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Comparative assessment on the prevalence of mutation in the *Plasmodium falciparum* drug-resistant genes in two different ecotypes of Odisha state, India

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Abstract

Considering malaria as a local and focal disease, epidemiological understanding of different ecotypes of malaria can help in devising novel control measures. One of the major hurdles in malaria control lies on the evolution and dispersal of the drug-resistant malaria parasite, *Plasmodium falciparum*. We herewith present data on genetic variation at the Single Nucleotide Polymorphism (SNP) level in four different genes of *P. falciparum* (*Pfcrt*, *Pfmdr1*, *Pfdhfr*, and *Pfdhps*) that confer resistance to different antimalarials in two different eco-epidemiological settings, *i.e.* Hilly-Forest (HF) and Riverine-Plain (RP), in a high malaria endemic district of Odisha state, India. Greater frequency of antimalarial resistance conferring SNPs and haplotypes was observed in all four genes in *P. falciparum*, and *Pfdhps* was the most variable gene among the four. No significant genetic differentiation could be observed in isolates from HF and RP ecotypes. Twelve novel, hitherto unreported nucleotide mutations could be observed in the *Pfmdr1* and *Pfdhps* genes. While the *Pfdhps* gene presented highest haplotype diversity, the *Pfcrt* gene displayed the highest nucleotide diversity. When the data on all the four genes were complied, the isolates from HF ecotype were found to harbour higher average nucleotide diversity than those from RP ecotype. High and positive Tajima's *D* values were obtained for the *Pfcrt* and *Pfdhfr* genes in isolates from both the HF and RP ecotypes, with statistically significant deviation from neutrality in the RP ecotype. Different pattern of Linkage Disequilibrium (LD) among SNPs

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3. Competing interests

The authors declare that they have no competing interests.

4. Authors' contributions

NPK, NN, JMC, and AK designed the study. NPK led the survey team, collected samples, and data and did experiments. NPK, AD, and KC analysed the data. NPK, NN, JMC, AD, AK, and KC wrote the manuscript. All authors read and approved the final manuscript.

located in different drug-resistant genes was found in the isolates collected from HF and RP ecotypes. Whereas in the HF ecotype, SNPs in the *Pfmdr1* and *Pfdhfr* were significantly associated, in the RP ecotype, SNPs located in *Pfcrt* were associated with *Pfmdr1*, *Pfdhfr* and *Pfdhps*. These findings provide a baseline understanding on how different micro eco-epidemiological settings influence evolution and spread of different drug resistance alleles. Our findings further suggest that drug resistance to chloroquine and sulfadoxine-pyrimethamine is approaching fixation level, which requires urgent attention of malaria control program in India.

Keywords

malaria; *Plasmodium falciparum*; ecotypes; drug resistant genes; Odisha

1. Introduction

Epidemiological outcome of malaria infection differs in different ecotypes of the globe (Das et al., 2012; Kaewwaen and Bhumiratana, 2015; Kar et al., 2014; Okwa et al., 2009; Sharma, V.P. et al., 2015), and therefore, malaria is considered as a local and focal disease (Conn et al., 2015; Dash et al., 2008; Rath, 2004). Several studies involving different ecological and climatic settings have provided evidence that malaria epidemiology can be significantly variable across small eco-climatic scales (Jambulingam et al., 1991; Kaga and Ohta, 2012; Schapira and Boutsika, 2012). For example, malaria epidemiological outcomes including distributional prevalence of mosquito vectors and malaria transmission were correlated with different ecotypes in Nigeria (Okwa et al., 2009), Kenya (Ingasia et al., 2015), Brazil (Rosa-Freitas et al., 2007), Southeast Asia (Seng et al., 1999) and India (Jambulingam et al., 1991; Ramar et al., 2014; Shukla et al., 2007; Singh et al., 2015). Moreover, malaria outcome was found to be significantly higher in forested ecotype in comparison to no-forest ecotype (Kar et al., 2014) as observed in Belize (Hakre et al., 2004), Bangladesh (Haque et al., 2011), Nepal (Reisen et al., 1993), and India (Nath and Mwchahary, 2012; Sharma et al., 2006; Shukla et al., 2008). In India, studies conducted in the Sundargarh districts of Odisha state (high malaria endemic) showed that villages in forest and plain areas (separated by short geographical distances) have distinct malaria transmission pattern (Sharma et al., 2006), which could have been a consequence of prevalence of different species and vectorial behavior of a particular species of the mosquito vectors (Das, 2015; Manguin et al., 2008; Nanda et al., 2000; Singh et al., 1996). Considering the evolution and spread of malaria parasites resistant to different antimalarials [*viz*. Chloroquine (CQ), Sulfadoxine and Pyrimethamine (SP) *etc*.] that highly influence malaria epidemiological outcome and pose strong impediment to malaria control programs (Das and Dash, 2007; Hastings, 2003; Mallick et al., 2013b; Singh, V. et al., 2009), whether different local micro eco-climatic factors have influenced genetic changes at the genes conferring resistance to different antimalarials, needs to be evaluated (Sorosjinda-Nunthawarasilp and Bhumiratana, 2014). Needless to mention, such information will be of enormous benefit to the local malaria control program (Dash et al., 2008). This is because, in high malaria transmission areas resistance against CQ and SP in the malaria parasite *Plasmodium falciparum* spreads fast, whereas in low transmission areas, drug pressure plays a much crucial role (Hastings and Watkins, 2005; Malisa et al., 2016; Mallick et al., 2013a; Mallick et al., 2013b; Talisuna et

al., 2002). It has further been proposed that the predominance of tribal groups along with unrestricted use of inappropriate antimalarials, population movements, resettlements, and presence of sylvatic mosquito vectors promote rapid evolution of antimalarial resistance and therefore high malaria transmission settings encompassing this type of ecotype were proposed to be centre of origin of drug resistance (Chareonviriyaphap et al., 2000; Kar et al., 2014; Keiser et al., 2005; Malakooti et al., 1998; Singh, N. et al., 2009).

