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Research article

Comparative analysis of Plasmodium falciparum dihydrofolate-reductase gene sequences from different regions of India

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ABSTRACT

Molecular surveillance of the drug resistance genes in parasite can be used for monitoring/surveillance of drug resistance in endemic malaria areas. Here we report the prevalence of single nucleotide polymorphisms (SNPs) in dihydrofolate reductase (*dhfr*) gene in nucleotide sequence of Plasmodium falciparum from different regions in India. We found markedly prevalent mutants evident in P. falciparum infections N51I, C59R, 108N and I164L. Our results indicate that P. falciparum populations in the regions show an increase in the prevalence of polymorphisms, most likely reflecting different selective pressures found in humans and mosquitoes. Molecular surveillance can serve as a useful tool to monitor the prevalence/emergence of resistant genotypes within endemic populations and can serve for determining the efficacy of antimalarial drugs.

1. Introduction

Resistance to anti-malarial drugs has complicated not only the treatment of malaria but have also threatened the control and elimination of the disease leading to increased malaria morbidity and mortality in the tropical countries [1]. The rampant usage of antimalarial drugs has led to the emergence of resistance [2]. Several molecular, clinical and epidemiological studies have clearly shown that resistance to pyrimethamine and sulfadoxine results from specific point mutations in the parasite genes *viz* dhfr and dihydropteroate synthase (dhps) respectively, responsible for reduced competitive drug binding at the enzyme's active site [3]. Molecular analysis of resistant and sensitive parasite isolates has revealed a clear rise in a particular order at codons 16, 50, 51, 59, 108 and 164 of *dhfr* gene associated with resistance to pyrimethamine the most widely used antifolate anti-malarial drug [4, 5]. An increase in the prevalence of the SNPs in the *dhfr* and *dhps* genes from Indian population have been documented [5, 6]. With the spread of chloroquine resistance in the country, the artemisnin combination therapy (ACT) is now used as first-line antimalarial treatment in P. falciparum in combination of sulfadoxine and pyrimethamine (SP) [7]. The goal of this study was to perform sequence analysis of *dhfr* gene sequences from different endemic regions of the country deposited in the public database to enhance the understanding of the existing polymorphisms and their distribution patterns in the parasite population of India [5, 6]. The present study provides a first glimpse of P. falciparum *dhfr* genotypes compared from eight different geographical regions in Indian P. falciparum isolates.

2. Materials and methods

In the study 295 *dhfr* gene sequences of Indian field isolates from northeastern regions (Assam, Arunachal Pradesh and Tripura), central region (Madhya Pradesh), eastern regions (Jharkhand, Chhattisgarh and Orissa) and northern region (Delhi) with 3D7 (ID 9221804) as the reference strain using the alignment tool in MEGA 7.0 [8]. The regions and NCBI accession number for the genomic sequence study included: Tripura [KX575511- KX575491], Assam [KX575490- KX575440], Arunachal Pradesh [KX575439- KX575395], Madhya Pradesh [HM582086-HM582082], Ranchi (Jharkhand) [KU041318- KU041290], Raipur (Chattisgarh) [KU041289- KU041264], Bissam Cuttack (Orissa) [KU041263- KU041246], Jagdalpur (Chattisgarh) [KU041245-KU041234], Rourkela (Orissa) [KU041233- KU041217] and Delhi [KU041216- KU041146] corresponding to locus positions from 755069 to 756895 bp and to amino acid residues from 2 to 218 amino acids. Pearson's independence chi square (χ^2) was used to compare proportion of different mutation codons between different study areas. The statistical package for social science (SPSS) version 16 for Windows was used

