J Vector Borne Dis 52, June 2015, pp. 116–121

Research Articles

Molecular determination of antifolate resistance associated point mutations in *Plasmodium falciparum* dihydrofolate reductase (dhfr) and dihydropteroate synthetase (dhps) genes among the field samples in Arunachal Pradesh

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ABSTRACT

Background & objectives: Antimalarial resistance in *P. falciparum* malaria parasite creates a serious obstacle in malaria control programme. Keeping this in mind, in the present study antifolate resistance associated point mutations in *P. falciparum* dihydrofolate reductase (*Pfdhfr*) and dihydropteroate synthetase (*Pfdhps*) genes among the field samples in Arunachal Pradesh were determined.

Methods: Blood samples were collected from 145 suspected malaria patients/healthy control subjects in malarious areas of Lohit and Changlang districts of Arunachal Pradesh, India during January 2012 to December 2013.

Results: In microscopic slide examination, 51.03% (74/145) were found malaria positive. *Plasmodium falciparum* mono-infection was observed in 62.16% (46/74) of total malaria positive cases. Polymerase chain reaction (PCR) was employed in all the *P. falciparum* positive samples for detection of 648 bp of *Pfdhfr* and 710 bp of *Pfdhps* genes. All the amplified products were analysed for detection of single nucleotide polymorphisms in *dhfr* and *dhps* genes. A total of four different genotypes of *Pfdhfr* gene were observed, of which double mutant allele ANRNI was mostly prevalent and it was found in 65.22% (30/46) cases. Likewise, four different haplotypes of *Pfdhps* gene were detected, of which triple mutant allele AGEAA shares 69.57% (32/46) followed by other haplotypes. In *Pfdhfr-Pfdhps* two locus mutations analysis, two isolates in Changlang district had shown quintuple mutant haplotype AIRNL-AGEAA, likely to be associated with treatment failure. The *P. falciparum* two locus *dhfr-dhps* haplotype (ANRNI-AGEAA) was observed in 56.52% (26/46) cases.

Interpretation & conclusion: Overall, high grade of sulphadoxine-pyrimethamine resistance associated genetic polymorphisms were observed among the *P. falciparum* parasite population in Arunachal Pradesh during the study period.

Key words Arunachal Pradesh; *dhfr-dhps*; haplotypes; locus; mutation; *P. falciparum;* sulphadoxine-pyrimethamine

INTRODUCTION

Currently, >106 countries/territories are affected by malaria, the world's most widespread infection[1]. It also remains a major public health problem in India. This is primarily because of the presence of malaria parasites having antimalarial resistance activity, pesticide resistant mosquito vectors and non-availability of suitable and effective malaria vaccines[2]. Hilly regions of Northeast (NE) India are known as highly endemic zone for malaria infection. The eight NE states record about 0.07–0.1 million *Plasmodium falciparum* (*P. falciparum*) malaria cases annually and these contribute 12% of total reported cases in India[3]. To reduce the malaria incidence in NE states, several antimalaria programmes are implemented under National Vector Borne Disease Control Programme (NVBDCP). Now, this region has seen substantial progress in alleviating the burden of malaria[4-5], but could not completely get rid of the saddle till now.

Resistance to antimalarial drugs by the *P. falciparum* malaria parasite is posing a serious impediment in malaria control agenda. Such antimalarial resistant *P. falciparum* malaria parasites are widespread across the globe[6]. Due to increased chloroquine (CQ) resistance, the antifolate drugs sulphadoxine-pyrimethamine (SP) are gaining importance in the treatment of uncomplicated *P. falciparum* malaria[7-9]. However, now-a-days resistance against SP antimalarials has also been reported in many