India is endemic to malaria and accounts for about 52% of the total malaria morbidity in Southeast Asia (Pradhan et al., 2016). Interestingly, majority of the malaria morbidity (about 26.9%) and mortality (about 17.6%) is contributed by Odisha state alone, although it comprises about 3% of Indian population (including some aboriginal tribes) (Pradhan et al., 2016). Intense and stable malaria has been reported from tribal areas of Odisha and neighbouring states (http://www.malariasite.com/tag/orissa/)(Das et al., 2012; Kumar et al., 2012; Kumar et al., 2007; Nanda et al., 2000). The state of Odisha consists of two highly malarious clusters; the North-Western (comprising of five districts, *viz*. Deogarh surrounded by Keonjhar, Sundergarh, Anugul and Sambalpur) and the South-Western (comprising of seven districts, *viz*. Koraput, surrounded by Malkangiri, Nawarangpur, Kalahandi, Raygada, Nuapada, and Kandhamal (Mohanty et al., 2009; Pradhan et al., 2016; Rao et al., 2015; Sahu et al., 2013), although other districts too contribute to the total malaria cases. Interestingly, the districts in both the clusters are rich in hills and forests and home for aboriginal tribes (Pradhan et al., 2016; Ramar et al., 2014; Sahu et al., 2013). The Deogarh district is one of the epicentres of high malaria endemicity (Pradhan et al., 2016); comprising of two distinct ecotypes [Hilly-Forested (HF) and Riverine-Plain (RP)], and therefore can serve as a model to understand the influence of micro eco-typical habitats on malaria epidemiological outcome (in this case mutational pattern in different genes conferring drug resistance in the malaria parasite, *P. falciparum*). This is important, as treatment in case of failure to the antimalarial CQ and SP has now become very common in almost all malaria endemic regions of the globe including India (Cui et al., 2015; Klein, 2013). In Odisha, high level of resistance to both CQ and SP has been reported from many malarious districts including Keonjhar, Sundargarh, Anugul and Sambalpur districts (Mohanty et al., 2009; Peterson et al., 1988; Srivastava et al., 2013; Sutar et al., 2011), with no report from the Deogarh district.

Tracking the patterns of mutations, estimating genetic diversities at the Single Nucleotide Polymorphism (SNP) level and asserting linkage among the SNPs in populations are the most efficient ways to understand the evolution of that particular gene (Carlton et al., 2015; Malisa et al., 2016; Pelleau et al., 2015; Sutar et al., 2013). Several studies following these methodologies in genes conferring resistance to antimalarials in *P. falciparum* have indicated evolutionary potential of these genes both at the global scale and also in India (Awasthi et al., 2011; Brown et al., 2015; Das and Dash, 2007; Kumar et al., 2015; Li et al., 2015; Rouhani et al., 2015). Mutations in the gene encoding a *P. falciparum* CQ resistance transporter (*Pfcrt*) and resulting change in single amino acid (AA) locus 76 from K to T (K76T) were proven a strong marker for CQ (Fidock et al., 2000; Valderramos et al., 2010). Similarly, mutations in the *P. falciparum* multi-drug resistance gene (*Pfmdr1*) conferring single AA change at point 86 from N to Y (N86Y) and/or multi-copy number of *Pfmdr1* was further reported to be linked with K76T and CQ resistance conferring synergistic increase in

resistance when combined (Babiker et al., 2001; Chauhan et al., 2014; Foote et al., 1990; Mwai et al., 2009; Price et al., 1999). Furthermore, mutations (S436A and A437G) in the *P. falciparum* dihydrofolate reductase enzyme coding gene singly pose mild resistance to SP drugs, and when linked with mutations A581G and /or K540E and /or A613S/T, confer high resistance (McCollum et al., 2012; Peterson et al., 1988; Rouhani et al., 2015). Moreover, polymorphism in the *Pfdhps* gene encoding S108N is the core mutation, but this confers comparatively lower resistance when present singly (Brooks et al., 1994; McCollum et al., 2012; Reeder et al., 1996; Triglia et al., 1997). Of particular importance is the correlation on the number of different mutations a parasite possesses to the ability to resist an antimalarial. For example, double mutant of the *Pfcrt*-$S_{72}V_{73}M_{74}N_{75}T_{76}$ and triple mutant $C_{72}V_{73}I_{74}E_{75}T_{76}$ haplotypes were more prevalent along with wild type $C_{72}V_{73}M_{74}N_{75}K_{76}$ and reflected more successful resistant haplotypes (Ghanchi et al., 2011; Nagesha et al., 2003). Moreover, accumulation of multiple mutations in *Pfdhfr* gene resulting in double mutants (C59R/S108N and N51I/S108N) shows moderate levels of resistance, the triple mutants N51I/C59R/S108N show a significant level, and the quadruple mutant parasite (N51I/C59R/ S108N/I164L) is considered to be completely resistant to pyrimethamine (McCollum et al., 2012; Sirawaraporn et al., 1997). Very similarly, co-accumulation of resistance-conferring mutations in both the *Pfdhfr* and *Pfdhps* genes synergistically diminishes the success of SP (Rouhani et al., 2015).

In the present study, we have performed DNA sequencing of four genes conferring drug-resistance in the malaria parasite *P. falciparum* in field isolates collected in the two different ecotypes in the Deogarh district of Odisha state, India. We identified SNPs in the four genes that are differentially segregating in these two populations and compared both the occurrence and distributional prevalence of different SNPs between the high endemic (HF) and moderately endemic (RP) areas. Pattern of genetic diversity between populations for each gene and linkage disequilibrium (LD) between different SNPs of a particular gene were also estimated. The results as a whole were interpreted in term of ongoing and past use of CQ and SP on evolution of drug resistance genotype of Indian *P. falciparum* in the two different malaria ecotypes in Deogarh district of Odisha, India.

2. Methodology

2.1. Study area, sample collection and malaria species diagnosis

The Deogarh district (21° 31' N Latitude and 84° 43' E Longitude) is located in the western part of Odisha (Figure 1). It covers a 2781.66 Sq. kilometre area with a total population of 27,41,08 (http://www.ordistricts.nic.in/district_profile/aboutus.php) and is highly endemic to *P. falciparum* malaria (Annual Parasite Incidence > 25) (http://nrhmorissa.gov.in/mis/SearchDetail.aspx). About 22% of the total land area of the Deogarh district is covered by forest, and therefore, this district is comprised of two distinct ecological settings (ecotypes); Hilly-Forest (HF) and Riverine-Plain (RP) (http://www.odishasampad.in/). We have collected isolates of *P. falciparum* from villages falling under these two ecotypes (HF and RP) that are under the two primary health centres (PHCs) located in about 40-kilometre distance (PHC Tileibani - HF and PHC Bampada – RP) (Figure 1).

Since malaria transmission in the Deogarh district occurs almost throughout the year, we have collected *P. falciparum* samples though active field collection during three different transmission periods [pre-monsoon (February-March), monsoon (July-August) and post-monsoon (September-November)] in the years 2011 and 2012 (Table 1). Malaria symptomatic individuals were finger-pricked and six drops of blood were collected from each patient. While three drops were used for diagnosis by (i) microscopic examination, preparation of thick and thin films and stained with Giemsa and (ii) rapid diagnostic test with bivalent kit, the rest three drops were put in a Whatman filter paper (for DNA isolation in the lab). The patients diagnosed with malaria with the rapid diagnosis test were provided appropriate treatment support. The blood samples in the slides and the filter paper (after dried) were brought to the laboratory in New Delhi for further analyses. In the lab, both the thick and thin films were stained with Giemsa and the results were matched with the observations on the rapid diagnostic test. Since diagnostic tests by PCR is considered to be highly sensitive (Gupta et al., 2010; Johnston et al., 2006), DNA was isolated from all the field collected samples using Qiagen kit. Considering high mixed parasite infections prevalent in India (Gupta et al., 2010), we used PCR diagnostic assay using published primers (Gupta et al., 2010) for determination of mono infection by *P. falciparum* in the collected samples. Monoclonality of *P. falciparum* infections were determined on the basis of determination of a single haplotype of each of the four genes conferring drug resistance (see below). In total, 1,000 samples have initially been collected from both the two ecological zones (354 from HF and 646 from RP), out of which only 229 were pure and single-clonal *P. falciparum* infections (118 from HF and 111 from RP) were used for DNA sequencing of the four genes conferring drug resistance in *P. falciparum*. The study was approved by the human ethics committee of the National Institute of Malaria Research, New Delhi, India and written informed consents have been obtained from each adult participant and from parents/guardians of patients below 18 years of age.