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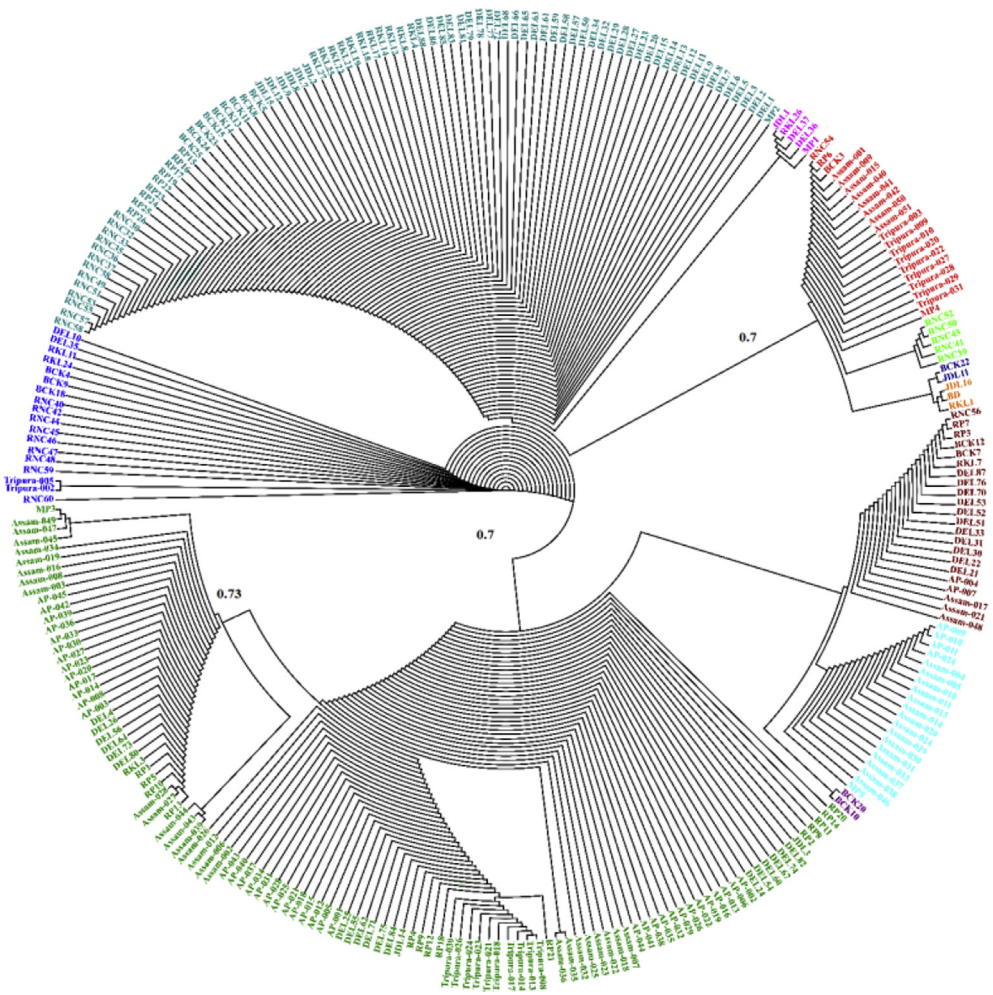

Figure 1. Radial cladogram depicts the distribution of P. falciparum dihydrofolate-reductase genotypes from different geographical isolates in India. The colour represents the mutation codon combination distribution: wild type, 108 codons, 51,59,108 codons, 51, 59 codons, 51, 108 codons, 51 codon, 59, 108, 164 codons, 51, 59, 108, 164 codons, 59, 164 codons, 59, 108 codons, 59 codons.

to perform statistical analysis (IBM, SPSS, Inc, Chicago, IL, USA). P-value less than 0.05 were considered statistically significant. Nucleotide diversity (ϴ) and pairwise difference (π), were also calculated between the sequences and Tajima's D, Fu and Li's D* and Fu and Li's F* indices were determined by DnaSp 5.10.1 software [9]. The radial cladogram network was constructed for all the studied sequences by using Dendroscope version 3.5.9 software [10]. The bootstrap values for the different clades have been given in the supplementary file as all the values could not be incorporated in the radial cladogram (Supplementary file 1).

