International Journal of Medical and Health Research
ISSN: 2454-9142
Received: 28-11-2019; Accepted: 29-12-2019
www.medicalsciencejournal.com
Volume 6; Issue 1; January 2020; Page No. 112-121

Prevalence of polymorphism in Pfdhps & Pfdhfr genes associated sulphadoxine-pyrimethamine resistance among clinical isolates

Dr. Priti Singh
Tutor, Department of Microbiology, Calcutta School of Tropical Medicine, Kolkata, West Bangle, India

Abstract
The present study was carried out to evaluate the prevalence of polymorphism of point mutations at position 16, 51,59,108 and 164 of *Pfdhfr* and at positions 436, 437, 540, 581 and 613 of *Pfdhps*genes in preserved blood samplesof patients with confirmed *P. falciparum*monoinfection, who were treated with SP only, in 2010, in Kolkata, West Bengal before introduction of ACT in West Bengal, India.This study may be used to assess the usefulness of these molecular marker in determining the future trends of SP resistance.

The prevalence of polymorphism of P*fdhfr* and P*fdhps* gene point mutation in preserved blood samples of patients with confirmed P. *falciparum* mono infection, who were treated with SP only in 2010, in Kolkata, West Bengal. W.H.O. now recommends use of ACT for the treatment of uncomplicated P. *falciparum* malaria. As SP monotherapy is no longer in use for *falciparum* malaria treatment, blood samples of patients with confirmed P. *falciparum*monoinfection, who attended the malaria clinics, of CSTM during July to December 2010 and who met the W.H.O. inclusion criteria and were treated with SP only, were kept preserved in EDTA vials at -20^{0}C in protozoology section of CSTM were used for this study.

Through this study, we have tried to characterize the prevalence of mutations in Pfdhfr and Pfdhps genes associated with SP resistance, from isolates collected in 2010 from Kolkata, before the implementation of ACT. We found a high prevalence of mutated allele for both the gene locus (Pfdhfr more than Pfdhps). The absence of quadruple mutation in either gene or absence of sextuple mutation or more in combined haplotypes assures that resistance was not at an alarming level to cause SP treatment failure and so majority of the cases were found to show ACPR. However, this high prevalence also points that if the drug pressure continues to increase (as SP is being used with ACT), it will lead to further increase in mutations and will finally cause treatment failure as has been seen in North Eastern states of India and many countries of world. Hence an ongoing monitoring is required to know the prevalence of these mutations in different regions, so that timely intervention in drug policy can be done.

Keywords: polymorphism of Pfdhps and Pfdhfr genes, sulphadoxine-pyrimethamine, ACPR, ACT etc

Introduction

A limited number of genes involved or potentially involved in *P. falciparum* antimalarial drug resistance have been identified: the genes encoding dihydrofolate reductase (P*fdhfr*), dihydropteroate synthase (P*fdhps*), the chloroquine resistance transporter (P*fcrt*), the multidrug resistance 1 protein (P*fmdr1*), Na^+/H^+ exchanger (P*fnhe-1*) and cytochrome *b*.

Parasite transfection experiments have proved the role of*Pfdhfr* and *Pfdhp*s gene mutations in resistance to pyrimethamine and sulfadoxine, respectively. In addition, in-*vitro* assays of parasite purified enzymes as well as Pfdhfr allele expression in the yeast *Saccharomyces cervisiae*supports the role of gene mutation in SP resistance [1, 2, 3].

Sulfadoxine/Pyrimethamine is a synergistic combination. The drugs act as folate pathway blockers in the malaria parasite and thus interfere with the synthesis of DNA from guanosine triphosphate (GTP). Pyrimethamine inhibits P*fdhfr*, whereas sulfadoxine inhibits P*fdhps*. A remarkable feature of *P. falciparum* P*fdhfr*mutations both in resistant and sensitive parasites is that the mechanism of resistance depends not on a single mutation but on the accumulation of point mutations. The critical mutation is a serine (S) to asparagine (N) change at position 108, which is found in all resistant isolates. Altering Cystine (C) to Arginine (R) at position 59 Asparagine (N) to Isoleucine (I) at position 51 and Isoleucine (I) to Leucine (L) at position 164 increases pyrimethamine resistance, while the mutation at codon 16 where alanine (A) changes to valine (V) is specifically associated with Cycloguanil resistance [1].

While resistance to sulfadoxine and sulfones results from mutations within P*fdhps*. Amino acid changes at four positions (Serine-436, Glycine-437, Lysine-540, Alanine-581, and Alanine-613) confer resistance to sulfadoxine and also cross-resistance to sulfones and sulfonamides [4, 5].

The evolution and spread of resistant lineages of SP are traced by the P*fdhfr*rather than P*fdhps*because, a minor role of P*fdhps*mutations have been reported in the development of SP resistance [25, 26, 27]. The increase of sulfadoxine resistance was assumed to have occurred several years after the appearance of Pyrimethamine resistance and few studies of molecular evolution have been focused in Pfdhps [6, 7, 8, 9].

In India, chloroquine-resistant malaria was first reported in 1973, and since then resistance to this drug has been on the rise. Accordingly, Indian drug policy was changed and the SP combination was introduced in 1982 as a second line of treatment. However, in light of increasing resistance to SP monotherapy and WHO recommendations, Artesunate (AS)+SP combination was first introduced in India in 2006

in areas showing chloroquine resistance. This was revised by NVBDCP Drug Policy in 2008 to include all high P. falciparum predominant districts. Again in 2010 Drug Policy was revised extending ACT for all *P. falciparum* cases. But even this ACT combination has come under threat due to increased resistance to partner drug SP being reported from North-Eastern states of this country. In view of this the combination of AS+SP is replaced with AS+Lumefantrine in these states.

The increased SP drug pressure in the ACT is predisposed to put rapid selection pressure on *Pfdhfr*and *Pfdhps* resistant parasites. Ongoing monitoring of these mutations for their prevalence and therapeutic efficacy of the AS+SP combination should be done routinely for effective policies in the country. Though SP failure may not be rampant in India (except the North- eastern states and Nicobar Islands) but the rate at which the the mutations are increasing could lead to SP treatment failure as reported elsewhere.

The present study was carried out to evaluate the prevalence of polymorphism of point mutations at position 16, 51, 59, 108 and 164 of *Pfdhfr* and at positions 436, 437, 540, 581 and 613 of *Pfdhps*genes in preserved blood samples of patients with confirmed *P. falciparum*monoinfection, who were treated with SP only, in 2010, in Kolkata, West Bengal before introduction of ACT in West Bengal, India. This study may be used to assess the usefulness of these molecular marker in determining the future trends of SP resistance.

