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Brief communication

Antifolate drug resistance: Novel mutations and haplotype distribution in *dhps* and *dhfr* from Northeast India

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Malaria is a major public health concern in Northeast India with a preponderance of drug-resistant strains. Until recently the partner drug for artemisinin combination therapy (ACT) was sulphadoxine pyrimethamine (SP). Antifolate drug resistance has been associated with the mutations at dihydropteroate synthase (*dhps*) and dihydrofolatereductase (*dhfr*) genes. This study investigated antifolate drug resistance at the molecular level. A total of 249 fever cases from Arunachal Pradesh, NE India, were screened for malaria, and of these, 75 were found to be positive for *Plasmodium falciparum*. Samples were sequenced and analysed with the help of BioEdit and ClustalW. Three novel point mutations were found in the *dhps* gene with 10 haplotypes along with the already reported mutations. A single haplotype having quadruple mutation was found in the *dhfr* gene. The study reports higher degree of antifolate drug resistance as evidenced by the presence of multiple point mutations in *dhps* and *dhfr* genes. The findings of this study strongly discourage the use SP as a partner drug in ACT.

Keywords. *dhfr*; *dhps*; Northeast India; *Plasmodium falciparum*

1. Introduction

The worldwide importance of malaria is enormous. It is the most widespread vector-borne disease in the world, affecting some 214 million people in more than 64 countries, ∼45% of the world's population, with 4,38,000 deaths (World Malaria Report 2015). Northeast India shares about 4% of the country's population but contributes 0.15–0.2 million cases of malaria and over 100 deaths annually (NVBDCP 2013). The widespread resistance to chloroquine (CQ) and increasing therapeutic failure of sulphadoxine/pyrimethamine (SP) have led to critical hindrances in controlling of *Plasmodium falciparum* (*Pf*) malaria in this part of India. Resistance to SP was first reported from the Thailand–Cambodia border in the 1960s (Bjorkman and Phillips-Howard 1990), while in India, the first report of SP resistance came from the northeastern state Assam in 1979 and later from Arunachal Pradesh in 1992 (Das *et al.* 1981). Key point mutations in the genes dihydrofolate reductase (*dhfr*) and dihydropretorate synthetase (*dhps*) are found to be associated with antifolate resistance. Sulphadoxine inhibits the binding of para-amino benzoic acid (PABA) in the active site of *dhps* which converts PABA to folic acid essential for the parasite's replication. However, the mutation at the *dhps* gene leads to reduced binding capacity of sulphadoxine to the enzyme. These (436, 437, 540, 581 and 613) are five important point mutations that have been found to be associated with SP resistance. The first mutation occurs at position 436 (Ser-Ala) or 437 (Ala-Gly), giving resistance to sulphadoxine and the level of resistance increases with additional mutations at positions 540, 581 and 613. Pyrimethamine inhibits the enzyme *dhfr* involved in the biosynthesis of purine and pyrimidine bases essential for nuclear division of the parasite in the RBC. Occurrence of point mutations at codons 51, 59, 108 and 164 requires higher amount of pyrimethamine to inhibit *dhfr*, subsequently making it resistant to pyrimethamine. The first

Figure 1. Map showing the collection site.

mutation was found to be at position 108 (Ser-Asn). The efficacy of the drug further decreases with mutations at positions 51 (Asn-Ile), 59 (Cys-Arg) or 164 (Ile-Leu). Double mutation in *dhfr* gene causes intermediate resistance, while triple mutation causes high resistance (Wu *et al.* 1996). On the other hand, quadruple mutation with 51, 59, 108 in *dhfr* and 436/437 in *dhps*, or quintuple mutation with 51,59,108 in *dhfr* and 436/437 with 540 in *dhps*, result in higher level of resistance to SP and also to chloroprogunail dapsone and artesunate dapsone progunail (Lynch *et al.* 2008). Mishra *et al*. (2014) also reported declining efficacy of artesunate-based sulphadoxine pyrimethamine combination therapy in NE India. In this study the drug resistance pattern of antifolate drugs against *P. falciparum* was studied using therapeutic efficacy and molecular markers. This will aid in developing proper strategies for controlling malaria in this area as it remains an endemic area for malaria transmission.

