

# Detection of point mutation in *Plasmodium falciparum* *ATPase6* gene associated with artemisinin resistance from Assam and Arunachal Pradesh

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## ABSTRACT

**Background & objectives:** Resistance against partner drugs of artemisinin has been reported from different parts of India. The study aims to find out the single nucleotide polymorphisms in *Plasmodium falciparum* *ATPase6* gene associated with artemisinin resistance.

**Methods:** Blood samples were collected from 141 patients with *P. falciparum* monoinfection in malaria endemic zones of Assam and Arunachal Pradesh. A 645 bp portion of *PfATPase6* gene was amplified and sequenced to determine the frequency of mutations associated with resistance to artemisinin.

**Results:** Mutations at codon S769N, which have been proposed to confer artemisinin resistance, were not detected in our study samples. Instead of that a novel non-synonymous mutation (C-T) at 1847 bp position resulting in serine to phenylalanine alteration at codon S616F was detected from the *P. falciparum* field isolates in Changlang district of Arunachal Pradesh, whereas no mutation was detected in any of the analyzed samples in Assam indicating that wild type *PfATPase6* genotype was found circulating in this region. Overall, based on the mutational pattern, two haplotypes of *PfATPase6* gene were observed during the study, the wild type and mutant S616F allele. The overall haplotype diversity (Hd) was found to be: 0.069 and nucleotide diversity (per site Pi): 0.00012. Highest haplotype diversity was recorded in Changlang district of Arunachal Pradesh having Hd value of 0.33333 along with single polymorphic site and nucleotide diversity (Pi): 0.00060. A pair-wise fixation index ( $F_{ST}$ ) value of 0.16667 indicates great genetic differentiation within the parasite population of Changlang district with the population of Karbi Anglong, Chirang, Tinsukia, Sivasagar, Jorhat, NC Hills, Lakhimpur, Golaghat and Dibrugarh districts of Assam and Lohit district of Arunachal Pradesh.

**Interpretation & conclusion:** A better understanding of the distribution of antimalarial drug resistance with malaria parasite may provide insight into some of the epidemiological determinants of the increasing case burden.

**Key words** Artemisinin; *ATPase6* gene; haplotype; nucleotide diversity; *P. falciparum*

## INTRODUCTION

Artemisinin and its derivatives are most effective antimalarial drugs which act upon by selectively inhibiting *Plasmodium falciparum* *ATPase6* protein, the only SERCA-type  $Ca^{2+}$  *ATPase* in the *P. falciparum* genome, believed to be the primary target for artemisinins. In 2001, the World Health Organization (WHO) recommended the use of artemisinin-based combination therapy (ACT), in which a long-acting partner is added to artemisinin to make it more effective, as first-line treatment for uncomplicated *P. falciparum* malaria in all malaria endemic countries<sup>1</sup>. Five combinations currently recommended by the WHO for uncomplicated *P. falciparum* malaria cases are artemether-lumefantrine (AM-LF), artesunate-mefloquine (AS-MQ), artesunate-sulfadoxine-pyrimethamine (AS-SP), dihydroartemisinin-piperaquine, and artesunate amodiaquine<sup>2</sup>. Many countries are now

starting to implement this regime<sup>3–11</sup>. However, AS-SP combination is being widely used in many of the countries<sup>1, 11–14</sup>. Further, certain mutations in *P. falciparum* *ATPase6* are detected and are found to be associated with artemisinin resistance in some countries<sup>8, 15–16</sup>. There are few molecular markers which are believed to reduce parasite sensitivity<sup>8, 15–16</sup>. It has been established in several studies that serine to asparagine mutation at codon 769 in *P. falciparum* *ATPase6* gene is associated with raised artemether half maximal inhibitory concentration ( $IC_{50}$ ), and suggested that the mutation may be used as a molecular marker for monitoring artemisinin resistance<sup>8</sup>. The combination of two additional single nucleotide polymorphisms (SNPs), E431K and A623E was defined in fresh isolates from Senegal with increased  $IC_{50}$  to artemether<sup>8</sup>. Besides this, it has been suggested that the mutations at codon L263E are associated with artemisinin resistance<sup>8, 15–16</sup>. The increase in *P. falciparum* multidrug re-

sistance-1 copy number is also believed to reduce parasite sensitivity to some quinoline antimalarials (typically an aminoquinoline) the partner drug of ACT<sup>17-18</sup>. However, artesunate resistance has not yet been reported from India<sup>1</sup>. Due to the prevalence of high level of resistance in partner drug of ACT in *P. falciparum* field isolates from northeastern part of India<sup>19-22</sup>, it is assumed that *PfATPase6* genotype could make early warning signals for emergence of ACT resistance.