Objectives

The present study was designed with following objectives:

- The prevalence of polymorphism of P*fdhfr* and P*fdhps* gene point mutation in preserved blood samples of patients with confirmed P. *falciparum* mono infection, who were treated with SP only in 2010, in Kolkata, West Bengal.
- The co-relation of mutant*Pfdhps* and *Pfdhr*genes with *in vivo* SP resistance.

Methodology

The study was carried out at protozoology section of the Department of Microbiology, Calcutta School of Tropical Medicine [CSTM].

Study Population

W.H.O. now recommends use of ACT for the treatment of uncomplicated P. *falciparum* malaria. As SP monotherapy is no longer in use for *falciparum* malaria treatment, blood samples of patients with confirmed P. *falciparum*monoinfection, who attended the malaria clinics, of CSTM during July to December 2010 and who met the W.H.O. inclusion criteria and were treated with SP only, were kept preserved in EDTA vials at -20^{0}C in protozoology section of CSTM were used for this study.

Inclusion criteria

1. Age group >6 months.
2. Mono-infection with P. *falciparum* detected by microscopy.
3. Asexual parasite count of 2000 - 200,000/microliter in areas of high transmission and 1000 - 100,000/microliter in areas of low to moderate transmission.
4. Axillary temperature > 37.5 or history of fever during the 24 hour before recruitment.
5. Absence of of general danger signs in children under 5 years or signs of severe falciparum malaria according to the definitions of W.H.O. (2000).
6. Absence of severe malnutrition according to W.H.O. Child growth standards (W.H.O., 2006).
7. Absence of febrile condition due to diseases other than malaria (e.g. Measles, acute lower respiratory tract infection, severe diarrhea with dehydration) or other known underlying chronic or severe diseases (e.g. Cardiac, renal or hepatic diseases, HIV/AIDS).
8. Absence of regular medication,which might interfere with antimalarial pharmacokinetics.
9. Absence of history of hypersensitivity reactions or contraindication to any medicine tested or used as an alternative treatment.

Exclusion criteria

1. Patients who did not comply with the complete SP treatment as per records.
2. The blood samples which got degenerated/ contaminated during preservation.

Sample Size

From the 100 preserved blood samples of the patients fulfilling the above criteria, 50 samples were selected randomly using the random number table.

The definite study end point (as per W.H.O.2009 protocol) like “adequate clinical and parasitological response (ACPR)”, “early treatment failure (ETF)”, “late clinical failure (LCF)” and “late parasitological failure (LPF)”, “loss to follow up”, “withdrawal from study” and “protocol violation” was noted as per records.

a. Early treatment failure (ETF) if the patient develops one of the four conditions during first three days of follow up:
   1. Development of danger signs of severe malaria on day 1, day 2 or day 3 in the presence of parasitaemia.
   2. Parasitemia on day 2 higher than day 0 count irrespective of axillary temperature.
   3. Parasitaemia on day 3 greater than or equal to 25% of the count on day 0; and
   4. Parasitaemia on day 3 with axillary temperature >37.5^{0}C

b. Late clinical failure (LCF): Development of danger signs or severe malaria in the presence of parasittaemia on any day between day 4 and day 28 in patients who did not previously meet any of the criteria of early treatment failure; and Presence of parasitaemia on any day between day 4 and day 28 with axillary temperature ≥ 37.5^{0}C in patients who did not previously meet any of the criteria of early treatment failure.

c. Lateparasitological failure (LPF): Presence of parasitaemia on any day from day 7 to day 28 and axillary temperature <37.5^{0}C, without previously meeting any of the criteria of early treatment failure or late clinical failure.

d. Adequate clinical and parasitological response (ACPR): Absence of parasitaemia on day 28 irrespective of axillary temperature without meeting any of criteria of early treatment failure or late clinical failure or late parasitological failure.

e. **Loss to follow up:** despite all reasonable efforts, an enrolled patient could not be found. Reasons include failure to find a patient in the community after he or she misses a scheduled follow-up visit or moves out of the study area.
f. **Withdrawal from study:** A study patient (or a study patient's parent or guardian) who decides not to participate any further in the study is referred to as voluntary withdrawal. An involuntary withdrawal would be due to development of a concomitant illness that would interfere with the clear interpretation of study outcomes.
g. **Protocol violation:** when a study patient is removed from the study because of an event that does not allow for continued accurate interpretation of response to treatment. Examples include missed treatment dose, detection of a mixed infection during follow-up, or a credible report of additional antimalarial drug use outside the study protocol (such as self-medication).

Study Period
The study was conducted in between April 2016 to March 2017.

Study Design
Descriptive, observational, cross-sectional study.

Study Tools
Preserved blood samples as described earlier, DNA extraction, PCR augmentation, gene sequencing and DNA sequence analysis.

Study techniques
This study was conducted at protozoology section of Department of Microbiology at CSTM, Kolkata, after obtaining approval from institution ethical committee, from preserved blood samples. 50 samples of SP treated patients having P. *falciparum* mono-infection were randomlyselected using random number table, out of 100 preserved samples in good condition, collected in June-December 2010.

DNA Extraction
Genomic DNA of *P. falciparum* was isolated from 200µl EDTA blood, that was collected on day 0, using QiaAmp DNA Mini kit (Qiagen, Hilden, Germany) as per following procedure.

- 20 µl QIAGEN Protease was pipetted out and poured into the bottom of a 1.5 ml micro centrifuge tube.
- 200 µl of blood sample was added to the micro centrifuge tube.
- 200µl Buffer AL was added to the sample. Mixture was then mixed well by pulse-vortex for 15 sec.
- The mixture was then incubated at 56°C for 10 min.
- After incubation, the 1.5 ml micro centrifuge tube was briefly centrifuged to remove drops from the inside of the lid.
- 200 µl ethanol (96–100%) was added to the sample, and mixed again by pulse-vortex for 15 s. After mixing, the 1.5 ml microcentrifuge tube was briefly centrifuged to remove drops from the inside of the lid.
- Then the mixture from microcentrifuge tube was carefully applied to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. After closing the cap, the QIAamp Mini spin column was centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded.
- The QIAamp Mini spin column was carefully opened and 500 µl Buffer AW1 was added without wetting the rim. After closing the cap, the QIAamp Mini spin column was centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded.
- The QIAamp Mini spin column carefully opened and 500 µl Buffer AW2 was added without wetting the rim. After closing the cap, the QIAamp Mini spin column was centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 min. The QIAamp Mini spin column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded.
- The QIAamp Mini spin column was placed in a clean 2 ml collection tube, and the old tube containing the filtrate was discarded. The QIAamp Mini spin column was then centrifuged at full speed (20,000 x g; 14,000 rpm) for 1 min.
- The QIAamp Mini spin column was then placed in a clean 1.5 ml micro centrifuge tube, and the collection tube containing the filtrate was then discarded. After opening the QIAamp Mini spin column carefully, 200 µl Buffer AE was added without wetting the rim. After closing the cap, the QIAamp Mini spin column was incubated at room temperature (15–25°C) for 5 min, and then centrifuged at 6000 x g (8000 rpm) for 1 min.The 1.5 ml micro centrifuge tube containing isolated DNA was stored in -20 °C freezer until further analysis.

