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Association between prevalence of pyrimethamine resistance and double mutation in *pfdhfr* gene in West Bengal, India

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ABSTRACT

Objective: To find whether antifolate drug (pyrimethamine) resistance has occurred in the two malaria endemic zones (Kolkata and Purulia) of West Bengal, India. **Methods:** Parasitic bloods were collected from patients of Kolkata and Purulia, *in vitro* susceptibility test were performed in those 90 isolates. Now parasitic DNA was isolated by phenol chloroform extraction method and then polymerase chain reaction and restriction fragment length polymorphism analysis of different codons of *pfdhfr* gene (51, 59, and 108) were assessed in *Plasmodium falciparum* isolates from patients in India. **Results:** Among 45 isolates from Kolkata *dhfr* mutant isolates at codons 108, 51 and 59 were found in 71.11%, 100% and 15.55% isolates respectively while in Purulia mutation found in those codons were 42.22%, 57.77% and 0%, respectively. In Kolkata, the isolate having double mutation (N108 + 51I) were resistant to pyrimethamine ($P < 0.01$) whereas in Purulia N108 mutation is occurred with both mixed and mutant 51I mutation. **Conclusions:** Our present findings implicate that due to enormous drug (pyrimethamine) pressure double mutation with *dhfr* S108N/T and N51I was highly correlated ($P < 0.01$) with *in vitro* pyrimethamine resistance in two malaria endemic region of West Bengal. The increase in the number of *dhfr* mutations was strongly correlated to resistance to pyrimethamine.

1. Introduction

Malaria is a major vector-borne disease in India based on vast geographic areas with associated topographic and climatic diversity; the variable malaria epidemiology in India is associated with high parasite genetic diversity and rapidly evolving drug resistance, differential distribution of vector species, emerging insecticide resistance, underlying human genetic diversity and past evolutionary histories. Further, changing climatic patterns have possibly changed malaria epidemiology to a great extent. The outcome of these changes is an increased incidence of *Plasmodium falciparum* (*P. falciparum*) over the *P. vivax* malaria in recent years[1]. Around 1.5 million confirmed cases are reported annually by the National Vector Borne Disease Control Programme (NVBDCP), of which 40-50% is due to *P. falciparum*[2].

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Drug resistant *P. falciparum* is the major factor for death of malaria mainly in children below the age of 5 and the pregnant women[3]. No new class of antimalarials has been introduced into clinical practice since 1996[4]. The spread of *P. falciparum* resistance to cheap drugs is a serious world wide problem, considering the limited number of drugs available, the lack of vaccine, the morbidity and mortality impact of malaria[5]. In India chloroquine is the first choice of drug for treatment of *P. falciparum* malaria but after evolution of chloroquine resistant *P. falciparum* strain, the combination of sulfadoxine-pyrimethamine (SP) is used as a second line of therapy for the treatment of uncomplicated chloroquine resistant *P. falciparum* malaria. Recently extensive use arouses resistance to SP due to certain point mutations in the dihydrofolate reductase (*dhfr* genes) of the parasite[6]. Pyrimethamine inhibits the *dhfr* present in *Plasmodium* as a bifunctional enzyme with thymidylate synthase (*DHFR-TS*). The target of sulfadoxine is dihydropteroate synthase (*DHPS*), also part of a bifunctional enzyme, the 7, 8-dihydro-6-hydroxymethylpterin pyrophosphokinase-*DHPS* (*PPK-DHPS*). The molecular basis of *P. falciparum* resistance to antifolates consists of point mutations in genes encoding for both *dhfr* and *dhps*. The understanding of resistance

molecular mechanisms is of utmost importance for both designing new drugs and providing molecular markers to monitor drug activity and treatment efficacy[5]. *In vitro* resistance to pyrimethamine is caused due to single and multiple mutations at residues 51, 59, and 108, *P. falciparum* dihydrofolate reductase (*pfdhfr*) whereas mutation of residue 16 specifically conferred resistance to cycloguanil[7-8]. In humans, many studies reported the poor predictive value of *dhps* mutations for SP treatment failure. Severe resistance in SP occurs when at least two *dhfr* mutations and one *dhps* mutation are present[9].

So the present study was aimed to find whether antifolate drug (pyrimethamine) resistance has occurred in the two malaria endemic zones (Kolkata and Purulia) of West Bengal India.