2. Methodology

The study was carried out in the northeastern state Arunachal Pradesh (27.1300°N, 95.7400°E) (figure 1). Samples were collected during the malaria transmission season of 2013, i.e. July–September from the primary health centre. A total of 249 fever cases were screened for malaria by blood slide examination under the microscope out of which 75 were found to be positive for *Plasmodium falciparum*. Patients having the following inclusion criteria were taken for the study: (1) >1 year of age, (2) initial parasite count of 1000–1,00,000 parasites/μL on Day 0, (3) devoid of severe malnutrition, (4) not having any other illness, (5) temperature >37.5°C, (6) no earlier history of allergy to sulpha drugs, and (7) informed written consent. Artesunate + Sulphadoxine Pyrimethamine (25 mg of sulphadoxine and 1.25 mg of pyrimethamine/kg body weight) was used to treat the patients. Blood slides were taken and examined on D0, D3, D7, D14, D21, D28 and D42 to determine the presence of parasite. Before the drug was given, approximately 2 mL of intravenous blood was drawn from each microscopically confirmed patient into cryo vials containing cryoprotectant and stored in liquid nitrogen until transport to the laboratory.

The study was approved by scientific advisory committee and the Ethical Committee of Regional Medical Research Centre for North East Region (Indian Council of Medical Research), Dibrugarh, Assam, India, and informed consent was taken from patients before they enrolled in the study.

The DNA of parasite was extracted from blood samples using the Qiagen DNA extraction kit (Germany) following the manufacturer's instruction with minor modifications. Species identity of the parasite was confirmed by targeting the 16S rDNA as described by Snounou *et al.* (1993). The regions of *dhfr* and *dhps* genes containing the mutations for antifolate resistance were amplified as described elsewhere (Plowe *et al.* 1995; Wang *et al.* 1997) and sequenced for detection of the mutations. The amplified PCR products were purified using High Pure PCR Product Purification Kit (Roche, Germany) and sequenced using the Sanger method (Genewiz INC, NJ, USA) with both primers.

Obtained DNA sequences were edited manually in BioEdit (Hall 1999) and aligned using ClustalW (Larkin *et al.* 2007). Multiple sequence alignment (MSA) was done

Table 1. Demographic characteristics of the patients

			Age (range 5–60)			
Female	Male	Male:female	n (<15 years)	n (>15–30 years)	n (>30 years)	Parasites count (μL^{-1}) (geometric mean)
47	28	1.67	10	21	44	8396.744

to detect intraspecific variation, if any, among the sequences. Laboratory-adapted strain 3D7 sensitive to both chloroquine and antifolate was taken as the reference sequence to study the presence or absence of mutations in both *dhps* and *dhfr* genes. The demographic data and patients' characteristics are given in table 1.

3. Results

Of the 75 cases, only 35 successfully completed the 42-day follow-up. Of these 35 cases, 1 was found to be early treatment failure (ETF), whereas 3 cases were found to be late clinical failure (LCF), and the remaining 31 cases were found to show adequate clinical and parasitological response (ACPR).

All the 75 samples were successfully amplified for analysis of *dhfr* and *dhps* genes. The samples showed mutations at *dhfr* codons 51 (Asn- Ile), 59 (Cys-Arg), 108 (Ser-Asn) and 164 (ILE-Leu) (table 2). Three novel mutations at codons 498 (Val-Leu), 404 (Asp-Glu), and 453 (Val-Leu) were also found along with the mutations associated with sulphadoxine resistance in *dhps* gene at codons 436 (Ser-Ala), 540 (Lys-Glu) and 581 (Ala-Gly) (table 2). The mutation at codon 540 (Lys-Glu) was found to be the most prevalent mutation.

Table 2. Status of *dhfr* and *dhps* mutations

Gene	Codons	Types	Total (n = 75)
dhfr	51	Wild (N)	0
		Mutant (I)	75 (100%)
	59	Wild (C)	0
		Mutant (R)	75 (100%)
	108	Wild (S)	0
		Mutant (N)	75 (100%)
	164	Wild (I)	0
		Mutant (L)	75 (100%)
dhps	**404**	Wild (D)	60 (80.0%)
		Mutant (E)	15 (20.0%)
	436	Wild (S)	38 (50.7%)
		Mutant (F/A)	37 (49.3%)
	453	Wild (V)	42 (56.0%)
		Mutant (L)	33 (44.0%)
	498	Wild (V)	49 (65.3%)
		Mutant (L)	26 (34.7%)
	540	Wild (K)	30 (40.0%)
		Mutant (E)	45 (60.0%)
	581	Wild (A)	53 (70.7%)
		Mutant (G)	22 (19.3%)

Numbers in bold represent novel mutations.