PCR Amplification of P*fdhfr* and P*fdhps* Genes
A portion of P*fdhfr*gene spanning codons 16, 51, 59, 108 and 164 and a portion of the P*fdhps*gene spanning codons 436, 437, 540, 581 and 613 will be amplified by two rounds of PCR using the primers.

Table 1: Primer sequence of *Pfdhfr* gene

Gene Name	Primer Sequence	
Pfdhfr	Primary PCR	F CCAACATTTTCAAGATTGATACATAA
		R ACATCGCTAACAGAAATAATTTGA
	Nested PCR	F GCGACGTTTTCGATATTTATG
		R GATACTCATTTTCATTTATTTCTGGA

Table 2: Primer sequence of *Pfdhps* gene

Gene Name	Primer Sequence	
Pfdhps	Primary PCR	F TTGTTGAACCTAAACGTGCTG
		R TTGATCCTTGTCTTTCCTCATGT
	Nested PCR	F TTTGAAATGATAAATGAAGGTGCT
		R TCCAATTGTGTGATTTGTCCA

PCR Amplification of P*fdhfr Gene*
Afragment 720-bp of the P*fdhfr*gene in located on chromosome 4, will be amplified by using oligonucleotide primer pairs AMP-1 (5_-TTT ATA TTT TCT CCT TTT TA-3) and AMP-2 (5-CAT TTT ATT ATT CGT TTT CT-3). PCR amplification was carried out in a final volume of 50µl which include 5µl of genomic DNA as template. The reaction mixture contained PCR buffer, 0.2mM dNTPs, 2.5

mM $Mgcl_2$, 0.3 µM of each of the primer and 1.5U of AmpliTaq polymerase (Perkin Elmer, Branchburg, NJ, USA). The cycling parameters used are as follows: denaturation at 94°C for 30 s, annealing at 45°C for 45 s, and extension at 72°C for 45 s. A total of 45 cycles are carried out.

PCR Amplification of P*fdhps Gene*

The P*fdhps*gene is located on chromosome 8. For amplification of *dhps*, a primary PCR were performed to amplify a 1,287-bp fragment of *P. falciparum* DNA by using primers M3717F (5_-CCA TTC CTC ATG TGT ATA CAA CAC-3_) and 186R (5-GTT TAA TCA CAT GTT TGC ACT TTC-3). Forty-five cycles are performed under the following conditions: denaturation at 94°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 90 s. A final extension will be performed at 72°C for 30 min for detection of the nucleotides at codons S436F/A, A437G, K540E, A581G, and A613S/T (Wang *et al*.,1997)[77].

Table 3: PCR Amplification Profile

Target Gene	Denaturation	Annealing	Elongation	Cycle No.
Pfdhfr (primary)	94^0C (30sec)	45^0C (45sec)	22^0C (45sec)	45
Pfdhps (primary)	94^0C (30sec)	54^0C (30sec)	72^0C (90sec)	40
Nested (Pfdhfr/*Pfdhps*)	94^0C (30sec)	57^0C (30sec)	72^0C (90sec)	40

Sequencing of PCR Products

The quality and concentration of PCR products for each sample were ascertained by agarose gel electrophoresis. 3% agarose was used. Agarose powder was dissolved in 1× TBE buffer by heating up the mixture in microwave until boiling and all agarose particles are dissolved. Then 5 µl of ethidium bromide was added in to the conical flask. The dissolved agarose was then cooled to 50^0C and poured in the gel casting mould. The cast gel was then allowed to set for 30 minutes at room temperature before removing the well forming combs. The gel plate was placed in the tank and covered with TAE. Then pre run (blank running) of gel was done to remove any obstacle in the groove of gel. Then the PCR products were loaded in to the gel groove using pipette. A DNA ladder of 100 base pair up to 1000 base pair was loaded in the corner grooves. Then gel was run and visualized under UV illumination and documented by Gel-Doc system. In gel electrophoresis 5 µl of the PCR products were loaded for visualization of the PCR products and remaining 45 µl samples were used for sequencing.The PCR products are purified from the agarose gel by use of the QIAEX-II Gel Extraction Kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions. The sequencing was done using the Big Dye terminator 3.1 (Applied Biosystems) with forward primer of the respective PCR products the sequencing protocol is as follows -

- **DNA Preparation:** Automated sequencing requires high -quality DNA. Sequencing directly from PCR products is also possible if the quality and quantity are sufficiently high. PCR products were purified using Qiagen QIAquick PCR Purification Kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions.
- **Primer Design:** The length and sequence of a primer determines its melting temperature and specificity. Most recommendations are for primer lengths of 18-25 bp and GC content between 40-60%. There should also be no runs of a single nucleotide greater than 3 within the primer. The primer should also be checked for potential self-annealing or hairpin formation, especially at its 3' end. We used the former primer of the respective gene to sequence the PCR products.
- **DNA Quantity:** The quantity ofPCR product needed varies with the size of the fragment. The amount of the DNA required for the sequencing reaction according to the PCR product size, is as follows-

Table 4: Amount of DNA Required According to PCR Product size

PCR size (in bp)	Amount of DNA (in ng)
150-250	25-40
250-500	40-70
500-700	70-90
700-900	90-110
900-1200	110-140

- **Sequencing Reaction:** Sequencing reactions were performed using ABI Big Dye Terminator V.3.1 chemistries. Because of the increased sensitivity of the new platform the recommended dilution for the terminator reaction kit as indicated in the table below. The manufacturer supplies the dilution buffer needed.

Table 5: Sequencing Reaction Mixture

ABI	1/2Rxn	1/4Rxn	1/8Rxn
Terminator Chemistry (2.5X)	4 µl	2 µl	1 µl
5X Dilution Buffer	2 µl	3 µl	3.5 µl
Primer	3.2 pMoles	3.2 pMoles	3.2 pMoles
DNA			
H_2O			
Rxn Total	20 µl	20 µl	20 µl

For ABI chemistry, program the thermo cycler as below:

- Inital denaturation of 96^0C for 1 min
- 25 cycles of:96^0C 10sec; 50^0C 5sec& 60^0C 4min
- Followed by a 4C hold until ready to purify

After the thermo cycler has finished, samples were stored at -20^0C in the dark or taken immediately to Terminator Removal.