2. Materials and methods

2.1. Chemicals and reagents

Chloroform, isoamyl alcohol, phenol, gentamycin, folate, agarose, *p*-amino benzoic acid-free RPMI 1640, HEPES, cell culture grade dimethylsulfoxide (DMSO) were purchased from Himedia, India. Tris-Hcl, Tris buffer, potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), ethylene diamine tetra acetate (EDTA), sodium dodecyl sulfate (SDS), sodium hydroxide (NaOH), sodium bicarbonate ($NaHCO_3$), sodium acetate, ammonium acetate, isopropanol, ethanol, boric acid were procured from Merck Ltd., SRL Pvt. Ltd., Mumbai, India. Albumax-II, hypoxanthine, proteinase K, RNase A, ethidium bromide, pyrimethamine were purchased from Sigma Chemical Co., USA. Oligonucleotide primers, restriction enzymes were purchased from New England Biolab, Baverly, USA. PCR grade nucleotide mixture, $MgCl_2$, dNTPs and Taq DNA polymerase were purchased from Roche applied science, USA. pLDH kit was purchased from Diatek, Kolkata, India. All other chemicals were from Merck Ltd., SRL Pvt., Ltd., Mumbai and were of the highest grade available.

2.2. Selection of subjects

Inclusion criteria for this experiment includes fever at consultation or history of fever within the past 24 h, a mono infection with *P. falciparum* based on the microscopic examination of Giemsa-stain thin and thick blood smears, a parasite density of 100-100 000 asexual parasites/μL of blood, and no recent history of self-medication with antimalarial drugs. Patients with signs and symptoms of severe and complicated malaria, as defined by the World Health Organization, were excluded (WHO, 2003).

2.3. Collection of sample

The study was carried out from May 2008 to April 2009. A total of ninety clinical isolates of *P. falciparum* were enrolled in this study. Each 45 isolates are respectively taken from the Kolkata (located at 22° 30′ N and 88° 30′ E) and Primary Health center, Purulia (lies at 22° 60′ N and 85° 75′ E), two malaria endemic zone of West Bengal, India. Informed consent was obtained from the respective patient and the patient's guardian both in case of adult and child patients. The experimental protocol of this study was followed the World Health Organization (WHO) and duly approved by the Institutional Ethical Committee.

2.4. Transport of sample

After collection of the sample from Kolkata, and Purulia of West Bengal, samples are transported to the laboratory as early as possible to carry out the different experiments.

2.5. Separation of red blood cell (RBC)

5-10 mL of venous blood samples were collected in a vacutainer (BD falcon) coated with an anticoagulant and washed in folate and *p*-amino benzoic acid-free RPMI 1640 medium for several times; followed by centrifugation at 2 000 ×g for 10 min at 4 ℃, an aliquot of 1.5-2 mL of the red blood cell pellet was obtained[10].

2.6. In vitro drug sensitivity assay

In vitro drug susceptibility assays were performed on the clinical isolates with prior adaptation to the *in vitro* parasite culture[11]. Here 3D7 (Antifolate sensitive) and W2 (Antifolate resistant) are taken as control strain. Infected erythrocytes were suspended in the complete folate and *p*-amino benzoic acid free RPMI 1640 medium consisting of 0.5% Albumax II, 25 mM HEPES, 25 mM $NaHCO_3$, 25 μg/mL gentamicin and 0.2% hypoxanthine at a hematocrit of 1.5% and an initial parasitemia of 0.2-1.0%. If the blood sample had a parasitemia >1.0%, fresh uninfected, type O^+ erythrocytes were added to adjust the parasitemia to 0.6% to 1% and cultured at 37 ℃ in 5% CO_2.

2.7. IC_{50} value

The 50% inhibitory concentration (IC_{50}) value means the inhibition of growth up to 50%. Ethanol was used to prepare stock solutions and dilutions of pyrimethamine. The final concentrations ranged from 50 to 40 000 nM for pyrimethamine. Twenty five microlitres of each concentration were distributed in culture plate and IC_{50} was determined using microscopic examination, detection of pLDH and hypoxanthine uptake assay[10]. The calculation was based on non linear regression analysis of the logarithm of concentrations plotted against the percentage growth inhibition. Isolates were defined as susceptible to pyrimethamine when IC_{50} values were <100 nM, moderate resistance when 100- 2 000 nM and resistant when >2 000 nM.