countries including various parts of India[10-15]. There are several molecular markers which were found to be associated with drug resistance profile. Analyses of these molecular markers associated with *P. falciparum* antimalarial drug can provide important information about resistance levels of different drugs. *P. falciparum* dihydrofolate reductase (*Pfdhfr*) mutations N51I, C59R, S108N, and I164L; and dihydropteroate synthetase (*Pfdhps*) mutations S436A, A437G, K540E, A581G, and A613S/T have been found to be associated with SP resistance[12, 16]. These point mutations cause a structural alteration in active site of the enzymes, so that the antimalarial drug is unable to recognize the binding site and consequently, the malaria parasite develops resistance capability to that drug. Due to the high incidence of antimalarial resistance (particularly CQ and SP) in *P. falciparum* malaria parasites in NE states, the development of fast, reliable and affordable methods for determination of drug resistance is necessary to reduce the burden at an early stage. In this aspect, molecular surveillance should be used for enhanced understanding of the drug-resistant *P. falciparum* parasite. Such information would be helpful in better implementation of the drug policies. Hence, the study was conducted with the objective of molecular determination of antifolate-resistance associated point mutations in *Pfdhfr* and *Pfdhps* genes among the field samples in Arunachal Pradesh.

MATERIAL & METHODS

Study site and sample collection

Arunachal Pradesh is the largest state (area-wise) situated in the NE region of India, sharing a long international border with Bhutan, China and Myanmar. This state is situated between latitude 26° 30' N and 29° 30 'N and longitude 91° 30' E and 97° 30' E. The presence of large forested land and many perennial streams creates suitable environment for rapid proliferation of malaria vectors, so that malaria remains a common problem in this state of India. Based on this information, a study was carried out in Changlang and Lohit districts (with maximum annual parasite incidence) of Arunachal Pradesh for molecular determination of antifolate-resistance associated point mutations in *Pfdhfr* and *Pfdhps* genes. Patients of all age groups and both the sexes were included in our study. Informed consent was obtained from all the patients or in case of children from their parents/legal guardians. Institutional ethical clearance was also obtained from the Institutional Ethical Committee, Regional Medical Research Centre (RMRC), Dibrugarh, Assam.

About 3 ml of blood sample (prior to treatment) was collected from each suspected malaria patient and asymptomatic patient through household visit in different malaria reporting areas from January 2012 to December 2013. Blood slides were immediately prepared and further confirmation was done in the Laboratory of RMRC, Dibrugarh, Assam. Patient's clinical and demographical history was also taken in a predesigned proforma.

Microscopic examination

Presence of *P. falciparum* malaria parasite in collected blood slides was confirmed by light microscopy with 100X microscopic fields. Further, for quality control, polymerase chain reaction (PCR) method was used for microscopic positive as well as negative cases as described by Snounou *et al*[17]. Number of parasites/µl of blood (thick film) was calculated by number of observed asexual parasites × total WBC count per µl against 200 WBC in 100 microscopic fields.

Amplification of Pfdhfr and Pfdhps genes by PCR

Parasite DNA was extracted from 200 µl of whole blood samples using the QIAamp DNA Mini spin columns kit (Qiagen, Hilden, Germany). A total of 648 bp portion of *Pfdhfr* gene and 710 bp portion of *Pfdhps* gene were amplified using specific primers (Table 1) as described elsewhere[18]. Five microlitres of purified DNA were added to a final reaction volume of 50 µl which consisted of 10 µl of 5× colourless Gotaq reaction buffer, 0.3 µM of each primer, 0.2 mM of each deoxyribonucleoside triphosphate (dATP, dTTP, dGTP and dCTP) and one unit of taq DNA polymerase. Amplification was performed under the following conditions: Initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 45 sec, and extension at 62°C for 1 min and a final extension period at 62°C for 10 min[18]. The amplification was performed in a Thermal cycler (Gene Amp®PCR System 9700 and Veriti, Applied Biosystems, Foster City, CA, USA). The PCR amplicons were run on 1.5% agarose gel using electrophoretic chamber (BioRad, USA). Positive amplicons were observed under gel docu-

