND	**Y**YSND	N**F**SND	**YF**SND	
Clonality of *Pfmsp1*	Monoclonal	19 (51.4)	52 (31.7)	10 (37.0)	0.148	19 (42.2)	16 (43.2)	43 (30.3)	3 (37.5)	0.314
	Polyclonal	18 (48.6)	112 (68.3)	17 (63.0)		26 (57.8)	21 (56.8)	99 (69.7)	5 (62.5)	
RO33	Absent	23 (62.2)	112 (68.3)	19 (70.4)	0.724	33 (73.3)	25 (67.6)	93 (65.5)	6 (75.0)	0.762
	Present	14 (37.8)	52 (31.7)	8 (29.6)		12 (26.7)	12 (32.4)	49 (34.5)	2 (25.0)	
MAD20	Absent	20 (54.1)	75 (45.7)	9 (33.3)	0.206	21 (46.7)	14 (37.8)	70 (49.3)	2 (25.0)	0.386
	Present	17 (45.9)	89 (54.3)	18 (66.7)		24 (53.3)	23 (62.2)	72 (50.7)	6 (75.0)	
K1	Absent	10 (27.0)	85 (51.8)	15 (55.6)	0.026*	16 (35.6)	17 (45.9)	75 (52.8)	4 (50.0)	0.243
	Present	27 (73.0)	79 (48.3)	12 (44.4)		29 (64.4)	20 (54.1)	67 (47.2)	4 (50.0)	
Clonality of *Pfmsp2*	Monoclonal	13 (35.1)	32 (19.5)	10 (37.0)	0.098	16 (35.6)	12 (32.4)	24 (16.9)	2 (25.0)	0.032*
	Polyclonal	24 (64.9)	132 (80.5)	17 (63.0)		29 (64.4)	25 (67.6)	118 (83.1)	6 (75.0)	
IC/3D7	Absent	4 (10.8)	87 (53.0)	6 (22.2)	<0.001*	6 (13.3)	12 (32.4)	79 (55.6)	3 (37.5)	<0.001*
	Present	33 (89.2)	77 (47.0)	21 (77.8)		39 (86.7)	25 (67.6)	63 (44.4)	5 (62.5)	
FC27	Absent	20 (54.1)	45 (27.4)	11 (40.7)	0.005*	23 (51.1)	13 (35.1)	39 (27.5)	3 (37.5)	0.034*
	Present	17 (45.9)	119 (72.6)	16 (59.3)		22 (48.9)	24 (64.9)	103 (72.5)	5 (62.5)	

Data are presented as frequency (*n*) and proportion (%); Pearson χ^2 test was used to compare proportion between different groups; *Statistically significant at *P*<0.05; Haplotypes CVMNT (*n*=2) and RVMNT (*n*=1) were removed from *Pfcrt* analysis due to small sample size in each of the groups.

Table 3. Association between the *Pfmsp1/2* allelic families and clonality of *Pf* infections with *Pfdhfr* and *Pfdhps* haplotypes.

Variable		*Pfdhfr* haplotype, *n* (%)				*P* value	*Pfdhps* haplotype, *n* (%)				*P* value
		Wild type	Single mutant	Double mutant	Triple mutant		Wild type	Single mutant	Double mutant	Triple mutant	
Clonality of *Pfmsp1*	Monoclonal	22 (43.1)	17 (44.7)	37 (27.8)	6 (54.5)	0.048*	60 (37.0)	13 (24.1)	2 (50.0)	5 (45.5)	0.255
	Polyclonal	29 (56.9)	21 (55.3)	96 (72.2)	5 (45.5)		102 (63.0)	41 (75.9)	2 (50.0)	6 (54.5)	
RO33	Absent	35 (68.6)	34 (89.5)	82 (61.7)	7 (63.6)	0.014*	128 (79.0)	18 (33.3)	4 (100.0)	7 (63.6)	<0.001*
	Present	16 (31.4)	4 (10.5)	51 (38.3)	4 (36.4)		34 (21.0)	36 (66.7)	0 (0.0)	4 (36.4)	
MAD20	Absent	28 (54.9)	11 (28.9)	61 (45.9)	6 (54.5)	0.094	61 (37.7)	42 (77.8)	1 (25.0)	2 (18.2)	<0.001*
	Present	23 (45.1)	27 (71.1)	72 (54.1)	5 (45.5)		101 (62.3)	12 (22.2)	3 (75.0)	9 (81.8)	
K1	Absent	17 (33.3)	12 (31.6)	82 (61.7)	2 (18.2)	<0.001*	70 (43.2)	34 (63.0)	1 (25.0)	7 (63.6)	0.041*
	Present	34 (66.7)	26 (68.4)	51 (38.3)	9 (81.8)		92 (56.8)	20 (37.0)	3 (75.0)	4 (36.4)	
Clonality of *Pfmsp2*	Monoclonal	14 (27.5)	10 (26.3)	25 (18.8)	6 (54.5)	0.044*	41 (25.3)	7 (13.0)	1 (25.0)	5 (45.5)	0.086
	Polyclonal	37 (72.5)	28 (73.7)	108 (81.2)	5 (45.5)		121 (74.7)	47 (87.0)	3 (75.0)	6 (54.5)	
IC/3D7	Absent	12 (23.5)	11 (28.9)	75 (56.4)	2 (18.2)	<0.001*	57 (35.2)	39 (72.2)	2 (50.0)	2 (18.2)	<0.001*
	Present	39 (76.5)	27 (71.1)	58 (43.6)	9 (81.8)		105 (64.8)	15 (27.8)	2 (50.0)	9 (81.8)	
FC27	Absent	25 (49.0)	17 (44.7)	33 (24.8)	3 (27.3)	0.006*	64 (39.5)	8 (14.8)	1 (25.0)	4 (36.4)	0.010*
	Present	26 (51.0)	21 (55.3)	100 (75.2)	8 (72.7)		98 (60.5)	46 (85.2)	3 (75.0)	7 (63.6)	