2.8. Isolation of parasitic DNA

Erythrocytes (infected and uninfected) were suspended in 15 mL of ice-cold NET buffer (150 mM NaCl, 10 mM EDTA, 50 mM Tris, pH 7.5) and lysed with 0.015% Saponin (Sigma). The lysate was centrifuged at 2 000 ×g for 10 min at 4 ℃ and the pellet was transferred to a 1.5-mL micro centrifuge tube and suspended in 500 μL of NET buffer. The mixture was treated with 1% N-lauroylsarcosine (Sigma) and RNAse A (100 μg/mL) at 37 ℃ for 1 h and proteinase K (200 μg/mL) at 50 ℃ for 1 h. Parasite DNA was extracted three times in equilibrated phenol (pH 8), phenol-chloroform-isoamyl alcohol (25:24:1), chloroform-isoamyl alcohol (24:1) and precipitated by the addition of 0.3 M sodium acetate and cold absolute ethanol. The extracted DNA was air-dried and resuspended in TE buffer (10 mM Tris, 1 mM EDTA). Parasite DNA was stored at -20 ℃ until use[10].

2.9. Primer designing of dhfr gene

The primer design was based on published *pfdhfr* sequences of *P. falciparum* (gene bank accession number *dhfr*, M22159, J04643) aided by primer designing software primer-3 (Table 1). First amplification was designed to harboring the single nucleotide polymorphisms (SNPs) present on the position 51, 59 and 108 of *pfdhfr*[12]. The protocol induces nest I followed by multiplex PCR reaction incase of *dhfr* gene,

Table 1
Sequences of the primers used for the detection of polymorphism in *dhfr* genes.

Nest	
dhfr, M1	5′ TTTATGATGGAACAAGTCTGC3′
M5	5′AGTATATACATCGCTAACAGA3′
Multiplex PCR	
dhfr, 108 F	5′ CAAAGAAACTGTGGATAATGTAAATGATATGC3′
108R	5′ AACAACGGAACCTCCTATAATAAAACATT3′
51/59F	5′ CTAGGAAATAAAGGAGTATTACCATGGAAATGgA3′
51/59R	5′ CATATTTTGATTCATTCACATATGTTGTAACTGCtC3′

2.10. Polymerase chain reaction of dhfr gene

The regions of the *Pfdhfr* gene surrounding the polymorphisms of interest were amplified by the polymerase chain reaction using the Eppendorf thermal cycler under the following conditions: approximately 200 ng of genomic DNA, 15 pmol of primers, reaction buffer (10 mM Tris, 50 mM KCl, pH 8.3), 2.5 mM $MgCl_2$, 250 μM dNTP, and 1 unit of Taq DNA polymerase in a 25 μL reaction mixture at 95 ℃ for 5 min for the first cycle and 30 sec in subsequent cycles, 50 ℃ for 30 sec in all cycles, and 72 ℃ for 1 min in all cycles, for a total of 40 cycles. 5 μL of the amplification product then mixed with 1 μL of bromophenol blue and loaded on a 2% agarose gel; subjected to electrophoresis and stained with ethidium bromide, and visualized under ultraviolet transillumination to confirm the presence of the particular amplicon according to base pair size[10,12].

2.11. RFLP analysis of dhfr gene

Single nucleotide polymorphism of the *pfdhfr* gene at their specific codon was determined by enzymatic digestion of specific restriction enzymes. In *dhfr* gene BsrI, BstNI and TaqI identify 108 Asn, 108 Thr and 59 Arg respectively, whereas AluI and EcoRI causes double digestion of the PCR products and identify 108 Ser and 51 Asn. 15 μL of the restriction enzyme treated product was mixed with 2 μL of bromophenol blue and finally loaded on a 2 % agarose gel; subjected to electrophoresis and stained with ethidium bromide, and visualized under ultraviolet trans illuminator to confirm the presence of the particular DNA fragment according to base pair size[13].

2.12. Statistical analysis

The data were expressed as mean ± SEM. The relationship between IC_{50} values of pyrimethamine and genotypes were assessed by Fisher's exact tests, and Mann-Whitney U-test. All the analysis as performed using a statistical package, Origin 6.1, Northampton, MA 01060 USA with multiple comparison *t*-tests, $P< 0.05$ as a limit of significance and GraphPad InStat software 3.0.