Table 1. List of primers used for amplification of *dhfr* and *dhps* genes

Primers	Sequences (5'-3')	Product size (bp)	Gene
PFDHFRM1	TTT ATG ATG GAA CAA GTC TGC	648	*Pfdhfr*
PFDHFRM5	AGT ATA TAC ATC GCT AAC AGA	648	*Pfdhfr*
PFDHPSRF	AAC CTA AAC GTC CTG TTC AA	710	*Pfdhps*
PFDHPSRR	AAT TGT GTG ATT TGT CCA CAA	710	*Pfdhps*

mentation system (Kodak). The amplified products were further purified with the QIAquick PCR purification kit (Qiagen) before sequencing and the nucleotide sequences were determined by automatic dideoxy cycle sequencing techniques (South Korea, *via* Anshul Biotechnologies, Hyderabad, India) by using the same primers of *Pfdhfr* and *Pfdhps* amplification, with standard protocols. The forward and reverse sequences of each sample were edited manually using BioEdit v 7.0.9 software (Hall TA, 1999). DNA sequences were aligned and single nucleotide polymorphisms (SNPs) of concerned genes were estimated by software DnaSP v. 5.10.01 and Mega 5.

Statistical analysis

The filled up questionnaire of the patients were directly entered into an electronic database, statistical package for social science (SPSS) to summarize the quantitative measurements (age, sex, blood groups, hemoglobin, *etc*).

RESULTS & DISCUSSION

A total of 145 blood samples were collected for microscopic examination of malaria parasite, of which 51.03% (74/145) samples were found malaria positive. It was observed that 52.39% (39/74) of malaria positive cases were asymptomatic and 47.61% (35/74) were having symptoms of high fever and headache. *P. falciparum* mono-infection was detected among 62.16% (46/74) malaria positive cases. Parasite count in microscopic examination revealed an average parasite count of 3.015% (range 0.1 to 11%, SD ±2.387 and the median value of 2). The two tailed *p*-value was found as <0.0001, considered very significant (one sample *t*-test) having $t = 10.863$ with 73 degree of freedom. Wilcoxon signed rank test also showed similar finding. All the age groups and both the sexes were found affected with malarial infection (Table 2). People with O^+ (27/74) and B^+ (25/74) blood groups were more susceptible to malaria infection. Hematological analysis revealed that 89.19% malaria positive patients were having low hemoglobin level, 74.32% low hematocrit, 95.95% with low mean corpuscular volume, 86.49% with low mean corpuscular hemoglobin and 78.38% with low monocyte count. In contrast, 24.32% were detected with high platelet count, 62.16% with high lymphocyte count, 93.24% having high red cell distribution width and 82.43% with high platelet distribution width (Table 3).

A partial fragment of *Pfdhfr* and *Pfdhps* genes containing major SNPs associated with SP resistance were amplified, sequenced and analyzed from 46 *P. falciparum* positive cases in Changlang and Lohit districts of Arunachal Pradesh. In *Pfdhfr* gene, four haplotypes (ANRNI, AIRNI, ANRNL and AIRNL) were observed with a value of haplotype (gene) diversity, H_d: 0.537, variance of haplotype diversity: 0.00533, and standard deviation (SD) of haplotype diversity: 0.073 (Table 4). The double mutant haplotype ANRNI was most prevalent (65.22%) followed by other haplotypes ANRNL (17.39%), AIRNI (13.04%) and AIRNL (4.35%). Mutation analysis at nucleotide level have shown the existence of four polymorphic sites with SNPs, *viz.* at nucleotide position 152, A-T mutation (N51I), at nucleotide position 175, T-C mutation (C59R), at nucleotide posi-

Table 2. Demographic characteristics among suspected/confirmed malaria positive cases in Arunachal Pradesh

Age group (in yr)	No. of suspected malaria cases	No. of malaria positive cases
0–10	25	21
11–20	34	23
21–30	34	13
31–40	22	9
41–50	17	6
>50	13	2
Gender		
Male	69	38
Female	76	36
Total	145	74

Table 3. Hematological parameters among suspected/confirmed malaria positive cases in Arunachal Pradesh

Hematological value	Suspected malaria patients			Malaria positive patients		
	High	Low	Normal	High	Low	Normal
HB	0	115	30	0	66	8
WBC	14	21	110	5	10	59
RBC	6	26	113	3	9	62
HCT	0	92	53	0	55	19
MCV	2	112	31	0	71	3
MCH	0	101	44	0	64	10
MCHC	2	30	113	0	18	56
PLT	25	34	86	18	12	44
GR	27	15	103	10	8	56
LY	69	7	69	46	1	27
MO	10	84	51	0	58	16
RDW	124	6	15	69	5	0
MPV	12	0	133	0	0	74
PCT	3	38	104	0	24	50
PDW	122	0	23	61	0	13

