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
countries including various parts of India\textsuperscript{10-15}. There are several molecular markers which were found to be associated with drug resistance profile. Analyses of these molecular markers associated with \textit{P. falciparum} antimalarial drug can provide important information about resistance levels of different drugs. \textit{P. falciparum} dihydrofolate reductase (\textit{Pfdhfr}) mutations N51I, C59R, S108N, and I164L; and dihydropteroate synthetase (\textit{Pfdhps}) mutations S436A, A437G, K540E, A581G, and A613S/T have been found to be associated with SP resistance\textsuperscript{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 \textit{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 \textit{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 \textit{Pfdhfr} and \textit{Pfdhps} genes among the field samples in Arunachal Pradesh.

\section*{MATERIAL & METHODS}

\subsection*{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 \textit{Pfdhfr} and \textit{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.

\subsection*{Microscopic examination}

Presence of \textit{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 \textit{et al}\textsuperscript{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.

\subsection*{Amplification of \textit{Pfdhfr} and \textit{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 \textit{Pfdhfr} gene and 710 bp portion of \textit{Pfdhps} gene were amplified using specific primers (Table 1) as described elsewhere\textsuperscript{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.\textsuperscript{18} The amplification was performed in a Thermal cycler (Gene Amp\textsuperscript{®}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 docum-

\begin{table}[h]
\centering
\caption{List of primers used for amplification of \textit{dbhfr} and \textit{dhps} genes}
\begin{tabular}{lll}
\hline
Primers & Sequences (5'-3') & Product Gene size (bp) \\
\hline
PFDHFRM1 & TTT ATG ATG GAA CAA GTC TGC & 648 \textit{Pfdhfr} \\
PFDHFRM5 & AGT ATA TAC ATC GCT AAC AGA & 648 \textit{Pfdhfr} \\
PFDHPSRF & AAC CTA AAC GTC CTG TTC AA & 710 \textit{Pfdhps} \\
PFDHPSRR & AAT TGT GTG ATT TGT CCA CAA & 710 \textit{Pfdhps} \\
\hline
\end{tabular}
\end{table}
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 \textit{Pfdhfr} and \textit{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. \textit{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 \textit{Pfdhfr} and \textit{Pfdhps} genes containing major SNPs associated with SP resistance were amplified, sequenced and analyzed from 46 \textit{P. falciparum} positive cases in Changlang and Lohit districts of Arunachal Pradesh. In \textit{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 (1164L) leading to the occurrence of above
mentioned haplotypes with a nucleotide diversity (per
site), \( \pi(t) \): 0.00126, sampling variance of \( \pi \): 0.0 and SD
of \( \pi \): 0.00020. The average number of nucleotide differ-
ences was \( k \): 0.64155, \( \theta \) (per sequence) from S, W:
0.45507 and \( \theta \) (per site) from S, \( \theta \)-W: 0.00089.

Depending upon the point mutations in \( Pf dhps \) 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. \( Pf dhps \) 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 \( Pf dhps \) gene a
total of four variable sites were observed with a nucle-
otide diversity (per site) of \( \pi \): 0.00251, sampling vari-
ance of \( \pi \): 0.0000002 and SD of \( \pi \): 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 nucle-
otide 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 \( Pf dhfr \) 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 respect-
ively. The value of Fu’s Fs statistic was –0.308 and
Strobeck’s S statistic was 0.801. Likewise in case of
\( Pf dhps \) 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 signifi-
cant, \( p \) > 0.10). The Fu’s Fs statistic and Strobeck’s S
statistic was 2.063 and 0.267, respectively.

This study reported four \( Pf dhfr \) haplotypes and four
\( Pf dhps \) 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 \( Pf dhfr \)
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 \( Pf dhfr \) 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 \textit{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

<table>
<thead>
<tr>
<th>District</th>
<th>( P. falciparum dhfr )</th>
<th>( P. falciparum dhps )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ANRNI</td>
<td>AIRNI</td>
</tr>
<tr>
<td>Changlang (n = 26)</td>
<td>14</td>
<td>6</td>
</tr>
<tr>
<td>Lohit (n = 20)</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 5. \( P. falciparum \) two locus \( dhfr-dhps \) genotypes in Arunachal Pradesh

<table>
<thead>
<tr>
<th>District</th>
<th>( P. falciparum dhfr )</th>
<th>( P. falciparum dhps )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ANRNI</td>
<td>ANRNL</td>
</tr>
<tr>
<td>Changlang</td>
<td>10</td>
<td>–</td>
</tr>
<tr>
<td>Lohit</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>26</td>
<td>4</td>
</tr>
</tbody>
</table>
eral other countries like Thailand, Kenya, Gabon, Malaysia, Indonesia, Mali, Iran, Malawi, South Africa, Cameroon, Vietnam, Oman, Afghanistan, Pakistan and Tanzania. In the current study, the AIRNL haplotype was observed only from Changlang district and is in line with the previous findings reported from Kamrup and Chirang districts of Assam. At least two isolates of \textit{P. falciparum} from Changlang district have shown quadruple-mutant allele AIRNL which was only reported from Car Nicobar Islands in previous consequences. 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. In \textit{Pfdhps} gene, to the best of our information all the three genotypes (except FGKAA) reported here seem to be specific to India. Besides this, a novel \textit{Pfdhps} haplotype (FGKAA) was found in Lohit district of Arunachal Pradesh. This haplotype was not reported earlier from Indian \textit{P. falciparum} field isolates. However, it was accounted from other countries like Indonesia, Malaysia, Thailand and Kenya.

In our study, a total of eight \textit{dhfr-dhps} two locus haplotypes were established in Arunachal Pradesh of which seven were circulated in Changlang district and two were common in Lohit district of Arunachal Pradesh (Table 5). It has been observed that 56.52\% \textit{P. falciparum} field isolates in Arunachal Pradesh had shown ANRNL-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 \textit{P. falciparum} malaria parasites having high grade of SP resistance ability is prevailing in Arunachal Pradesh. Nowadays, artemisinin-based combination therapy (ACT) is widely used in different parts of India. However, depending upon the prevalence of high grade SP resistance in \textit{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 \textit{Pfdhfr} and \textit{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.

**REFERENCES**


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