2.2. PCR Amplification of the four drug-resistant genes

In this study, we have considered four genes (*Pfcrt, Pfmdr1, Pfdhfr*, and *Pfdhps*) that are reported to be associated with resistance to antimalarials, chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) in *P. falciparum*. For all the four genes, the genetic regions containing SNPs that are associated with *in vitro* drug-resistance in *P. falciparum* were only sequenced (for details of the genetic regions, primers for each gene fragment, see supplementary Table 1). For this, nested PCR protocols have been followed (for details of PCR protocols for each individual gene, please refer to the supplementary Table 1). For example, two different reaction volumes (15μl and 30μl) were used in primary and nested PCR. All PCR amplifications were performed by using “AmpliTaq Gold™” polymerase (PE Applied Bio Systems, Foster City, CA) and for visual quantification, only 3μl PCR products were used in 1.5% Agarose with 100bp DNA ladder (Bangalore Genei, Bengaluru, India). Details of protocols for PCR amplification of each gene have been provided in supplementary Table 1.

2.3. DNA Sequencing and population genetic analyses

All PCR products were purified using shrimp alkaline phosphatase (*SAP*) and exonuclease I (*Exo I*) enzymes (Fermentas, USA) before processed for DNA sequencing. For each 25 μl PCR product, one unit of *Exo I* and one unit of *SAP* with 10X SAP buffer were used and

final reaction volume was made up to 30.0 μl with autoclaved, nuclease-free water (Ambion, Life Technologies). The reaction mixtures were incubated in Eppendorf Master Cycler Pro gradient thermal cycler for 50 min at 37 °C (digestion) and then for 20 min at 85 °C (inactivation of enzymes). Purified PCR products were sequenced commercially (Macrogen Inc., Seoul, Korea, http://dna.macrogen.com/english).

Multiple DNA sequences from each gene were collectively imported to the DNADynamo computer program (Blue Tractor Software, North Wales, United Kingdom; (http://www.bluetractorsoftware.co.uk/) along with respective reference sequence of the wild type (*Pf*3D7) for viewing the sequence chromatogram, manual editing, and multiple sequence alignment. The edited sequences were deposited in GenBank with accessions XXXXXX–YYYYY. Single nucleotide polymorphisms (SNPs) were spotted by scanning mismatch highlights from the split window of DNADynamo base-call alignment window and reconfirmed by referring aligned chromatograms in lower split window. Since all the four sequenced DNA fragments are located in the coding regions (exon) of the genes, the aligned sequences were translated to amino acid sequences and synonymous and non-synonymous mutations, if any, were spotted. Any change in the amino acid was ascertained by looking at the responsible SNP in comparison to the standard reference sequence. Since specified SNPs in designated genetic regions have been implicated with drug-resistance phenotype of *P. falciparum*, SNPs located in those specific regions were specifically looked at. For example, SNPs causing amino acid changes in the *Pfcrt*-$C_{72}V_{73}M_{74}N_{75}K_{76}$ (capital letters representing wild type amino acids (AA) with their positions in the gene subscripts), *Pfmdr1*-N_{86}, *Pfdhfr*-$A_{16}N_{51}C_{59}S_{108}I_{164,}$ and *Pfdhps*-$S_{436}A_{437}K_{540}A_{581}A_{613}$ were noted. Similarly, mutations in other regions of the sequenced DNA fragments of each gene and corresponding change in amino acid were also noted.

Differential arrangements of SNPs for each gene in every *P. falciparum* isolate form a particular haplotype. In order to know genetic differences between the HF and RP ecotypes for each independent gene, first the frequencies of different haplotypes were calculated using the Statistical Package for Social Sciences 16.0 (SPSS, Inc., Chicago, IL, USA) for each ecotype separately. Further, in order to know if differential distribution of different haplotype in a particular gene exists between isolates from HF and RP ecotypes, chi-square tests were performed independently for each gene. In addition, haplotype diversity (Nei, 1987) and two measures of nucleotide diversity (θ_w and π) were estimated for each of the HF and RP ecotypes. While the nucleotide diversity parameter π is estimated based on average number of pair wise nucleotide difference per site, (Tajima, 1989) the θ_w estimate is dependent on the number of segregating sites (Watterson, 1975). All these parameters were estimated using the DnaSP 5.02 computer program (Librado and Rozas, 2009). In order to ascertain if the four genes conferring drug resistance in *P. falciparum* follow neutral equilibrium model of molecular evolution, the Tajima's *D* test was performed for each gene for the isolates from each ecotypes separately using the DnaSP 5.02 computer program (Librado and Rozas, 2009). The Tajima's *D* (Tajima, 1989) statistic calculates the normalized differences between the two measures of nucleotide diversity (θ_w and π) for isolates from each ecotype. Whereas an excess of low frequency polymorphism generates negative value of TD indicating directional selection or population size expansion, low level of high frequency polymorphism generates positive value indicating balancing selection or population size

reduction (Das et al., 2004). Moreover, in order to determine if differential association exists among SNPs segregating in all the four gene for isolates from a particular ecotype (either HF or RP), linkage disequilibrium (LD) tests were performed for each possible pair-wise SNP implicated as drug-resistant marker in the four genes by calculating the r^2 values using the Haploview computer program (Barrett, 2005).

1. Results

Out of the total 1000 malaria positive blood samples collected (354 from HF and 646 from RP), only 229 isolates (118 from HF and 111 from RP) were found to be infected with *P. falciparum* employing three different types of malaria diagnosis, *viz*. microscopy, RDT and PCR. In addition, in order to ascertain if all the 229 isolates are monoclonal infections, sequence chromatograms of the four genes conferring drug resistance (*Pfcrt*, *Pfmdr1*, *Pfdhfr*, and *Pfdhps*) of all the 229 isolates were carefully checked for double peaks. For every gene, mono-clonality was established based on single peaks, resulting fractions of the 229 isolates independently in each of the genes (*Pfcrt*-64/229; *Pfmdr1*-73/229; *Pfdhfr*-29/229 and *Pfdhps*-84/229) to be monoclonal. The distribution of single clonal infections was however not found to be significantly different between the HF and RP ecotypes based on the data of single peak in the four genes (for HF; *Pfcrt*-34, *Pfmdr1*-36, *Pfdhfr*-13 and *Pfdhps*-41 and for RP; *Pfcrt*-30, *Pfmdr1*-37, *Pfdhfr*-16 and *Pfdhps*-43). In total, only 24 isolates (12 from HF and 12 from RP) were found to be monoclonal based on single peak of nucleotide chromatogram when all the four genes were considered.