3. Results

3.1. In vitro susceptibility to pyrimethamine

In vitro assay for pyrimethamine yield interpretable result on all 45 isolates from Kolkata and 45 isolates from Purulia respectively. Using the *in vitro* responses to pyrimethamine 11 (24.44%) isolates from Kolkata were pyrimethamine-sensitive (Geometric mean IC_{50}= 65 nM, range= 10-90 nM) (Figure 1), while 13 (28.88%) isolates are sensitive to pyrimethamine in Purulia. (Geometric mean IC_{50}= 57.08 nM, range= 15-90 nM) (Figure 2). Out of 45 isolates from Kolkata, 7 (15.56%) (Mean IC_{50}= 987.86 nM, range= 150-1 675 nM) and 15 (33.33%) isolates from Purulia were intermediately susceptible to pyrimethamine (Mean IC_{50}= 1 022.67 nM, range= 115-1 950 nM). 27 (60.00%) isolates were highly resistant to pyrimethamine (Mean IC_{50}= 2 842 nM, range= 2 000-4 600 nM) in Kolkata, whereas 17 (37.78%) isolates from Purulia were also highly resistant (Mean IC_{50}= 2 544.12 nM, range= 2 000-3 500 nM to pyrimethamine.

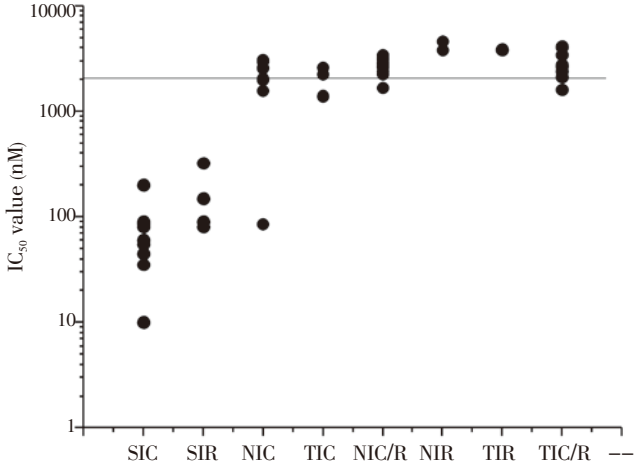

Figure 1. Different types of *dhfr* mutation in Kolkata.

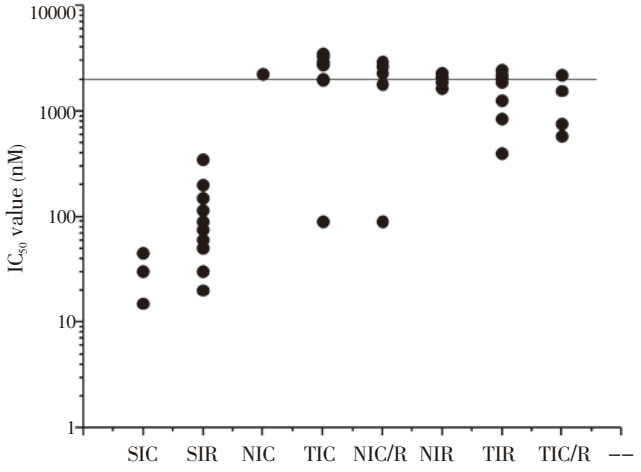

Figure 2. Different types of *dhfr* mutation in Purulia.

3.2. pfdhfr genotypes

The region of *pfdhfr* genes flanking the polymorphism of interest were amplified by PCR (at first nested PCR and followed by multiplex PCR) and finally digestion with specific restriction enzyme to detect each variant.

3.4. PCR/ RFLP – test for dhfr gene

During the SNP analysis of *pfdhfr*, amplification with the primer M1 and M5 giver a 262 bp PCR product that run in 2% agarose gel and visualized under UV transilluminator. As presented in Table 2 and Table 3, the frequency of samples composed of *dhfr* mutant isolates at codons 108, 51 and 59 were 71.11%, 100% and 15.55%, respectively in Kolkata while in Purulia mutation found in those codons were 42.22%, 57.77% and 0% respectively. The three alternative forms of codon 108; ser, asn, thr were discriminated by digestion with BsrI, AluI and BstNI.