## MATERIAL & METHODS

The study was conducted in different malaria endemic areas of Assam and Arunachal Pradesh during the period from January 2012 to February 2014 (Fig. 1). Two millilitre of blood sample was collected from malaria suspected patients (before treatment) irrespective of age, sex and disease severity. Prior to collection of blood samples, a concise enlightenment was given to the participants about the purpose of the study. Simultaneously consent was taken from each participant. In case of children, informed consent was provided by their guardians. Human ethical clearance was obtained from the Institutional Ethical Board of the Regional Medical Research Centre (RMRC), ICMR, Dibrugarh. After collecting blood samples, microscopic slide examination was done as a gold standard for laboratory confirmation of malaria and species identification. Parasite counts were done by Giemsa staining and the number of asexual parasites against 200 WBC was counted in 100 × microscopic fields, assuming an average of WBC count 8,000/μl. Parasitaemia was calculated and expressed as percentage. Genomic DNA was extracted from 200 μl of whole blood by using QIAamp DNA mini kit as per manufacturer's instruction (Qiagen, Hilden, Germany). Initially, a 1793

bp fragment spanning the coding region of exon 1 of the *PfATPase6* gene was amplified by primary polymerase chain reaction (PCR). The primary amplification was performed by using the oligonucleotide primers *PfATPase6-P1* and *PfATPase6-P2* as described elsewhere<sup>4</sup> in a reaction mixture of total volume 20 μl which consisted of 5 μl of genomic DNA, 4 μl of 5 × colourless Gotaq reaction buffer, 0.3 μM (0.6 μl in 20 μl of reaction volume) of each primer, 0.2 mM (0.165 μl in 20 μl of reaction volume) of each deoxyribonucleoside triphosphate (dATP, dTTP, dGTP, dCTP) and 1.2 units of taq DNA polymerase (0.24 μl in 20 μl of reaction volume). Amplification was performed under the following conditions: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 47°C for 1 min, and extension at 72°C for 1 min and a final extension period at 72°C for 10 min. In the nested reaction, a 645 bp fragment was amplified from the primary amplicons. The nested amplification was done by using the oligonucleotide primers *PfATPase6-N1* and *PfATPase6-N2* as described elsewhere<sup>23</sup> in a total volume 50 μl consisted of 3 μl of primary PCR product, 10 μl of 5 × colourless Gotaq reaction buffer, 0.3 μM (1.5 μl in 50 μl of reaction volume) of each primer, 0.2 mM (0.45 μl in 50 μl of reaction volume) of each deoxyribonucleoside triphosphate (dATP, dTTP, dGTP, dCTP), 0.48 units of taq DNA polymerase (0.24 μl in 50 μl of reaction volume) and 1 mM of MgCl<sub>2</sub> (2 μl in 50 μl of reaction volume). The reaction settled with an initial hold (94°C/3 min), 40 cycles (94°C/1 min, 51°C/1 min, 72°C/1 min and 72°C/10 min). The amplified products were further purified before sequencing and the sequencing products were edited in BioEdit software and aligned through ClustalW in Mega 5 software. Single nucleotide polymorphisms were analysed in DnaSP software version v.5.10.01.

## RESULTS & DISCUSSION

A partial fragment of *PfATPase6* gene, believed to be a target for the artemisinin drugs was amplified and sequenced in 141 *P. falciparum* positive samples from malaria endemic areas of Assam and Arunachal Pradesh (Fig. 1). The nucleotide sequences were submitted to National Centre for Biotechnology Information (NCBI) gene bank data base and accession numbers obtained afterwards (KJ510660-KJ510800). The nucleotide sequences were analysed for detection of single nucleotide polymorphisms in *PfATPase6* gene. Novel non-synonymous mutation (C-T) at 1847 bp position of *PfATPase6*

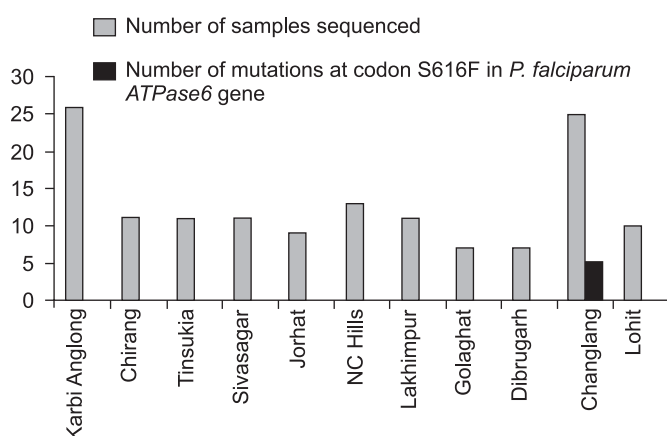


Fig. 1: Area-wise number of samples sequenced from *P. falciparum*-infected patients.

gene that leads serine to phenylalanine alteration at codon S616F was observed in 3.55% *P. falciparum* field isolates. The mutant S616F allele was detected only in the *P. falciparum* field isolates in Changlang district of Arunachal Pradesh, however, this mutant genotype was not observed in any other parts of the study area.