All the mutations implicated in conferring resistance to different antimalarials in *P. falciparum* were sequenced in multiple isolates from both the HF and RP ecotypes. In addition, with multiple sequence alignment, additional mutations (some reported and some novel) were also detected. The distribution of different haplotypes (due to combinations of different mutations implicated in drug resistance in four genes) in both the HF and RP is depicted in Table 2. Whereas in three genes similar haplotypes (*Pfcrt* three haplotypes, in *Pfmdr1* two and on *Pfdhfr* two haplotypes) were found between the HF and RP ecotypes, in case *Pfdhps*, six haplotypes in HF and four in RP were detected (Table 2). Although the frequency of different haplotypes of the four genes was different between isolates from two ecotypes, but no marked difference could be seen (Table 2). The distribution of the number of haplotypes were subjected to chi-square test for all the four genes between the isolates from HF and RP ecotypes, which indicate statistically significantly deviation from the expected distribution only in case of *Pfcrt* gene. Therefore, the data in general on the frequency distribution of different haplotypes that are associated with phenotype drug resistance in *P. falciparum* indicate no significant difference between the two ecotypes.

Interestingly, many other mutations (other than the mutations implicated in drug resistance) were found to be segregated in the two (*Pfmdr1* and *Pfdhps*) out of the four genes. Whereas the *Pfmdr1* harbours four mutations (one in HF and three in RP), only one mutation (C228T) was common between the isolates of two ecotypes and the rest two were confined to RP ecotype (Table 3). While the A269G mutation (found in the RP ecotype) has been reported elsewhere, the other two mutations were novel (Table 3). On the other hand, the *Pfdhps* gene possessed as many as 11 mutations with one mutation common to the isolates from two

ecotypes and the rest are unique to an ecotype (five in HF and five in RP) (Table 3). Surprisingly, the mutation that is common in both the ecotypes (T1632Z) was found in multiple isolates (seven in each ecotype) and identified to be a stop codon. The rest 10 mutations were found in individual *P. falciparum* isolates in two ecotypes of Deogarh district (Table 3).

In this study, Single Nucleotide Polymorphisms (SNPs) were identified by direct DNA sequencing of the DNA fragments in the four genes conferring drug resistance in *P. falciparum*. In order to ascertain if genetic differentiation exists between the isolates from two ecotypes, we segregated the DNA sequences of the four genes according to ecotypes and performed different population genetic analyses of DNA sequence variation. In general, the *Pfdhps* gene was found to be highly polymorphic among the four genes (Table 4). This pattern was more pronounced in the HF ecotype than in the RP. The TD values were found to be variable across the genes and between the ecotypes. Surprisingly, for *Pfdhfr*, high and positive values of TD were observed in isolates from both the ecotypes (Table 4). However, in no case statistically significant deviation from neutral expectation could be observed. However, in case of the *Pfcrt* gene, positive value of TD with statistically significant deviation from neutral model of molecular evolution could be observed in isolates from RP ecotype. The data therefore suggest that DNA sequence polymorphisms do exist among different genes conferring drug resistance in *P. falciparum* and also between the isolates from two different ecotypes.

Finding of 12 isolates from each of the two ecotypes to be monoclonal in all the four genes and determination of 12 SNPs (variably present in four genes) to be segregating in the two ecotypes provides us an opportunity to determine if any two SNPs either present in a single gene or in two different genes are associated with each other. For this, we performed LD test and determined the R^2 value of each SNP pair-wise association (Figure 2). In general, more number of statistically significant associations between SNP pairs was found in the RP in comparison to the HF ecotype. As expected, several statistically significant associations were found among SNPs located in the *Pfcrt* gene in both the ecotypes (Figure 2). Similarly, significant associations were found between SNPs located in both *Pfdhfr* and *Pfdhps* genes. Surprisingly, several cases of statistically significant associations were found between the SNPs present inside the *Pfcrt* gene and *Pfdhfr* gene and *Pfdhps* in RP ecotype. However, no such association could be detected in the HF ecotype. In contrast, in HF ecotype, the sole SNP of the *Pfmdr1* gene was significantly associated with both the SNPs of the *Pfdhfr* gene; and no such association was found in the RP ecotype. The result on the whole thus indicate that while association of SNPs present in a particular gene is a common phenomenon in both the ecotypes, association of SNPs between different genes seems to be ecotype-specific.

2. Discussion

Considering malaria as a highly local and focal disease, local ecological conditions play vital role in malaria transmission (Conn et al., 2015; Das, 2015; Dash et al., 2008; Rath, 2004). To this extent, what amount of micro ecological settings influence malaria epidemiological outcome is poorly understood. We herewith have considered differential prevalence, frequency, and evolutionary pattern of mutations in the four genes that are

known to confer drug resistance in the most dreadful malaria parasite, *P. falciparum* prevalent and predominant in two ecotypes (HF and RP). We have chosen these two ecotypes placed very closely (about 40 kilometres apart) to each other but with different ecological and topographical settings in a single district of Odisha state of India, which significantly contributes (about 26.9%) to malaria in India (Pradhan et al., 2016). Furthermore, it is reported that intense malaria transmission occurs in the HF, whereas the RP is moderately endemic for malaria (Kar et al., 2014; Pradhan et al., 2016). Since (i) resistance to different antimalarials is highly prevalent in India (and in Odisha), (ii) such conditions contribute considerably to malaria epidemiology, and (iii) genetic basis of drug resistance in malaria is widely established, we attempted to retrieve epidemiological information from population genetic studies of the genes conferring resistance and compare with ecological and other micro-variables between the two different ecotypes (HF and RP).