Terminator Removal & Sample preparation

Best sequencing results are obtained when excess dye terminators are removed prior to electrophoresis.

Hot SDS Treatment

2.2% SDS in deionized waterwas prepared.

An appropiate amount of the 2.2% SDS solution was added to each sample to bring the final SDS concentration to 0.2%. Tubes were sealed and mix thoroughly.

The tubes were heated to 98^0C for 5 min and then allowed to cool to ambient temperature before proceeding to the next step.

- **Dye Terminator Removal:** The unincorporated dye terminators were removed by using Edge Bio systems Performa DTR Gel Filtration Cartridges, including precolumn SDS treatment.
- After terminator removal purified samples were transferred

Sequence Analysis

The sequences will be analyzed using the software BioEdit Sequence Alignment Editor version 7.0.5.2. The sequences will be then aligned using the online multiple sequence alignment tool ClustalW (available at: http: //www.ebi.ac.uk/clustalw). The amino acid sequences were compared with the wild type amino-acid sequences (GenBank accession numbers, XM_001351443 for *Pfdhfr*gene, and Z30654 for *Pfdhps*gene).

Plan for Analysis of Data

Out of 50 randomly selected stored blood samples of SP treated patients, the prevalence of mutations at various codons of *Pfdhps*and *Pfdhfps*genes were recorded and tabulated. The various two locus mutations in the genes were noted and the associated resistance level was tried to understood, as described in earlier field studies.

Results & Discussion

This study was conducted with preserved blood samples not with patients, so demographic data of age sex were as per records preserved.

The DNA from 50 samples were PCR amplified and sequenced for *Pfdhps* and *Pfdhfr*gene. Out of these 50 samples, PCR was successfully done for both genes in only 45 samples. (n=45).

As per records, subjects were divided into three age groups, 0-5 years, 5-15 years and >15 years (adults). In total samples, 1 subject (2%) was in 5-15 years age group, 44 (98%) subjects, were adults and no subjects in 0-5 years age group. Out of 50 samples, 30 (60%) were males and 20 (40%) were females.

Out of the 45 samples, 5 were recorded to have treatment failure with SP monotherapy (11%), while the rest were found to have ACPR. Of the 5 treatment failure cases, 4 were found to be LCF and only one as LPF. None of the sample was recorded as ETF.

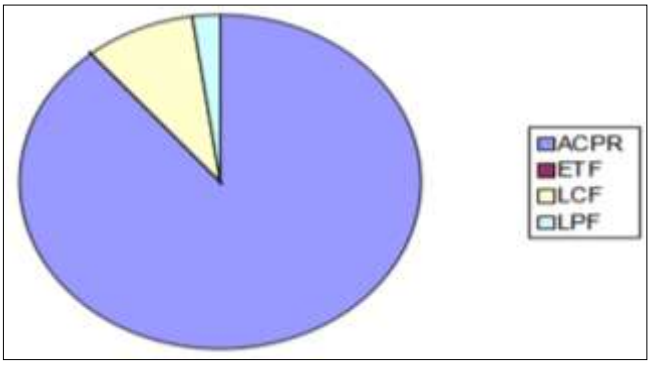


Chart 1: Response to SP Treatment

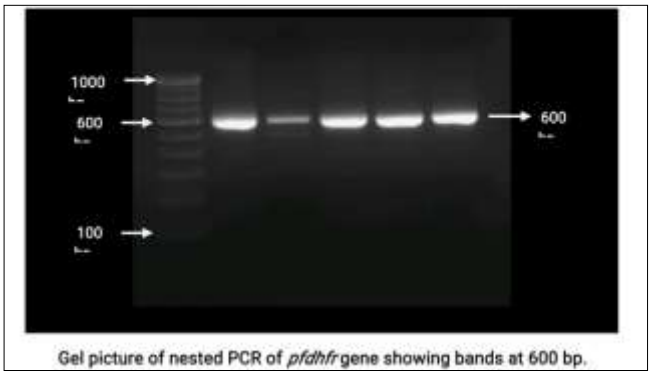
After sequencing, the results were analysed for mutations in five codons of the *Pfdhfr*gene (A16V, N51I, C59R, S108N/T, and I164L) and five codons of the *Pfdhps*gene (S436A/F, A437G, K540E, A581G, and A613S/T) to know the prevalence of mutated haplotypes of both gene in this region.

Mutations of *Pfdhfr*Gene

In *Pfdhfr*gene the most common mutant codon was found to be S108N (93.33%) followed by C59R mutation (91.11). Mutant allele for N51I was 6.67% and for I164L was 8.89%. None of the isolates showed mutant allele at position 16 of *Pfdhfr* gene in our study. Also there was no S108T mutation.

Table 6: Mutations of *Pfdhfr*Gene

Codon Position	16		51		59		108		164	
Allele (W/M)	45	0	42	3	5	40	3	42	41	4
% Mutation		0		6.67		8.89		93.3		8.89



Gel picture of nested PCR of *pfdhfr* gene showing bands at 600 bp.

Fig 2

Our study found, six different haplotypes of *Pfdhfr*gene. The wild type haplotype ANCSI was seen in 2 cases (4.44%). Among them, $A_{16}N_{51}R_{59}N_{108}I_{164}$ was the the most prevalent (73%) (Mutated amino acids are red coloured). Prevalence of single mutant ANCNI and ANRSI was 4.44% and 2.22% respectively. Triple mutant haplotypes were of two varieties ANRNL (8.89%) and AIRNI (6.67%). No quadruple mutant genotype was found in *Pfdhfr*gene of any isolate.

Table 7: Frequency of *Pfdhfr* Haplotypes

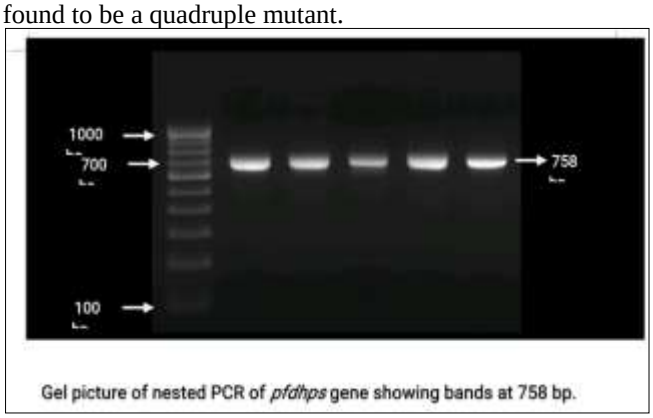
Haplotypes of *Pfdhfr*	ANCSI (wild type)	ANCNI	ANRNI	ANRNL	AIRNI
Total no.	3	2	33	4	3
%	6.67	4.44	73.33	8.89	6.67

Mutations of *Pfdhps* Gene

In case of *Pfdhps* gene wild type allele (SAKAA) were predominant at all codons. Mutation was found to be most common at position 437 where A replaces G (60%). This is followed by by mutation at K540E (31.11%). The prevalence of mutation at positions S436A and A581G were 17.78% and 13.33% respectively. None of the isolates showed any mutation at position 613. Also no S436F mutation was found in this study.