HB–Hemoglobin; WBC–White blood corpuscle; RBC–Red blood cell; HCT–Hematocrit; MCV–Mean corpuscular volume; MCH–Mean corpuscular hemoglobin; MCHC–Mean corpuscular hemoglobin concentration; PLT–Platelet; GR–Granulocyte; LY–Lymphocyte; MO–Monocyte; RDW–Red blood cell distribution width; MPV–Mean platelet volume; PCT–Plateletcrit; PDW–Platelet distribution width.

tion 323, G-A mutation (S108N) and at position 490, A-T mutation (I164L) leading to the occurrence of above mentioned haplotypes with a nucleotide diversity (per site), π(Pi): 0.00126, sampling variance of π: 0.0 and SD of π: 0.00020. The average number of nucleotide differences was *k:* 0.64155, θ (per sequence) from S, W: 0.45507 and θ (per site) from S, θ-W: 0.00089.

Depending upon the point mutations in *Pfdhps* gene, four haplotypes (FAKAA, AGKAA, FGKAA and AGEAA) were observed with haplotype (gene) diversity H_d: 0.487, variance of haplotype diversity: 0.00606, and SD of haplotype diversity: 0.078. The wild type allele SAKAA was not found from any of the isolates in the study area; however, 69.57% field isolates have shown AGEAA (S436A+A437G and K540E) haplotypes. Single mutant allele FAKAA (S436F) was detected in 17.39% (8/46) cases. *Pfdhps* double mutation allele FGKAA (S436F+A437G) was found in 8.70% (4/46) and AGKAA (S436A+A437G) was seen among 4.35% (2/46) *P. falciparum* field isolates (Table 4). During nucleotide analysis of 1282 to 1884 bp portion of *Pfdhps* gene a total of four variable sites were observed with a nucleotide diversity (per site) of π: 0.00251, sampling variance of π: 0.0000002 and SD of π: 0.00039. The average number of nucleotide differences was *k*: 1.51498. The *P. falciparum* field isolates have shown that at nucleotide position 1306, T-G mutation (S436A); at 1307 position, C-T (S436F) mutation; C-G (A437G) mutation at nucleotide position 1310 and at nucleotide location 1618, A-G (K540E) mutation were accountable for occurrence of haplotypes FAKAA, AGKAA, FGKAA and AGEAA associated with different levels of sulphadoxine resistance. Neutrality tests were done by using DnaSP v5 software. For *Pfdhfr* gene analysis Tajima's D value was 0.74630 (Not significant, $p > 0.10$). The Fu and Li's D-test and Fu and Li's F-test statistics were statistically not significant at $p > 0.10$ with a value of 0.75596 and 0.87367 respectively. The value of Fu's Fs statistic was –0.308 and Strobeck's S statistic was 0.801. Likewise in case of *Pfdhps* gene analysis, the Tajima's D value was 1.53599 (statistically not significant, $p > 0.10$). Similarly, the value of Fu and Li's D-test statistic was 1.01271 and Fu and Li's F-test statistic was 1.36745 (statistically not significant, $p > 0.10$). The Fu's Fs statistic and Strobeck's S statistic was 2.063 and 0.267, respectively.

This study reported four *Pfdhfr* haplotypes and four *Pfdhps* haplotypes among the isolates of Arunachal Pradesh. All these haplotypes found here, have also been reported elsewhere in Indian states and other countries[12-13, 16]. Previous studies revealed that Indian parasite populations have shown seven different *Pfdhfr* alleles besides the wild-type allele NCSI at amino acid positions 51, 59, 108 and 164 [12-13, 16, 19-21].