A general observation on the prevalence and distribution of different mutations associated with drug resistance in the four genes (*Pfcrt*, *Pfmdr1*, *Pfdhfr*, and *Pfdhps*) of *P. falciparum* is the presence of all the mutations that too in appreciable frequency in samples from both the HF and RP ecotypes. However, some deviations exist; for example, the *Pfdhps* gene harbours high diversity in both the type and number of mutations and therefore the corresponding haplotypes. All the four commonly reported amino acid mutations that confer resistance to sulfadoxine-pyrimethamine ($S_{436}A_{437}K_{540}A_{581}$) could be found in the present study, except one ($A_{613}S$) that is rare in India (Biswas et al., 2000; Kumar et al., 2015). The present findings therefore substantiate similar outcomes from other Indian states including Odisha with respect to the mutational pattern at the *Pfdhps* gene (Sharma, D. et al., 2015). For the *Pfcrt* gene, three different haplotypes were found including the wild type (chloroquine sensitive) haplotype ($C_{72}V_{73}M_{74}N_{75}K_{76}$), which reconfirm its prevalence in Odisha. However, in other Indian states this haplotype is very rarely found (Mishra et al., 2006; Mixson-Hayden et al., 2010). Similarly, the $C_{72}V_{73}I_{74}E_{75}T_{76}$ haplotype is highly prevalent, and the frequency of the $S_{72}V_{73}M_{74}N_{75}T_{76}$ haplotype is comparatively low (Table 2), reconfirming previous observations from Odisha (Okombo et al., 2014; Ramani et al., 2016; Sutar et al., 2013; Sutar et al., 2011). The skewed distributional prevalence of the $C_{72}V_{73}I_{74}E_{75}T_{76}$ haplotype is reflected by the observation of statistically significant χ^2 value (Table 2). To be noted that the $C_{72}V_{73}I_{74}E_{75}T_{76}$ type is known to confer higher resistance to CQ than the $S_{72}V_{73}M_{74}N_{75}T_{76}$ type (Mittra et al., 2006), suggesting high level of CQ resistance in *P. falciparum* in Odisha (Ramani et al., 2016). The principal mutation conferring resistance to antimalarial at the *Pfmdr1* gene (Y_{86}) could be found in high frequency (73% in RP and 77.8% in HF; Table 2). Interestingly, not all the four amino acid substitutions ($I_{51}R_{59}N_{108}L_{164}$) associated with drug resistance could be found in the present study, whereas, these have been reported from India including Odisha (Sharma, D. et al., 2015). In the present study, we could find higher prevalence of the $R_{59}N_{108}$ combination (haplotype) in comparison to the $C_{59}S_{108}$ (drug sensitive type). These two mutations are considered to be highly dominate ones across many malaria endemic populations of the globe including India (Sharma, D. et al., 2015) and the other two mutations ($I_{51}L_{164}$) are reported to be surfacing in India relatively recently (Sharma, D. et al., 2015). Since it is argued that *P. falciparum* parasites with triple and quadruple mutations in the *Pfdhfr* gene are highly resistant to SP, and currently sulfadoxine-pyrimethamine is used in a combination

therapy with Artemisinin, it can be noted that *P. falciparum* isolates in Deogarh district of Odisha is less resistant to SP. However, recent finding on the prevalence of triple mutations [($I_{51}R_{59}N_{108}$) or ($R_{59}N_{108}L_{164}$)] in Odisha (Sharma, D. et al., 2015) indicate that resistance to SP is emerging in Odisha. The overall pattern of mutations associated with resistance to different antimalarials in *P. falciparum* in Deogarh district of Odisha indicates that (i) haplotypes associated with drug resistance in all the four genes are prevalent in isolates from both HF and RP ecotypes, (ii) the *Pfdhps* gene harbours a comparatively larger number of haplotypes than other three genes and (iii) no significant differences could be observed for the patterns of mutations associated with drug resistance (and their corresponding haplotypes) between the HF and RP ecotypes.

Interestingly, only two (*Pfmdr1* and *Pfdhps*) out of the four genes harbour mutations other than the ones that are associated with drug resistance (Table 3). Based on the observed pattern of mutations in the *Pfcrt* gene in Indian *P. falciparum*, it has been previously demonstrated that this gene is under massive genetic reconstruction (Chauhan et al., 2013; Das and Dash, 2007). For the *Pfdhfr* gene, although we have sequenced a larger DNA fragment in comparison to the *Pfcrt* gene, occurrence of mutations other than the ones conferring to SP resistance is reported to be minimal (Sharma, D. et al., 2015) For the *Pfmdr1* gene, three amino acid substitutions (other than the mutations associated with drug resistance) could be found; one common in isolates from both the HF and RP ecotypes, and two only confined to RP (Table 3). While the common one ($C_{228}T$) and the $T_{317}C$ (confined to RP) are entirely novel, the $A_{269}G$ (confined to RP) has been reported earlier in Kenya (Okombo et al., 2014). For the *Pfdhps* gene, as many as 11 nucleotide substitutions (resulting in two synonymous substitutions and nine non-synonymous, including three stop codons) could be found. Out of these two synonymous substitutions one has been recently reported from Odisha (Kumar et al., 2015). On the whole, the HF contains two stop codons; two synonymous and three non-synonymous substitutions and the RP comprises two stop codons (one common with HF) and six non-synonymous substitutions. All the three non-synonymous in HF and five (out of six) non-synonymous substitutions and the three stop codons are novel and unique to that particular ecotype (Table 3); whereas with the $T_{544}Z$ non-synonymous substitution is common between the two ecotypes. Such an observation indicates that (i) pattern of mutations (other than the ones associated with drug resistance) in the four genes (*Pfcrt*, *Pfmdr1*, *Pfdhfr* and *Pfdhps*) are highly gene-specific, and (ii) no significant differentiation in the overall pattern could be observed between the isolates from HF and RP ecotypes.

In order to know if differential patterns of nucleotide diversity and signature of molecular evolution and association of commonly occurring SNPs in the four genes implicated in providing drug resistance in *P. falciparum* exist between the isolates from HF and RP ecotypes, we have conducted population genetic analyses of DNA sequence data. As found in case number and prevalence of different mutations (see above), the *Pfdhps* gene harbours the highest number of haplotypes (13 in HF and 7 in RP) as well as haplotype diversity among the other genes (Table 4). However, the *Pfcrt* gene displays the highest nucleotide diversity as measured by π (0.02297 in HF and 0.03218 in RP) among the four genes. High and positive TD values could be observed in case of the *Pfcrt* and *Pfdhfr* genes in both the ecotypes, with highest values of 2.28081 (*Pfcrt*-RP) (Table 4). This value is statistically

significantly deviated from the expectation under neutral model of molecular evolution. Since positive TD values indicate evidence of balancing selection (Chauhan et al., 2014), it seems that alleles of both the *Pfcrt* and the *Pfdhfr* genes are maintained in stable frequencies. In case of *Pfdhps*, the TD values are high and negative in both the ecotypes, but not statistically significantly deviated from the neutral equilibrium model. This might indicate an initial genetic hitchhiking in the presence of large effective population size of *P. falciparum* (due to high malaria transmission in Odisha) as observed earlier in *Pfmdr1* gene in Odisha (Chauhan et al., 2014) and in case of microsatellite polymorphisms in case of *Pfcrt* gene in India (Chauhan et al., 2013). To be noted that such evolutionary events (invoked by both natural selection and demography) might have created high haplotype diversity in the *Pfdhps* gene, as suggested by Chauhan et al. 2013.