Seven different *Pfdhps* haplotypes were recorded, of which the wild variety $S_{436}A_{437}K_{540}A_{581}A_{613}$was the most prevalent one, being 35.56% of total isolates. Among the mutant variety, there were two single mutant haplotypes SGKAA (17.78%) and SAEAA (4.44%). Prevalence of double mutant variety SGKGA and SGEAA were 13.33% and 8.89% respectively. Only one variant of triple mutant haplotype $A_{436}G_{437}E_{540}A_{581}A_{613}$ was found with a

dominating presence of (17.78%). None of the isolates were found to be a quadruple mutant.



Gel picture of nested PCR of *pfdhps* gene showing bands at 758 bp.

Fig 3

Table 8: Mutations in *Pfdhps* Gene

Codon position	436		437		540		581		613	
Allele(W/M)	37	8	18	27	31	14	39	6	45	0
% mutation	17.78		60		31.11		13.33		0	

Table 9: Frequency of *Pfdhps* Haplotypes

Haplotype of *Pfdhps*	SAKAA	SGKAA	SAEAA	SGKGA	SGEAA	AGEAA
Total no	16	9	2	6	4	8
%	35.56	20	4.44	13.33	8.89	17.78

Pfdhfr-Pfdhps Two locus combined Mutation Analysis

Table 10: Frequency of *Pfdhfr/Pfdhps* Combined Haplotype

Haplotype	Pfdhfr/Pfdhps allele combinations	Total no. (n)	Percentage
Wild type	ANCSI/SAKAA	1	2.22
Single mutant	ANCSI/SGKAA	1	2.22
	ANCNI/SAKAA	2	4.44
Double mutant	ANCSI/SGKGA	1	2.22
	ANRNI/SAKAA	9	20
Triple mutant	ANRNI/SGKAA	6	13.33
	ANRNI/SAEAA	1	2.22
	AIRNI/SAKAA	3	6.67
	ANRNL/SAKAA	1	2.22
Quadruple mutant	ANRNI/SGKGA	5	33.33
	ANRNI/SGEAA	3	6.67
	ANRNL/SAEAA	1	2.22
	ANRNL/SGKAA	1	2.22
Quintuple mutant	ANRNL/SGEAA	1	2.22
	ANRNI/AGEAA	8	17.78

Wild type two locus haplotype (ANCSI/SAKAA) was observed in a single isolate (2.22%). Also 33.33% isolates had only mutation in *Pfdhfr*gene with wild type *Pfdhps* combination. There were two different types of single mutated two locus combinations ANCNI/SAKAA and ANCSI/SGKAA which were present in 4.44% and 2.22% of isolates respectively. Two type of double mutation combination was seen as ANRNI/SAKAA (20%) and ANCSI/SGKGA (2.22%).

For triple mutation four different combinations were seen. Out of these majority had a double *Pfdhfr*mutation and a single *Pfdhps* mutation (7 out of 11). While in 8.89% cases there was a triply mutated *Pfdhfr* gene with a wild variant *Pfdhps* gene.

Quadruple mutation was seen in 22.22% cases. There were four different combinations here with majority carrying a two point mutation in each of *Pfdhfr* and *Pfdhps*genes (8 out of 10). Only 2 isolates carried a triple mutated allele for *Pfdhfr*and only a single mutation for *Pfdhps.*

We also found quintuple mutations in 20% cases while only one of them had a triple muation in *Pfdhr* gene rest all had a triple mutation in *Pfdhps*gene with a double mutation of *Pfdhfr* gene.

It was seen that all the resistant samples had either quadruple or quintuple mutation. Of the 4 LCF cases, 3 had that the same triple mutant variant of*Pfdhfr*genotype (ANRNL) with either a single or a double mutant variant of *Pfdhps*. The lone LPF sample was found to have a double mutant variant of *Pfdhfr*gene (ANRNI) and a triple mutant variant of *Pfdhps (*AGEAA).

Table 11: Dstribution of Mutations

Total no of mutations	Wild(0)	single	double	triple	quadruple	quintuple	sextuple or more
(n)	1	3	10	11	10	9	0
%	2.22	6.67	22.22	24.44	22.22	20	0

Discussion

High prevalence of malaria and significant mortality and morbidity attached to it, has made epidemiology of malaria a subject of continuous investigation. Moreover control of drug resistant P. *falciparum*malaria has become a challenging task globally.

With the increasing incidence of resistance of falciparum malaria to Artemisnin and its derivatives in India as well as S.E. Asia, it is important to closely monitor the epidemiology and dynamics of the drug resistant P. falciparum [10, 11, 12]. Availability of easy and rapid molecular markers would greatly facilitate this process and will allow overcoming difficulties in the use of traditional methods for characterizing anti-malarial drug resistance.

Various studies from the past have identified *Pfdhfr*and *Pfdhps* gene as molecular markers to be associated with anti-folate drug resistance. Several reports on in *vitro* drug assays and clinical therapeutic assessment generally agrees with this hypothesis suggesting that genotyping of molecular marker may provide valuable information about trends of SP resistance in *falciparum* malaria [13, 14, 15].

My study was based on the use of PCR based molecular diagnostic tests to characterize mutations in *Pfdhps* and *Pfdhfr*genes of P. *falciparum*isolates collected from the blood samples of patient's with falciparum monoinfection on SP treatment from Kolkata.

Pyrimethamine inhibits the dihydrofolate reductase enzyme of P. *falciparum*which is involved in folate biosynthesis pathway. We studied point mutations at codon position 16, 51, 59, 108 and 164 of *Pfdhfr*gene.

This study found that majority of the isolates (93.33%) had mutation at position 108 were Serine was replaced by Asparagine (S108N).

All previous studies done in relation to *Pfdhfr* gene from India and around the world has shown a similar high prevalence of mutation in malaria endemic areas with SP in use (60,100). Infact, the highly endemic areas of world like African countries, Thailand, Malaysia and some parts of India (N-E India and Nicobar islands) have almost 100% mutation at this particular position (59, 61). It has also been hypothesized that S108N is the first step in the drug resistance selection process and that it causes upto 100 fold

decrease in sensitivity of parasite to Pyrimethamine.