In this study, we have found four different haplotypes of *Pfdhfr* gene, of which ANRNI haplotype was most predominant in both Lohit and Changlang districts of Arunachal Pradesh. Earlier studies have revealed that these haplotypes were circulated in Delhi, Uttar Pradesh, Odisha, Goa and Assam of India[12-13, 16, 18-21]. Recently, Sharma *et al*[15], carried out an extensive study in Assam. They found that ANRNI haplotype was widespread in Karbi Anglong, NC Hills, Chirang, Golaghat, Dibrugarh, Sivasagar, Tinsukia, Lakhimpur and Jorhat of Assam[15]. Additionally, this haplotype was also reported from sev-

Table 4. Distribution of *P. falciparum dhfr* and *dhps* haplotypes

District	*P. falciparum dhfr* and *dhps* genotypes							
	P. falciparum dhfr				*P. falciparum dhps*			
	ANRNI	AIRNI	ANRNL	AIRNL	FAKAA	AGKAA	FGKAA	AGEAA
Changlang (n = 26)	14	6	4	2	8	2	–	16
Lohit (n = 20)	16	0	4	–	–	–	4	16
Total	30	6	8	2	8	2	4	32

Table 5. *P. falciparum* two locus *dhfr-dhps* genotypes in Arunachal Pradesh

District	*P. falciparum dhfr*				*P. falciparum dhps*			
	ANRNI-AGEAA	ANRNL-FGKAA	ANRNL-FAKAA	ANRNI-FAKAA	AIRNI-FAKAA	AIRNI-AGEAA	AIRNL-AGEAA	ANRNI-AGKAA
Changlang	10	–	4	2	2	4	2	2
Lohit	16	4	–	–	–	–	–	–
Total	26	4	4	2	2	4	2	2

eral other countries like Thailand, Kenya, Gabon, Malaysia, Indonesia, Mali, Iran, Malawi, South Africa, Cameroon, Vietnam, Oman, Afghanistan, Pakistan and Tanzania[12-13, 16]. In the current study, the AIRNI haplotype was observed only from Changlang district and is in line with the previous findings reported from Kamrup and Chirang districts of Assam[12, 15]. At least two isolates of *P. falciparum* from Changlang district have showed quadruple-mutant allele AIRNL which was only reported from Car Nicobar Islands in previous consequences[12-13, 16, 19-21]. It may be noted here that the parasite population with this mutant allele is highly resistant to pyrimethamine. The haplotype ANRNL was circulated in both the districts of Arunachal Pradesh. Previously, these alleles were reported from Odisha and Assam[12-13, 16]. In *Pfdhps* gene, to the best of our information all the three genotypes (except FGKAA) reported here seem to be specific to India[12-13, 16, 19-21]. Besides this, a novel *Pfdhps* haplotype (FGKAA) was found in Lohit district of Arunachal Pradesh. This haplotype was not reported earlier from Indian *P. falciparum* field isolates. However, it was accounted from other countries like Indonesia, Malaysia, Thailand and Kenya [12-13, 16].

In our study, a total of eight *dhfr-dhps* two locus haplotypes were established in Arunachal Pradesh of which seven were circuited in Changlang district and two were common in Lohit district of Arunachal Pradesh (Table 5). It has been observed that 56.52% *P. falciparum* field isolates in Arunachal Pradesh had shown ANRNI-AGEAA haplotype which were believed to be associated with high grade of SP resistance. Two isolates from Changlang district of Arunachal Pradesh had shown quintuple mutant haplotype AIRNL-AGEAA, which has not been reported from any other part of India till now to the best of our knowledge. This result indicates that *P. falciparum* malaria parasites having high grade of SP resistance ability is prevailing in Arunachal Pradesh. Now-a-days, artemisinin-based combination therapy (ACT) is widely used in different parts of India. However, depending upon the prevalence of high grade SP resistance in *P. falciparum* malaria parasite, the partner drug of artemisinin needs to be replaced in this part of India.

CONCLUSION

High grade of antifolate resistance associated point mutations in *Pfdhfr* and *Pfdhps* genes was observed among the field samples in Arunachal Pradesh. Continuous molecular surveillance is not only useful for better implementation of the drug policy, but also to restrict the development of higher level of resistance.

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Received: 5 February 2014 *Accepted in revised form:* 26 February 2015