The test of linkage disequilibrium between a pair of SNP yielded interesting results (Figure 2). As expected, all the four mutations in the *Pfcrt* gene are found to be strongly linked in *P. falciparum* isolates sampled from both the HF and RP ecotypes. However, in the RP ecotype, all the four mutations of the *Pfcrt* gene were statistically significantly associated with mutations of the *Pfmdr1*, *Pfdhfr*, and *Pfdhps* genes. In the HF ecotype, however, significant LD was observed between SNPs of the *Pfmdr1* and *Pfdhfr* genes. The $N_{86}Y$ mutation is widely prevalent in almost all malaria endemic countries of the globe including India (Mita et al., 2009; Thomsen et al., 2013) and often found to be associated with mutations in the *Pfcrt* gene (Chauhan et al., 2014). Therefore, differential associations of mutations among the four genes implicated in drug resistance in the two ecotypes seem to be highly ecotype-specific. Different ecological and topographical conditions prevailing in the two different ecotypes might have contributed to the observed differential association of SNPs present in different genes. However, distributional prevalence of different species of *Anopheles* (vectors to malaria parasites) (*An. fluviatilis* in HF and *An. culicifacies* in RP) might also have contributed to the observation (Nanda et al., 2000; Sharma et al., 2006; Sorosjinda-Nunthawarasilp and Bhumiratana, 2014). It is known that individuals adjust their genomes through new genetic associations for adaptation to adverse eco-environmental conditions (Van Tyne et al., 2011). Whether the observed patterns of mutations and their evolution in the isolates from HF and RP ecotypes are propelled by adaptation by natural selection could not be established from the present study, but it could be ascertained that differential evolutionary forces (exerted by drug pressure in the field) are in operation at the four different genes implicated in drug resistance in the malaria parasite *P. falciparum* in the Deogarh district of Odisha.

In conclusion, the results of the present study, although limited to a single district of a state that contributes the highest number of cases and deaths due to malaria in India, provide many meaningful insights into the patterns of mutations in the four genes implicated in drug resistance in the malaria parasite, *P. falciparum*. Although in many aspects, there were no significant differences between the two ecotypes (HF and RP), but some underlying differences could be noted. Considering the amount of genetic diversity to be associated with the intensity of malaria transmission in India (Chauhan et al., 2014; Sharma, D. et al., 2015), the isolates from HF ecotype harbours more average genetic diversity (as estimated by nucleotide diversities, π and θ) than those from the RP. Further, the observed associations between pairs of SNPs present in four different genes could be due to different drug pressure

applied in the field due to different treatment practices. The results from genetic studies indicate that CQ resistant genotypes are approaching fixation level and SP resistant genotypes are evolving very fast, and the existing drug policy needs to be reviewed, as SP is a partner drug administered with artemisinin. However, genetic studies of this kind in wide areas (e.g. the whole Odisha state) including different micro ecological settings with different malaria transmission patterns are needed before a conclusive decision on change in drug policy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Patterns of genetic variations in genes conferring drug-resistance in micro eco-epidemiological settings in malaria are not known
- We performed SNP analyses in four different drug-resistant genes in *P. falciparum* form two different eco-epidemiological locations of Odisha state of India.
- Results revealed higher genetic variation in general in all the four genes in the Hilly-Forest ecosystem than the Riverine-Plain one
- More cases of genetic linkage between different drug-resistant genes were evident in Riverine-Plain ecosystem.
- High observed genetic variation and genetic linkage suggest strong resistance capabilities of *P. falciparum* in Odisha, calling for change in drug policy