The prevalence of mutation at position 59 where Cysetine is replaced by Arginine (C59R) closely follows S108N being 8.89% of the total isolates.

These two mutations are the key mutations in *Pfdhfr* sequence. Any other mutation which would lead to resistance, must be associated with them [16, 17].

In this study, we did not get even a single isolate with A16V and S108T mutations. This correlates with other studies done from Indian subcontinent which have similar findings from other regions. The above two mutations are assumed to provide resistance against Cycloguanil more than SP. So the absence of these two mutations corborates with the fact that Cycloguanil is not being used in india as a first line of treatment and so no associated drug selection pressure [18].

In this study we found another two point mutations in*Pfdhfr* gene at positions 51 where asparagine (R) replaces isoleucine (I)(6.67%) and 164 where isoleucine (I) is replaced by leucine (L) (8.89%).

The mutation at position 51 is a newly developing mutation. In fact studies done in multiple states have shown its presence only in regions with high transmission rate like Arunchal Pradesh, Assam, Orissa and Nicobar islands. As location of West Bengal puts it in close vicinity of these places, our finding relates to it [19].

From previous studies, seven different haplotypes for *Pfdhfr* gene were found. This study found five of these haplotypes. All these haplotypes have been reported previously from India and around the world. We found only 6.67% isolates with wild type haplotype ANCSI. While there was one single mutant variant ANCNI (4.44%) which has been reported from almost all states of India including West Bengal in previous studies. However, the most common haplotype of *Pfdhfr* was ANRNI in our study, which forms the majority (73.33%). This predominance was also seen in earlier studies done by Ahmed *et al* 2004 [19] and Sharma *et al*. 2015 [20]. Not only this the temporal studies from various regions have shown a dramatic rise in this double mutant variant because of constant use of SP for last two decades in India. A study done by Gatton *et al* 2004 [21] predicts that the long half-life of SP provides strong, selective pressure against parasites carrying wild type *Pfdhfr* gene, potentially resulting in the rapid decline of the wild type *Pfdhfr* genotype in high transmission areas. So, predominance of this double mutant haplotype shows continuous emergence of SP resistance at the study site.

In this study, we got two triple mutant haplotypes of *Pfdhfr* gene ANRNL (8.89%) and AIRNI (6.67%). Both these haplotypes have been reported from other states like Orissa, North eastern part of the country and from studies done in West Bengal in various frequencies [19, 22, 23]. Studies done in other endemic countries like Malaysia, Vietnam have also shown these mutations [15, 24].

The sequence analysis of the isolates of known drug susceptibility profile have revealed that a single or double *Pfdhfr* mutation alone will not cause SP treatment failure but triple or quadruple mutations will certainly provide a higher level of drug resistance. Furthermore some studies have shown that association of I164L mutation give rise to a higher level of resistance. So a parasite with triple mutant ANRNL will be more resistant than the parasite with AIRNI mutation [25, 26]. Although a single or double mutant variant of *Pfdhfr* will not cause resistance in itself, presence of a triple or quadruple mutant is believed to provide various degrees of resistance independent of *Pfdhps* haplotype.

The present study also found that the triple mutant variant of *Pfdhfr* ANRNL when associated with either a single or double mutant variant of *Pfdhp*s showed LCF. However, when ANRNL was combined to wild variant of *Pfdhps* (SAKAA), result was ACPR.

However my study did not show any quadruple or quintuple mutations of *Pfdhfr* gene. Other studies have shown there increasing presence in known resistant zones of country like north eastern states as well as Nicobar Islands [22, 23]. The quadruple mutations are also seen in high frequencies from countries like Thailand, Vietnam, Tanzania etc where SP has been withdrawn as first line management due to high drug failure rate [15, 27].

Previous studies done have shown SP treatment failure rate below the accepted 10% so our finding corroborates with others for the study site as there is absence of these resistant haplotypes present.

My study showed six different haplotypes of *Pfdhps* gene. The most common of which is the wild haplotype SAKAA (35.56%) at positions 436,437,540, 581 and 613 respectively. This predominance is seen in all regions of India except the Car-Nicobar islands [19, 22, 23, 28]. Although there are comparative studies done over a period of time which have shown that the total percentage of this particular haplotype is falling. These results also points to the fact that in two locus mutation of *Pfdhfr*/*Pfdhps* gene, mutation first settled in *Pfdhfr* followed by *Pfdhps* gene.

Among the mutated codons, most common is the mutation is at position 437 where alanine (A) is replaced by glycine (G). According to Wernsdorfer and Noedl, 2003 [29] this is the key point mutation in *Pfdhps*gene and additional mutations appears to increase the degree of resistance. We found that 60% of our isolates had this point mutation. Other studies have also got similar results. The next common point mutation in *Pfdhps* gene is at position 540 where Lysine (K) is replaced by glutamine (E) (31.11%) and then at codon 436 where alanine (A) replaces serine (S) (17.78%). In 13.33% cases alanine (A) is replaced by glycine (G) at codon 581 but none of these isolates showed mutation at position 613. This is similar to other findings done in recent past from this region [19, 22, 23]

After the wild haplotype SAKAA the most common haplotype in our study for *Pfdhps*gene was SGKAA (20%).There was another single mutated haplotype SAEAA (4.44 %/) in our study. The presence of this mutation essentially points to the fact that, though mutation at codon 437 is key mutation, though other mutations can occur in *Pfdhps* gene independent of it [30]. There two double mutated type of variant SGKGA and SGEAA (13.33% and 8.89% respectively). Both these haplotypes have been reported in earlier study done by Kumar *et al*. 2015 comprising several states of our country [30]. This haplotype is also common among isolates from Thailand [16] while the triple mutated haplotype AGEAA was seen in 17.78% cases. This type of mutation was previously reported from Orissa, Assam, Jharkhand as well in some parts of West Bengal. In fact this was the second most common mutated haplotype of *Pfdhps* gene after SGKAA. A recent Kolkata based study also came to a similar conclusion [11]. This study found that when AGEAA is present with ANRNI variant of *Pfdhps* resistant may be present. This was seen in two out of eight such combination. One of this was LCF while other was LPF.

As SP is a combination drug, the resistance associated with it is measured by studying mutations for both the gene locus (*Pfdhfr* and *Pfdhps)* simultaneously. A working hypothesis has been proposed by many studies done in different countries to correlate certain mutation at two locus genotype and its association with *in vivo* SP resistance. Higher the number of combined point mutations higher is the susceptibility of resistance.