3. Results and discussion

For this comparative study four regions *i.e.* north eastern regions (Assam, Arunachal Pradesh, Tripura), eastern regions (Jharkhand, Chhattisgarh, Orissa), central region Madhya Pradesh and capital region Delhi were included to understand the existing genotype in the endemic and the non-endemic malaria regions. The north-eastern, eastern and the central regions are endemic but Delhi is the non-endemic region for malaria in the country. Figure 1 describes the radial cladogram which

Table 1. Distribution of the four codons of *dhfr* gene studied in the various regions of the country. #n = number of isolates analyzed.

Regions (n)#	Codon			
	51	59	108	164
Assam (51)	47% (24) #	100% (51) #	100% (51) #	35% (18) #
Arunachal Pradesh (45)	8.8% (4) #	100% (45) #	100% (45) #	13.3% (6) #
Tripura (21)	40% (9) #	95% (20) #	86.3% (19) #	0% (0) #
Jharkhand (29)	24.1% (7) #	55% (16) #	6.8% (2) #	3.4% (1) #
Chattisgarh (38)	12.8% (5) #	48.7% (19) #	53.8% (21) #	5.1% (2) #
Orissa (35)	8.5% (3) #	30% (12) #	22.8% (8) #	14.2% (5) #
Delhi (71)	12.6% (9) #	35.2% (25) #	32.3% (23) #	12.6% (9) #
Madhya Pradesh (5)	40% (2) #	60% (3) #	80% (4) #	20% (1) #
P-value (Chi-square test)	<0.0001*	<0.0001*	<0.0001*	<0.0001*

*Statistically significant at P-value < 0.05.

Table 2. Neutrality test parameter of the nucleotide sequence from different geographical regions used to measure a correlation between the sequence diversity among the places. Tajima D, Fu, Li's D* and Li F* test used to compare the diversity analysis (*P value).

Parameter Tests	Assam	Arunachal Pradesh	Jharkhand	Chhattisgarh	Orissa	Delhi	Tripura	Madhya Pradesh
Nucleotide diversity: Θ	0.8	0.45	1.01	0.9	0.9	0.6	1.3	1.9
Pairwise difference: π	1.3	0.4	1	1.3	1.2	1.2	1.9	2
Tajima's D	0.5 (.4) *	(0.4) *-0.2	0.09 (0.5)*	0.9 (0.8)*	0.5 (0.6)*	1.9 (0.9)*	1.1 (0.8)*	0.2 (0.5)*
Fu &Li's D*	0.7 (0.6) *	1 (0.7)*	0.5 (0.4)*	0.9 (0.6)*	0.9 (0.5)*	0.9 (0.5)*	0.9 (0.6)*	0.3(0.4)*
Fu and Li F*	0.4 (0.5) *	1 (0.6) *	1.7 (0.5)*	0.9 (0.7)*	0.9 (0.5)*	0.9 (0.8)*	0.96 (0.7)*	0.4 (0.3)*

yields two main clades/branches further subdividing into subclades. The genetic relatedness is determined by clade in each phylogeny tree which signifies the similarity in the various isolates sequence. In the cladogram it was found that the isolates are divided in two clades: the first clade consists of wild type isolates with the reference strain 3D7 and the second clade comprises of isolates having mutant alleles which is further sub-divided into subclades. The second clade further differentiates into three sub-clades of which the first sub-clade is formed by the mutations of 51, 59 and 108 codons, second sub-clade consists of 51, 59 codons. The third sub-clade further divides in two branches with one branch made up of 51, 108 codons and the other of 51 codons. The part of second sub clade (of third clade) again divides into two subclades one with 59 codon and other comprised of 59, 108 codons. The analysis of phylogenetic trees showed relatedness among these polymorphic genes which formed different clade groups. The sequences analysed from different regions revealed single nucleotide polymorphisms (SNPs) present in different proportions as seen and the SNPs were distributed in the studied regions with no particular pattern (Figure 1). The most predominant mutant allele for *dhfr* gene was 59 followed by 108 in all the regions and also SNP at 164 was seen emerging in the analysed parasite populations [5, 11]. Pearson's independence chi square (χ^2) revealed statistically significant values among different mutations with respect to the study areas for *dhfr* gene as P-value was found to be < 0.05 for all the studied codons (Table 1). Mutations at codon 51 was found to be higher in Assam (47%) followed by Tripura (40%) and Madhya Pradesh (40%) regions whereas mutations at codon 59, 108 were maximum in Assam and Arunachal Pradesh (both at 100%) followed by Tripura (95%) areas (Table 1). Though mutations at codon 51 was seen in Assam (35%), Madhya Pradesh (20%) and Orissa (14.2%) regions but no mutations at codon 16 was seen in the sequence comparisons. Multiple sequence alignment of the *dhfr* gene revealed eleven (A1-A11) type of haplotypes among which A11 (wild type) followed by A3 (59R 108N) haplotypes were the most prominently distributed in the studied regions.