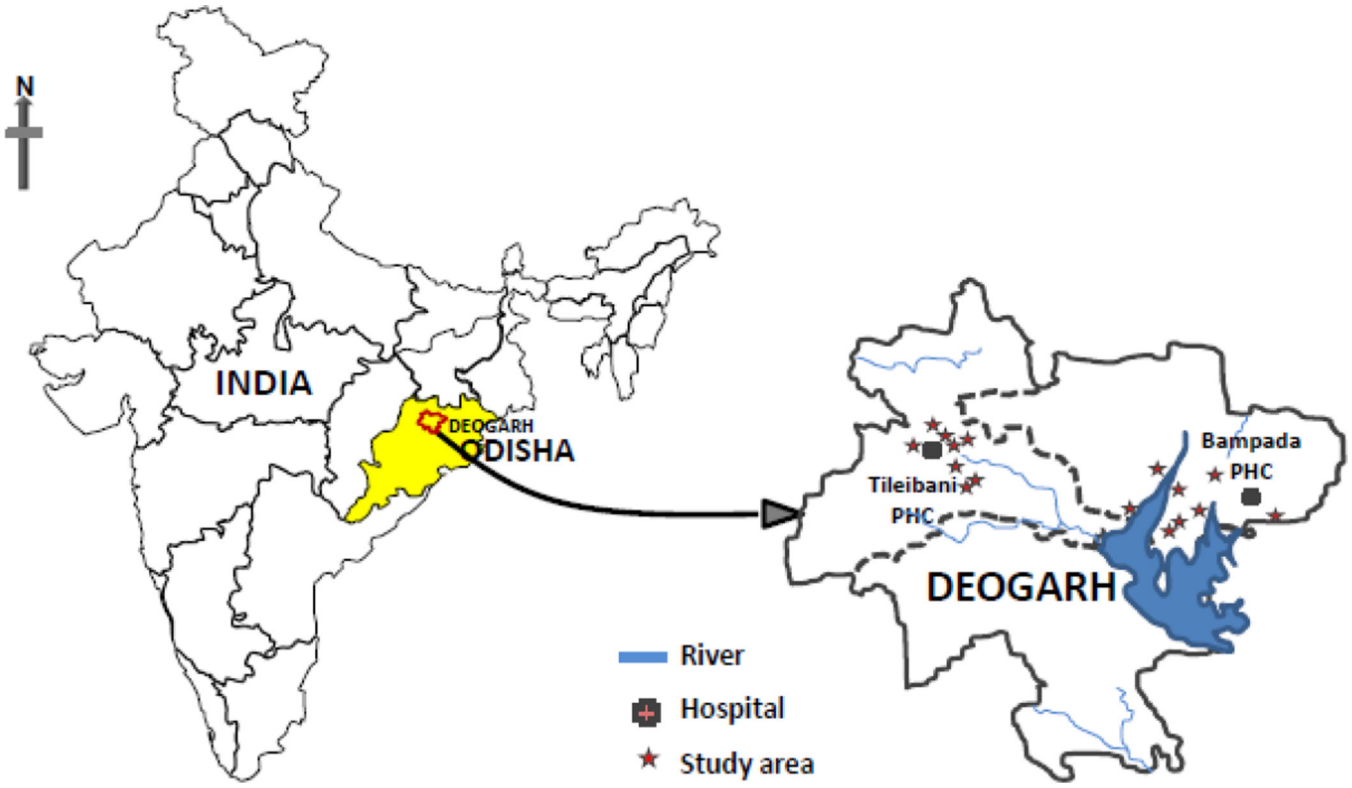
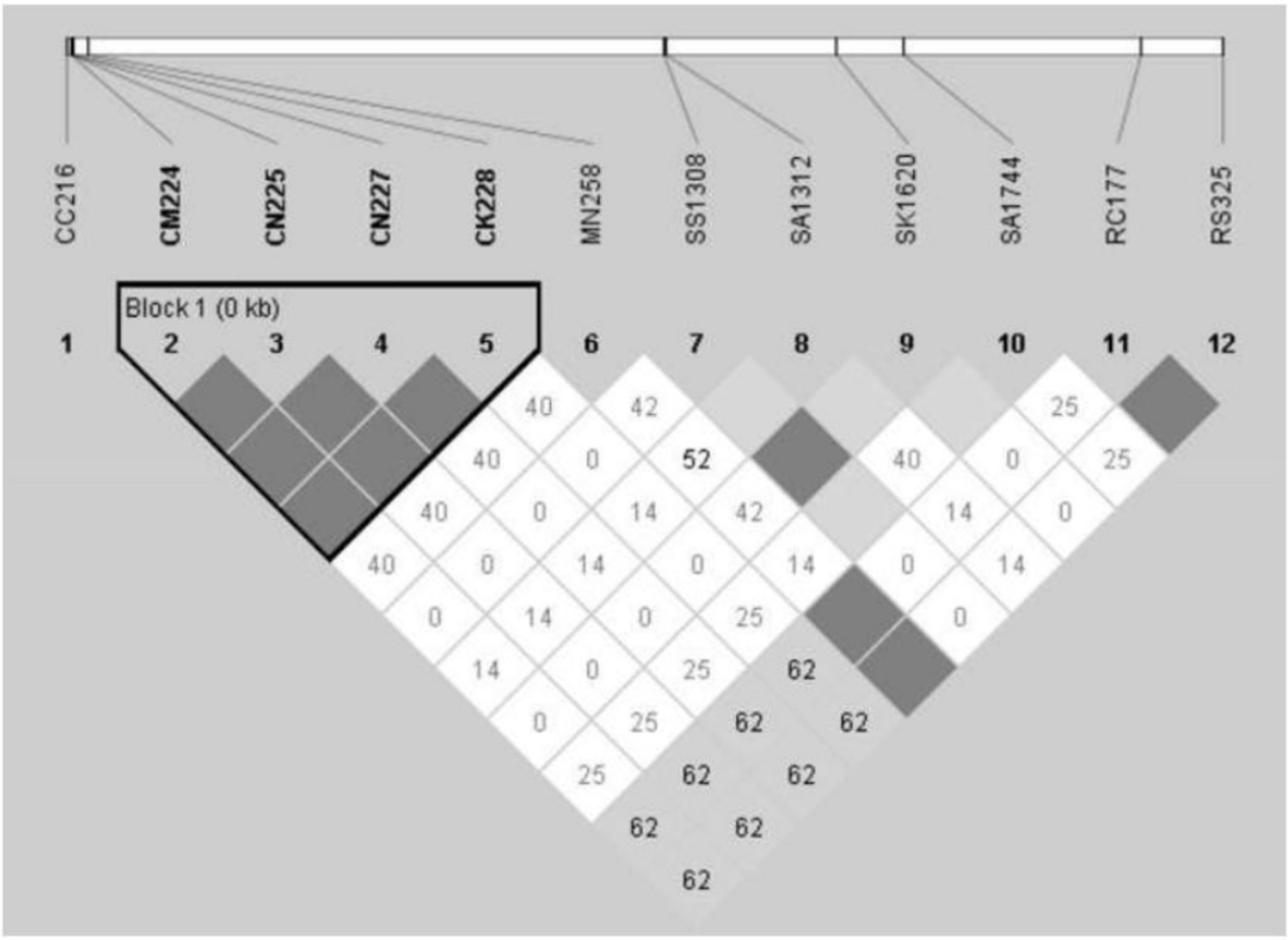
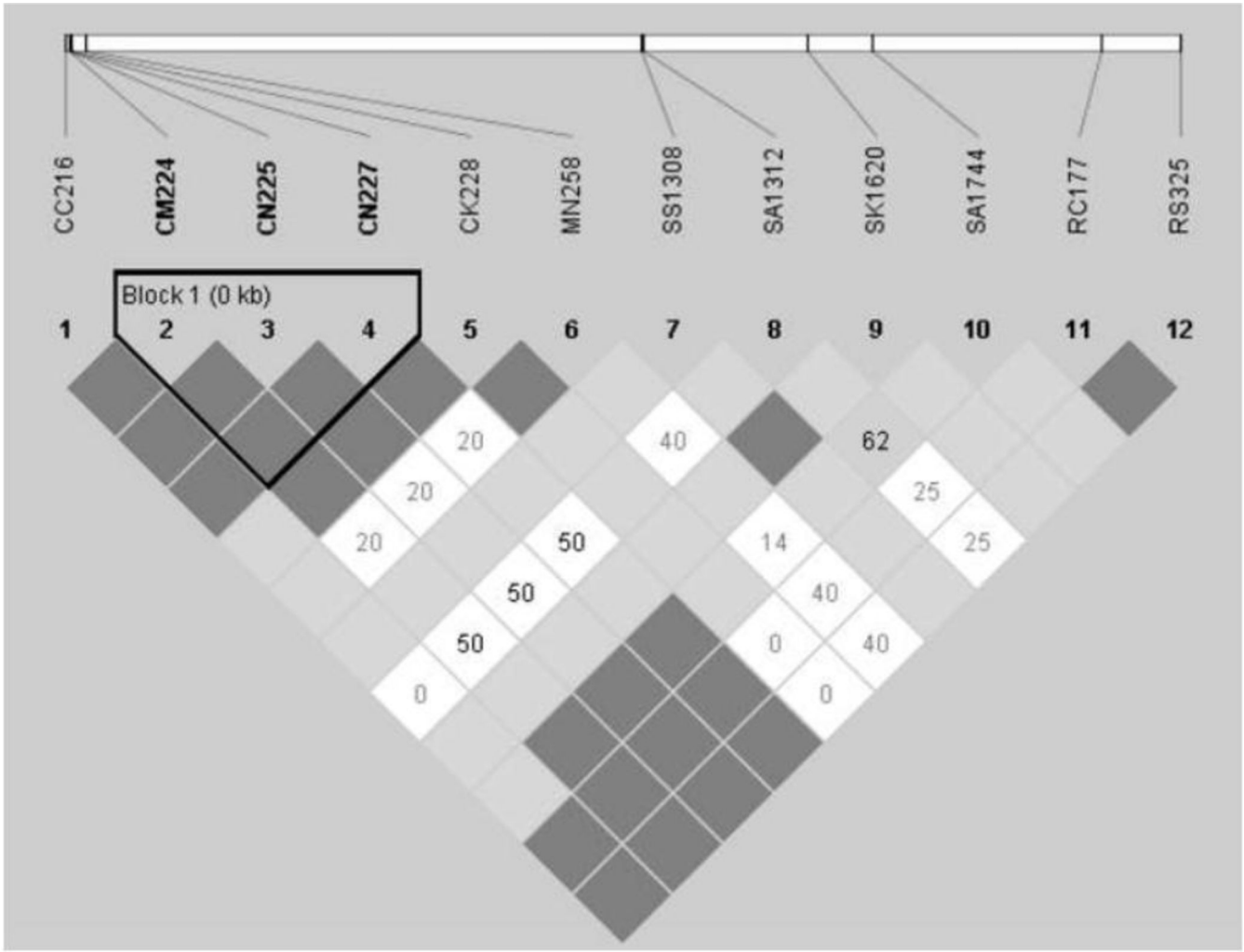


Figure 1.