In this study, we got fifteen different two locus combinations for *Pfdhfr* and *Pfdhps*genes. Only a single isolate showed wild type haplotype for both the genes. The commonest mutated form was a double mutant *Pfdhfr*and wild*Pfdhps* (ANRNI/SAKAA). This is in fact the most common finding from all the endemic countries of world were SP is in use. But this double mutant variant in itself is not capable of providing resistance. So the previous field studies done by Ahmed *et al* 2004 [11] had put this combined haplotype as the S/RI type and predicted them to be on verge of losing sensitivity to SP. Its high prevalence shows that the study area may develop resistant strains in future. The past studies have given a hypothesis that, the initial resistance to SP does not arise equally from a mutation in either gene associated with the folate metabolism of P. *falciparum*. It is proposed by by various workers that, first mutations in *Pfdhfr* gene occurs, and then mutations in *Pfdhps* takes place when most parasites in the population also carry at least a double and usually a triple mutant allele of *Pfdhfr*. These triple mutated alleles with or without mutated *Pfdhps*alleles are associated with RI and RII resistance of SP. While those with quintuple mutations (triple *Pfdhfr* and double *Pfdhps* or *vice-versa*) were categorized as RII/RIII. Parasites with total six mutations (triple mutations in each gene) were placed in category RIII (associated with treatment failure) i.e. these parasites will not be cleared by SP treatment [15, 31].

In this study we got four different combination of triple mutation in two locus combination. All the triple mutated AIRNI haplotype of *Pfdhfr* were associated with wild type of *Pfdhps* haplotype SAKAA in this study. So according to previous field studies, although level RII/III category (based on above mentioned criteria) is assigned to it, this may not actually result in treatment failure. Similarly ANRNL is also combined to SAKAA so again, though parasite clearance would be affected but the outcome is supposed to be ACPR. Rest two triple mutant haplotypes have same double mutant *Pfdhfr* variant (ANRNI) with a single *Pfdhps*mutation were proposed to be in RI category.

Next we got four types of quadruple mutants (22.22%). Two of these had double mutation in each of the genes (ANRNI/SGKGA and ANRNI/SGEAA). The first one of this forms the majority of quadruple mutant (5 out of 10). While the other two types contained a triple mutant *Pfdhfr* gene (ANRNL) and a single mutated *Pfdhps* haplotypes. Each of these had a single isolate. Again from the previous study these mutations were categorized into RII. Both of these mutated form were associated with LCF in present study emphasizing the importance of this triple mutated variant of *Pfdhp*s gene in evolution of resistance to SP.

Finally, we had nine isolates (20%) with quintuple mutation. One of them had a triple mutation of *Pfdhfr* gene and double mutation of *Pfdhps*gene (ANRNL/SGEAA) which was found to be associated with LCF. While the other eight had a double mutation of *Pfdhfr*gene and triple mutation of *Pfdhps*gene (ANRNI/AGEAA). One of them showed LCF and one was found to be associated with LPF. Kublin *et al*. 1998. [32]. Had found that such quintuple mutation would lead to SP treatment failure, but the same was not found to be true for Indian subcontinent (Ahmed *et al*. 2004) [19].

Other studies from our country have shown that presence of triple mutation in each of the two genes or more may show treatment failure. But the incidence of such mutation is negligible in our country except north eastern states and Nicobar Islands, where SP treatment as monotherapy as well as in combination with artemisinin derivatives has already been inhibited [19, 22, 23, 28]. None of the isolates in our study showed any such mutation. This correlates with the fact that parasites from the study sites were not totally resistant to SP during the period of blood collection. In fact studies done even after this period from this region have not reported treatment failure from this region.

So, our study shows, that though there is high prevalence of mutated alleles in study area, it seems to have not reached a treatment failure level. Further this high mutation prevalence may be explained from the fact that SP was being used for almost twenty years as monotherapy to combat *falciparum* malaria. Also the geographic location of West Bengal places it near high transmission areas like Orissa and Assam which have shown quintuple and more mutations in past which are related with higher degrees of resistance and *in vivo* will cause treatment failure.

Conclusion

Through this study, we have tried to characterize the prevalence of mutations in Pfdhfr and Pfdhps genes associated with SP resistance, from isolates collected in 2010 from Kolkata, before the implementation of ACT. We found a high prevalence of mutated allele for both the gene locus (Pfdhfr more than Pfdhps). The absence of quadruple mutation in either gene or absence of sextuple mutation or more in combined haplotypes assures that resistance was not at an alarming level to cause SP treatment failure and so majority of the cases were found to show ACPR. However, this high prevalence also points that if the drug pressure continues to increase (as SP is being used with ACT), it will lead to further increase in mutations and will finally cause treatment failure as has been seen in North Eastern states of India and many countries of world. Hence an ongoing monitoring is required to know the prevalence of these mutations in different regions, so that timely intervention in drug policy can be done.

References

1. Sirawarporn W, Sathitkul T, Sirwaraporn R, Yuthavong Y, Santi DV. Antifolate resistant mutant's ofPlasmodium falciparum dihydrofolate reductase. Proceedings of the National Academy of Sciences of the United States of America, 1997; 94:1124-1129.
2. Wooden JM, Hartwell LH, Vasquez B, Sibley CH. Analysis in yeast of anti-malaria drugs that target the dihydrofolate reductase of Plasmodium falciparum. Molecular and Biochemical Parasitology, 1997; 85:25-40.
3. Cortese JF, Plowe CV. Antifolate resistance due to new and known Plasmodium falciparum dihydrofolate reductase mutations expressed in yeast. Molecular and Biochemical Parasitology, 1998; 94:205-214.
4. Triglia T, Menting JG, Wilson C, Cowman AF. Mutations in dihydropteroate synthase are responsible