The nucleotide diversity (ɵ) and pairwise difference (π) of the four studied SNPs in 295 *dhfr* gene sequences were found to be in the range of 0.45–1.9 and 0.4 to 2 respectively (Table 2). The parasite population of 164 codon had the lowest genetic diversity and was highest in 59, 108 codons. Three neutrality tests were applied on the sequences and a varied range of statistics were found from negative value in some to positive in other regions but non-significant (Table 2). The results of neutrality test support the view that diversifying selection might be contributing to the existing/emerging diversity in the *dhfr* gene. Results on testing deviation from neutral evolution of sequences are presented in Table 2. The P value was found to be statistically insignificant for the neutrality tests undertaken for the *dhfr* gene.

This study revealed high frequency of mutant codons at *dhfr* loci in Indian parasite population as reported earlier [5, 12]. The results indicate that in natural P. falciparum infections, these mutations are present in different combinations, each of which confers different levels of resistance as documented earlier [1]. Although the extent of contribution of the different mutations to resistance remains to be determined with monitoring of the emergence of drug-resistant parasites by assays. It is now generally accepted that the major mechanism which gives rise to drug resistance is the introduction of point mutations in defined residues close to the active site of the enzyme [13]. In this study increase in the triple mutants (51I, 59R, 108N) in *dhfr* gene was seen as also reported from Africa and South East Asia where SP resistance is well established in the last two decades [2, 14]. Similarly, haplotypes with mutations at codons 51, 59, 108 in *dhfr* gene from Iran and African isolates have also been documented [15, 16, 17]. The *dhfr* gene sequences analysed among the different populations revealed the presence of SNPs among the parasite population suggestive of similar selective forces acting in different geographical regions. The distribution of these mutations in the studied gene may not be varying only in the studied regions but it also indicates that differences in the genotype within the regions does exist. The cladial tree of the *dhfr* gene indicates clearly depending upon their boot strap distance that the double and triple mutations are fast evolving due to drug pressure in Indian parasite populations. For comparison of relatedness to parasites among the various regions, genomic sequencing of *dhfr* gene from Indian field isolates delivers a comprehensive set of variations present in parasite populations which in turn, can provide an accurate approximation of associations between gene samples [18]. Globally, findings from Tajima's D, Fu and Li's D* and F* (i.e., positive values and not statistically significant) stand for the absence of any selective driven forces such as bottleneck, population reduction or expansion, balancing selection and selective sweep on the evolution of gene irrespective of study area [19]. Several studies have predicted the possibility of SP treatment failure with presence of mutations in *dhfr* and *dhps* genes but in this study no correlation with the SP failure could be included as no clinical data was available for the other reported studies [20]. The high rate of existing *dhfr* mutations observed in the eight regions of different endemicity for malaria, studied is a cause of concern for planning drug treatment. It is also recommended that continued surveillance and computational studies should be undertaken regularly as it provides valuable context to interpret population sequences differences of P. falciparum.

Declarations

Author contribution statement

Amit Kumara: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

S. K. Gahlawat: Contributed reagents, materials, analysis tools or data.

Vineeta Singh: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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