Data is presented as frequency (*n*) and proportion (%); Pearson χ^2 test was used to compare proportion between different groups; *Statistically significant at *P*<0.05.

4. Discussion

The reported study explored the associations between drug resistant haplotypes and *Pfmsp1/2* infection clonality and allelic families, shedding light on their potential linkages within *Pf* populations. The significant associations observed between *Pfcrt* **S**VMN**T** haplotype and *Pfmsp2* FC27 allele suggests a shared evolutionary origin. Both **S**VMN**T** haplotype and FC27 allele are believed to have originated in Papua New Guinea (PNG), where FC27 allele was named after the PNG FCQ27 isolate[20,21]. This common ancestry likely explains their strong association. Similarly, the strong association between *Pfcrt* wild-type CVMNK haplotype and IC/3D7 allelic family can be attributed to their shared evolutionary background as IC/3D7 family is named after Indochina 1 (IC1) and 3D7 isolates from which the allele was first identified[22]. For *Pfmdr1*, significant associations were identified between wild-type NYSND haplotype and *Pfmsp2* IC/3D7 subfamily. This relationship can also be explained by their shared 3D7-related ancestry, reinforcing the idea that genetic backgrounds play a crucial role in shaping the distribution of drug resistance and allelic family structures. In contrast, associations observed with *Pfdhfr* and *Pfdhps* genes cannot be directly linked to specific evolutionary lineages, as our analysis categorized them as single, double or triple mutants rather than specific haplotypes. This broader classification likely masked any distinct lineage-based associations that may exist. However, despite this limitation, significant associations were observed between *Pfdhfr* and *Pfdhps* haplotypes and specific *Pfmsp1* and *Pfmsp2* allelic families, emphasizing their potential role in shaping *Pf* genetic diversity.

Some of the observed relationships can be explained through shared ancestry although certain associations remain unclear. The relationship between the IC/3D7 family and the CV**IET** haplotype, for example, does not have a well-defined evolutionary explanation. Similarly, certain observed patterns in drug-resistant haplotypes and allelic family distributions may be influenced by additional, yet unidentified, genetic or ecological factors. In contrast to previous studies conducted globally and in India that reported no significant associations, these preliminary findings suggest a potential association between drug-resistant haplotypes and both *Pfmsp1* and *Pfmsp2* infection clonality and their specific allelic families across four key drug resistance genes[7,10–12]. While certain associations can be attributed to shared evolutionary ancestry, others require further investigation to understand the underlying genetic or selective mechanisms driving these relationships. More studies of this nature with larger dataset are needed to achieve greater statistical confidence for more detailed and accurate understanding of these associations.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval

The ethical approval of the study was obtained from Institutional Ethics Committee (IEC) of ICMR-National Institute of Malaria Research (NIMR), New Delhi (Letter no.: NIMR/IEC-M/2023/970/v3-Nov/01, approved on 8 November, 2023). All procedures were conducted in accordance with the ethical principles of the Declaration of Helsinki.

Availability of data and materials

All the data supporting the study findings are within the manuscript. Additional detailed information and raw data will be shared upon request addressed to the corresponding author.

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Author's contributions

Conceptualization: VS; Data generation and curation: GN; Formal analysis: GN; Writing-original draft: GN; Writing-review & editing: VS,GN and JJ; Resources: VS Validation and Supervision: VS.

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