for sulfone and sulfonamide resistance in Plasmodium falciparum. Proc Natl Acad Sci USA, 1997; 94:13944-13949.
5. Triglia T, Wang P, Sims PF, Hyde JE, Cowman AF. Allelic exchange at the endogenous genomic locus in Plasmodium falciparum proves the role of dihydropteroate synthase in sulfadoxine-resistant malaria. Embo J, 1998; 17:3807-3815.
6. McCollum AM, Mueller K, Villegas L, Udhayakumar V, Escalante AA. Common origin and fixation of Plasmodium falciparum dhfr and dhps mutations associated with sulfadoxine–pyrimethamine resistance in a low-transmission area in South America. Antimicrob. Agents Chemother, 2007; 51:2085-2091.
7. McCollum AM, Basco LK, Tahar R, Udhayakumar V, Escalante AA. Hitchhiking and selective sweeps of Plasmodium falciparum sulfadoxine and pyrimethamine resistance alleles in a population from central Africa. Antimicrob. Agents Chemother, 2008; 52:4089-4097.
8. Zhou Z, Griffing SM, De Oliveira AM, McCollum AM, Quezada WM, Arrospide N, *et al*. Decline in sulfadoxine–pyrimethamine-resistant alleles after change in drug policy in the Amazon region of Peru. Antimicrob. Agents Chemother, 2008; 52:739-741.
9. Pearce RJ, Pota H, Evehe MS, Ba el H, Mombo-Ngoma G, Malisa AL, *et al*. Multiple origins and regional dispersal of resistant *dhps* in African *Plasmodium falciparum* malaria. PLoS Med, 2009; 6:e1000055.
10. Mohapatra PK, Sarma DK, Prakash A, Bora K, Ahmed MA, *et al*. Molecular Evidence of Increased Resistance to Anti-Folate Drugs in *Plasmodium falciparum* in North-East India: A Signal for Potential Failure of Artemisinin Plus Sulphadoxine-Pyrimethamine Combination Therapy. PLoS ONE. 2014; 9(9):e105562. doi:10.1371/journal.pone.0105562
11. Mishra N, Kaitholia K, Srivastava B, Shah NK, Narayan JP, Dev V, *et al*. Declining efficacy of artesunate plus sulphadoxine-pyrimethamine in northeastern India. Malar J, 2014.
12. Saha P, Guha SK, Das S, Mullick S, Ganguly S, Biswas A, *et al*. Comparative efficacies of artemisinin combination therapies in *Plasmodium falciparum* malaria and polymorphism of pfATPase6, *pfcrt, pfdhfr, and pfdhps* genes in tea gardens of Jalpaiguri District. India Antimicrob Agents Chemother, 2012; 56:2511-7.
13. Guidelines for treatment of malaria, second edition. WHO, 2010. Document available at: http://www.who.int/malaria/publications.
14. Cowman AF, Morry MJ, Biggs BA, Cross GA, Foote SJ. Amino acid changes linked to pyrimethamine resistance in the dihydrofolate reductasethymidylate Synthase gene of Plasmodium falciparum. Proc Natl Acad Sci. USA, 1988; 85:9109-9113.
15. Wang P, Lee CS, Bayoumi R, Djimde A, Doumbo O, Swedberg G, *et al*. Resistance to antifolate in Plasmodium falciparum monitored by sequence analysis of dihydropteroate synthetase and dihydrofolate reductase alleles in a larger number of field samples of diverse origin. Mol. Biochem. Parasitol, 1997; 89:161-177.
16. WHO. World malaria report: 2010. World Health Organization, Geneva, Switzerland Foote SJ, Galatis D. and Cowman AF. (1990). Amino acids in the dihydrofolatereductase-thymidylate synthase gene of Plasmodium falciparum involved in cycloguanil resistance differ from those involved in pyrimethamine resistance. Proc Natl Acad Sci. USA, 2010; 87:3014-3017.
17. WU Y, Kirkman LA, Wellems TE. Transformation of Plasmodium falciparummalaria parasites by homologusintegration of plasmids that cofer resistance to Pyrimethamine. Proceedings of the National Academy of Sciences of the United States of America, 1996; 93:1130-1134.
18. Gyang FN, Peterson DS, Wellems TE. Plasmodium falciparum: rapid detection of dihydrofolate reductase mutations that confer resistance to cycloguanil and pyrimethamine. Exp. Parasitol, 1992; 74:470-472.
19. Ahmed A, *et al*. Plasmodium falciparum isolates in India exhibit a progressive increase in mutations associated with sulphadoxine-pyrimethamine resistance. Antimicrob. Agents Chemother, 2004; 48:879-889.
20. Jitendra Sharma, Khan SA, Prafulla Dutta, Jagadish Mahanta. 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 PradeshJ Vector Borne Dis 52, June, 2015, pp. 116-121.
21. Gatton ML, Martin LB, Cheng Q. Evolution of resistance to sulfadoxine-pyrimethamine in Plasmodium falciparum. Antimicrobial Agents and Chemotherapy, 2004; 48:2116-2123.
22. Ahmed A, Lumb V, Das MK, Dev V, Wajihullah Sharma YD, *et al*. Prevalence of mutations associated with higher levels of sulphadoxine-pyrimethamine resistance in Plasmodium falciparum isolates from Car Nicobar Island and Assam, India. Antimicrob. Agents Chemother, 2006; 50:3934-3938.
23. Biswas S, Sharma A, Joshi H, Mishra NC, Valecha N, Kabilan L, *et al*. Characteristics of clones derived from Indian Plasmodium falciparum isolates. J. Parasit. Dis, 1996; 20:23-28.
24. Masimirembwa CM, Phuong-Dung N, Phuc BQ, Duc-Dao L, Sy ND, Skold O, *et al*. Molecular epidemiology of Plasmodium falciparum antifolate resistance in Vietnam: genotyping for resistance variants of dihydropteroate synthase and dihydrofolate reductase. International Journal of Antimicrobial Agents, 1999; 12:203-211.
25. Wongsrichanalai C, Pickard AL, Wernsdrofer WH, Meshnick SR. Epidemiology of drug resistant malaria. The Lancet Infectious Disease, 2002; 2:209-218.
26. Wichmann O, Jelinek T, Peyerl-Hoffmann G, Muhlberger N, Grobusch MP, Gascon J, *et al*. Molecular surveillance of the antifolate-resistant mutation I164L in imported African isolates of Plasmodium falciparum in Europe: sentinel data from TropNetEurop. Malaria Journal, 2003; 2:17-14
27. Hastings MD, Bates SJ, Blackstone EA, Monks SM, Mutabingwa TK, Sibley CH, *et al*. Highly pyrimethamine-resistant alleles of dihydrofolate reductase in isolates of Plasmodium falciparum from Tanzania. Transactions of the Royal Society of Tropical Medicine and Hygiene, 2002; 96:674-676.
28. Sharma YD. Molecular survelliance of drug-resistant malaria in India, Current Science. 2012; 102(5):10-701.

29. Wernsdorfer WH, Noedl H. Molecular markers for drug resistance in malaria: use in treatment, diagnosis and epidemiology. Current Opinion in Infec-tious Diseases, 2003; 16:553-558.
30. Amit Kumar, Romilla Moirangthem. Emergence of sulfadoxine–pyrimethamine resistance in Indian isolates of *Plasmodium falciparum* in the last two decades, Infection, Genetics and Evolution, 2015; 36:190-198.
31. Sibley CH, Hyde JE, Sims PFG, Plowe CV, Kublin JG, Mberu EK, *et al*. Pyrimethamine–sulfadoxine resistance in Plasmodium falciparum: what next? Trends in Parasitology, 2001; 17:582-588.
32. Kublin JG, Witzig RS, Shankar AH, Zurita JQ, Gilman RH, Guarda JA, *et al*. Molecular assays for surveillance of antifolate-resistant malaria. Lancet, 1998; 351:1629-1630.