None of the samples were found to have the sensitive haplotype D404, S436, V453, V498, K540, and A581 at the *dhps* gene. A total of 10 haplotypes were detected at the *dhps* gene. Of the 75 samples, 30 (40%) exhibited 3 haplotypes with single mutations (**D**404**E**SVVKA, DS**V**453**L**VKA and DSVVK**A**581**G**), 12 samples (16%) had double mutations with 2 haplotypes (D**S**436**A**VV**K**540**E**A, DSVV**K**540**E** **A**581**G**), 8 samples (10.6%) had triple mutations with 4 haplotypes (**D**404**E** **S**436**A**VV**K**540**E**A, **D**404**E**SVV**K**540**E** **A**581**G**, D**S**436**A**V**V**498**L** **K**540**E**A, and D**S**436**A**VV**K**540**E** **A**581**G**). and 25 samples (33.3%) had quadruple mutation with single haplotype (D**S**436**A** **V**453**L** **V**498**L** **K**540**E**A) (figure 2). However, in the *dhfr* gene only a single haplotype was found across the 75 samples (**N**51**Y** **C**59**R** **S**108 **N** **I**164**L**).

4. Discussion

Molecular epidemiological studies have been conducted in this region to monitor the frequency of mutations associated with drug resistance (Mohapatra *et al.* 2014). Surveillance at the molecular level along with therapeutic efficacy studies allow local health authorities to plan and adjust anti-malarial drug policies and to act efficiently when changes in prevalence of certain mutations occur. The diversity at the genetic level further complicates the situation of drug resistance. The development of resistance of *P. falciparum* to antifolate drugs such as sulphadoxine and pyrimethamine is conferred by several point mutations in the parasite *dhfr* and *dhps* genes (Brooks *et al.* 1994; Triglia and Cowman 1999; Shaio *et al.* 1998). The *dhfr* alleles had mutations at codons 51 (Asn-Ile), 59 (Cys-Arg), 108 (Ser-Asn) and 164 (Ile-Leu) in all the samples examined in this study, indicating high resistance in this area. Sulphadoxine resistance is mediated by a single point mutation at codon 436/437 (Ser-Ala or Ala-Gly) of the *dhps* gene. Higher level of sulphadoxine resistance is associated with double mutation at codons 437 and 581 or triple mutations at codons 436,437 and 613. Three novel mutations at codons 498, 404 and 453 were found. The presence of key mutations in *dhfr* and *dhps* genes along with emergence of new mutations suggests clinical

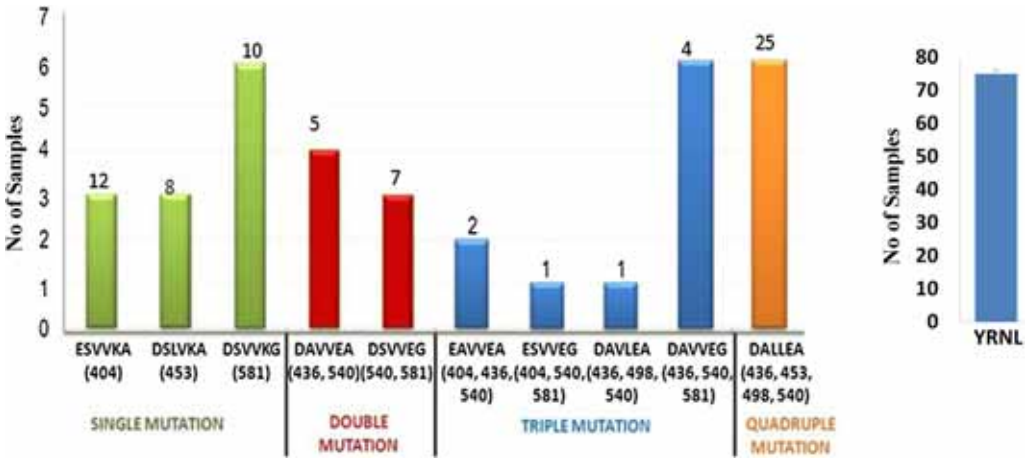

Figure 2. *dhps* haplotype distribution for 75 samples.

resistance of pyrimethamine and sulphadoxine in this region as evidenced by our therapeutic efficacy results. One case in our study showing ETF had quadruple mutation at *dhfr* gene and triple mutation at *dhps*, whereas three LCF were found to have quadruple *dhfr* mutation and double *dhps* mutation. Furthermore, these findings prohibit the use of antifolate drugs as partner drug for artesunate for the treatment of *P. falciparum* malaria. On the contrary, the emergence of new mutations might have potential of developing even higher antifolate resistance to *P. falciparum*, and hence it should be taken into consideration when using antifolate drugs nationwide. The artemisinin-based combination drug (lumefantrine–artemether) has been in use in the treatment of multi-drug-resistant *P. falciparum* malaria in other parts of Asia and no large-scale resistance has been reported thus far (Lefèvre *et al.* 2001; Mishra *et al.* 2014). Based on the presence of multiple mutations in *dhps* and *dhfr* genes in this region, lumefantrine–artemether has been in use as a first-line therapy for treatment of *P. falciparum* malaria in NE region of India.

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