The mutations at codon S769N, which have been proposed to confer artemisinin resistance were not detected from the analyzed samples. A number of molecular studies have been carried out in different countries for detection of point mutation in *PfATPase6* gene that was believed to be the main target of artemisinins. Previous study conducted in French Guyana confirmed the existence of S769N or L263E mutant alleles in the *P. falciparum* field isolate<sup>8, 15–16</sup>. Reduced efficacy of the AS-MQ combination therapy and *in vitro* resistance to AS have also been reported on the Thai-Cambodia border and in western Cambodia, respectively<sup>3, 24–25</sup>. Although in our study, no SNP's were detected in any of the analyzed samples from Assam, but five numbers of *P. falciparum* field samples in Changlang district of Arunachal Pradesh showed novel non-synonymous mutation (C-T) at 1847 bp position of *PfATPase6* gene. Further evidence regarding this mutation is not available from any part of the world, whether this mutation is associated with artemisinin resistance or not. In previous consequence, non-synonymous mutations were detected at codons E431K, K649E and N683K, and synonymous mutations were noted at codons G468G, N483N and I898I in *PfATPase6* gene from *P. falciparum* isolates in tea garden areas of Jalpaiguri district, India<sup>1</sup>. However, future studies will provide a hint regarding the role of this mutation with artemisinin resistance/sensitivity.

Based upon the mutational pattern, two haplotypes of *PfATPase6* gene were observed during the study period. The wild type haplotype was circulating in Assam, whereas the mutant S616F allele was predominant in Changlang district of Arunachal Pradesh. The overall haplotype diversity (Hd) was: 0.069, whereas the overall nucleotide diversity (per site Pi) was 0.00012. Highest haplotype diversity was recorded in Changlang district of Arunachal Pradesh having Hd value of 0.33333 along with single polymorphic site and nucleotide diversity (Pi): 0.00060. High value of haplotype diversity indicates the high probability that two randomly chosen haplotypes are different. No significant haplotype diversity and genetic differentiation was observed among the *P. falciparum* field isolates in Karbi Anglong, Chirang, Tinsukia, Sivasagar, Jorhat, NC Hills, Lakhimpur, Golaghat and Dibrugarh districts of Assam and Lohit district of Arunachal Pradesh with a value of Hd: 0.00000. The population genetic struc-

Table 1. Value of neutrality tests and their significance

Neutrality test	Value	Statistical significance
Tajima's D	-0.66531	Non-significant ( $p>0.10$ )
Fu and Li's D test statistics	0.47228	Non-significant ( $p>0.10$ )
Fu and Li's F test statistics	0.14604	Non-significant ( $p>0.10$ )
Fu's Fs statistics	-0.782	
Strobeck's S statistics	0.947	

ture was determined within the parasite population of Assam and Arunachal Pradesh. The parasite population of Changlang in comparison to any of other district had a pair-wise fixation index ( $F_{ST}$ ) value of 0.16667 indicating great genetic differentiation within the population<sup>26</sup>. This greater genetic distance value within populations indicates some isolation between parasite population and most likely mean that the populations are not currently breeding with one another. Conventionally, the Nm: 1.25 signifies that there is sufficient gene flow between localities to negate the effects of genetic drift. The overall values of neutrality test are given in Table 1. The negative values of Tajima's D indicate an excess of low frequency alleles relative to expectation that can result to population expansions or positive selection. However, as these values are not statistically significant, we cannot ascribe that these allelic frequencies lead to the outcome of population expansion.

The combination of AS+SP was introduced by Government of India since 2010 as the first line agent to treat all diagnosed uncomplicated *P. falciparum* malaria<sup>1, 4</sup>. Now-a-days, AS + SP remains efficacious for the treatment of *P. falciparum* across diverse sites in central and eastern India. Although, Government of India has supplied ACT (AS+SP) combination for treatment purpose but due to the occurrence of high grade of sulfadoxine-pyrimethamine resistance (SPR) malaria parasite from northeastern parts of India, it should be a reason of concern because the life-span of ACTs depend largely on the partner drug, and any pre-existing resistance to SP could endanger the new combination. Since pre-existing resistance to SP exists in India, especially along the border with Myanmar where reduced artemisinin sensitivity has been reported, constant monitoring of the efficacy of ACT is essential in this aspect<sup>3, 19–21</sup>.

## CONCLUSION

Novel mutations at codon position 616 in *P. falciparum ATpase6* gene was detected from the field isolates in Changlang district of Arunachal Pradesh. In Assam the wild type *PfATPase6* genotype is found to circulate

which indicates that the use of ACT is still effective for treatment of *P. falciparum* cases. Continuous *in vitro* assays as well as clinical efficacy trials along with molecular study can facilitate to monitor drug sensitivity which helps to confer early warning signal of an impending ACT resistance.

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