Map of India highlighting Deogarh district (Odisha state). Sample collection sites in Hilly-Forest ecotype (PHC Tileibani) and Riverine-Plain ecotype (PHC Bampada) are demarcated.



a



b

Figure 2.

Linkage Disequilibrium (LD) between pairs of SNPs located in 4 different genes (*Pfcrt*, *Pfmdr*, *Pfdhfr* and *Pfdhps*) implicated in drug resistance in *P. falciparum* in Hilly-Forest (a) (HF) and (b) Riverine-Plain (RP)

Table 1

Sampling detail of *P. falciparum* isolates in two different ecotypes in Odisha, India.

	Seasonal collection of *P. falciparum* isolates				
Ecotype	**February and March 2011**	**September and November 2011**	**July and August 2012**	**Total**	
Hilly-Forested	20	69	29	118	229
Riverine-Plain	24	47	40	111	

Table 2

Frequency (in percent) of different drug resistant marker haplotypes in four genes implicated in drug resistance in two ecotypes of *P. falciparum*

Ecotypes	Genes							
	Pfcrt		***Pftndr***		***Pfdhfr***		***Pfdhps***	
Hilly-Forested	$C_{72}V_{73}I_{74}E_{75}T_{76}$	25(73.5%)	N_{86}	8 (22.2%)	$C_{59}S_{108}$	4(30.8%)	$A_{436}G_{437}E_{540}A_{581}$	3 (7.3%)
	C_{72} $V_{73}M_{74}N_{75}K_{76}$	7 (20.6%)	Y_{86}	28 (77.8%)	$R_{59}N_{108}$	9 (69.2%)	$A_{436}G_{437}K_{540}A_{581}$	1 (2.4%)
	S_{72} $V_{73}M_{74}N_{75}T_{76}$	2(5.9%)					$S_{436}A_{437}K_{540}A_{581}$	28 (68.3%)
							$S_{436}A_{437}K_{540}G_{581}$	1 (2.4%)
							$S_{436}G_{437}K_{540}A_{581}$	1 (2.4%)
							$S_{436}G_{437}K_{540}G_{581}$	7(17.1%)
	Total	34		36		13		41
Riverine-Plain	$C_{72}V_{73}I_{74}E_{75}T_{76}$	15 (50.0%)	N_{86}	10 (27.0%)	$C_{59}S_{108}$	7(43.8%)	$A_{436}G_{437}E_{540}A_{581}$	4 (9.3%)
	C_{72} $V_{73}M_{74}N_{75}K_{76}$	6 (20.0%)	Y_{86}	27(73.0%)	$R_{59}N_{108}$	9(56.3%)	$S_{436}A_{437}K_{540}A_{581}$	33 (76.7%)
	$S_{72}V_{73}M_{74}N_{75}T_{76}$	9 (30.0%)					$S_{436}A_{437}K_{540}G_{581}$	1 (2.3%)
							$S_{436}G_{437}K_{540}G_{581}$	5(11.6%)
	Total	34		37		16		43

For *Pfcrt:* χ^2=6.808 *, P=0.033; *Pfmdr*: χ^2=0.227, P=0.634; *Pfdhfr:* χ^2=0.513, P=0.474; *Pfdhps:* χ^2=2.840, P=0.725

*Statistically significant at 0.05 level.

Table 3

List of novel mutations (other than the marker mutations) in the *Pfmdr1* and *Pfdhfr* genes in the two different ecotypes of Indian *P. falciparum*. No such novel mutations could be detected in the *Pfcrt* and *Pfdhfr* genes in the present study.

Genes	Nucleotide mutations and resulting changes in amino acids (in parentheses)	
	Hilly Forested	**Riverine Plain**
Pfmdr	$C_{228}T$ ($S_{76}S$)	$C_{228}T$ ($S_{76}S$)
		$A_{269}G$ ($D_{90}G$) *$
		$T_{317}C$ ($F_{106}S$)$
	$T_{1632}G$ ($Y_{544}Z$)	$T_{1632}G$ ($Y_{544}Z$)
Pfdhps	$C_{l305}A$($S_{435}S$) **#	$C_{l378}T$ ($Q_{460}Z$)$
	$A_{1335}G$ ($K_{445}K$)#	$A_{1454}G$ ($N_{485}S$)$
	$T_{1373}C$ ($L_{458}S$)#	$A_{1508}G$ ($D_{503}G$)$
	$A_{1375}C$ ($F_{459}L$)#	$A_{1537}G$ ($I_{513}V$)$
	$A_{1426}I$ ($K_{476}Z$)#	$T_{1685}C$ ($L_{562}P$)$

*Reported in earlier studies *(Okombo et al., 2014)

**(Kumar et al., 2015),

#Unique to HF;

$Unique to RP; 'Z' represents a stop codon.

Table 4

Summary statistics of DNA sequence polymorphism in four different genes containing drug resistance in two ecotypes of *P. falciparum*.

Ecotypes	Genes	No. of isolates	Number of haplotypes	Haplotype diversity	Nucleotide diversity		Test of neutrality
					π	θ	Tajima's *D*
Hilly-Forested	*Pfcrt*	34	3	0.426	0.02297	0.01698	0.93263
	Pfmdr	36	3	0.367	0.00106	0.00124	−0.28681
	Pfdhfr	13	3	0.5	0.0029	0.00221	1.89943
	Pfdhps	41	13	0.745	0.00307	0.00479	−1.08329
	Average			0.5095	0.0075	0.006305	
Riverine-Plain	*Pfcrt*	30	3	0.641	0.03218	0.01753	2.28081*
	Pfmdr	37	5	0.495	0.00146	0.00247	−0.9918
	Pfdhfr	16	2	0.525	0.0036	0.00206	1.89943
	Pfdhps	43	7	0.607	0.0026	0.00387	−0.94829
	Average			0.567	0.00996	0.006483	

*Statistically significant at 0.05 level.