The polymorphism in codon 59 was discriminated by double digestion with TaqI that allows the 94 bp amplicon to 57 and 37 bp fragment in presence of 59 Arg. Interestingly mutant 51 codon has been discriminated by digestion with EcoRI, that produce 33 and 61 bp fragment in presence of Asn 51 (wild type allele).

In kolkata we found about 40% of mixed infection in 59 codon, whereas no mixed infection was found either with 108 or 51 codon. In Purulia 24.44% isolated have mixed 108 mutations while large number of mixed 51I (33.33%) was also found. Interestingly no single mutation was observed with 59 codon in Purulia.

Table 2
Distribution of *pfdhfr* genotype in 45 blood sample form Kolkata, with *P. falciparum* infection.

Codons	*n*	Wild	Mutant	Mixed
*dhfr*108	45	13 (28.88%)	32 (71.11%)	0 (0%)
dhfr 51	45	0 (0%)	45 (100.00%)	0 (0%)
dhfr 59	45	20 (44.44%)	7 (15.55%)	18 (40.00%)

Table 3
Distribution of *pfdhfr* genotype in 45 blood sample form Purulia, with *P. falciparum* infection.

Codons	*n*	Wild	Mutant	Mixed
*dhfr*108	45	15 (33.33%)	19 (42.22%)	11 (24.44%)
dhfr 51	45	4 (8.88%)	26 (57.77%)	15 (33.33%)
dhfr 59	45	45 (100.00%)	0 (0%)	0 (0%)

3.5. Relation between dhfr genotypes and in vitro data

The presence of *dhfr* point mutations was linked to *in vitro* resistance to pyrimethamine. The phenotype of *in vitro* susceptibility to pyrimethamine was associated with *dhfr* genotypes at positions 108, 51 and with mixed 59 codon in Kolkata (Fisher's test, pyrimethamine: $P< 0.01$ for codons 108 and $P< 0.01$ for codon 51; and also $P< 0.05$ for mixed 59 mutation) (Table 4) whereas in Purulia *in vitro* susceptibility to pyrimethamine was associated with *dhfr* 108 and 51 codon but not with 59 codon (Fisher's test, pyrimethamine: $P< 0.01$ for codons 108 and $P< 0.01$ for codon 51; and P was not significant at the level of 0.05 for 59 mutation as there was no 59 mutation found) (Table 4). Figure 1 and Figure 2 shows that low IC_{50} values was associated with wild *dhfr* genotypes more specifically in S108, as opposed to mixed and mutant genotypes. In Kolkata N108 mutation is always occurred with 51I mutation. The isolate having double mutation (N108 + 51I) were resistant to pyrimethamine ($P<0.01$) whereas in Purulia N108 mutation is occurred with both mixed and mutant 51I mutation, although there was a single isolate found where N108 mutation occurred with wild 59 allele. In Purulia double mutation was associated with N108 + 51I having very high IC_{50} value for pyrimethamine and also resistant to pyrimethamine (Fisher's test $P <0.01$) while isolate having double mutation with S/N108 + N/I51 were intermediately susceptible to pyrimethamine ($P <0.05$) (Figure 1, 2).

Table 4
Distribution of *pfdhfr* genotype in relation to pyrimethamine *in vitro* susceptibility to chloroquine in Kolkata and Purulia.

No	*In vitro* susceptibility to PYR			*pfdhfr* genotype		
	108	51	59	S	I	R
K 9	S**	I	C	8	1	0
K 4	S	I	R	2	2	0
K 8	N**	I	C**	1	1	6
K 3	T	I	C	0	1	2
K 11	N**	I**	C/R*	0	1	10
K 2	N	I	R	0	0	2
K 1	T	I	R	0	0	1
K 7	T**	I**	C/R*	0	1	6
P 3	S	N	C	3	0	0
P 12	S*	I	C	8	4	0
P 1	N	N	C	0	0	1
P 8	N**	I**	C	1	1	6
P 6	T	I	C	1	1	4
P 4	N	N/I	C	0	2	2
P 7	S/N	N/I	C	0	4	3
P 4	S/T	N/I	C	0	3	1

K- Kolkata, P- Purulia; PYR-pyrimethamine. *$P<0.05$, **$P<0.01$.

4. Discussion

Genetic diversity in *Plasmodium* is well known but how the diversity affects clinical manifestation and the epidemiology of malaria is still under continuous investigation. This study was based on the use of PCR-based molecular diagnostic tests to characterize mutations in *dhfr* gene of *P. falciparum* and their distribution in places where the antifolate combination of pyrimethamine and sulfadoxine has shown treatment failure. In my study *P. falciparum* isolates from Kolkata and Purulia districts were analyzed for the drug resistance may be associated with genetic mutations.

The parasite isolates from both the places demonstrated a variable range of mutations in *dhfr* where resistance to PS is not well established. *dhfr* mutation profile demonstrated several variants over wild type. *dhfr* Asn 108 was very common followed by Ile 51. Among isolates from Kolkata the most common mutations in *dhfr* gene are at position 108 and 51. The presence of all these changes at key mutation sites reflects the resistance to available antifolates[14].

Clinical symptoms and profile of *P. falciparum* malaria also differ among age groups. Early recognition of symptoms and treatment of malaria is paramount especially in children who tend to have higher rates of complications compared to adults[1].

Adult persons frequently take antibiotics or antimalarial drugs without completing the course of the drug in general. So there is a high risk of drug resistance and development of mutant drug resistant parasites. Sometimes multi drug resistance is likely to be a consequence of multi-factors and enzymes in GSH system also[16].

Among 45 isolates from Kolkata *dhfr* mutant isolates at codons 108, 51 and 59 were, found in 71.11%, 100% and 15.55% isolates, respectively. Interestingly, here 40% cases have been found with mixed 59 mutations. From our study we found 60% isolates from Kolkata are *in vitro* pyrimethamine resistant. As we know from Basco *et al*, *in vitro* resistance to pyrimethamine has been associated with the key

mutation of *dhfr* S108N; additive mutations in *dhfr* N51I and C59R conferred higher levels of resistance, here we observed higher level of *in vitro* pyrimethamine resistance is associated with tipple NIR and NIC/R mutation as well as with double NIC and TIC mutation. From these results in Kolkata we may conclude that extensive and haphazard use of SP combination therapy arouses severe pyrimethamine resistance in Kolkata. Whereas in Purulia among 45 isolates only 37.78% isolates are *in vitro* resistant to pyrimethamine but large number of (33.33%) isolates are intermediately resistant to pyrimethamine. Another interesting result we found here was that there was absence of 59 mutation and presence of large number (57.77%) of 51I allele, instead of this we also found here 33.33% mixed 51I mutation. Beside this we also found 24.44% of mixed S108N and S108T mutation. So this large number of intermediately resistant to pyrimethamine cases may be due to these mixed infection found in 51 and 108 codon (P <0.05).

PCR-based methods do not detect minor clones in a mixed population, although a wild-type clone may remain undetected, this is unlikely for *in vitro* susceptibility, as IC_{50} mainly reflects the susceptibility of the major clone(s) present in the blood sample. Mutations *dhfr* N51I and C59R are thought to increase *in vitro* resistance to pyrimethamine[9].

Our results confirmed that *dhfr* S108N/T and N51I are both key mutation and it caused severe *in vitro* resistance to pyrimethamine in two malaria endemic region of West Bengal because out of 46 isolates with 108N/T + 51I, 37 isolates showed resistant property (P <0.01).

We conclude that sever *in vitro* resistance of pyrimethamine in this area of India is related mainly to the combination of two *dhfr* (108N/T and 51I) mutation. The increase in the number of *dhfr* mutations was strongly correlated to resistance to pyrimethamine. Pyrimethamine resistance is alarming in Kolkata as population mix up and pyrimethamine drug pressure are maximal. Whereas in Purulia pyrimethamine resistance is also highly alarming due to presence of large number of intermediately resistant parasite population as well as the presence of large number of mixed S108N/T and N51I mutation indicates the tremendous drug pressure over the population.

So changes in antimalarial policies in favor of the use of SP in these areas of India are likely to increase SP drug pressure, and the clinical efficacy of SP may rapidly fade. New cheap antimalarial combinations (as treatment with ACT is much expensive to third world country) should be tested for treating the drug resistant *P. falciparum*.

Conflict of interest statement

We declare that we have no conflict of interest.

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